INFECTIOUS DISEASE SURVEILLANCE: APPLYING COOPERATIVE RESEARCH TO RECENT OUTBREAKS INCLUDING COVID-19

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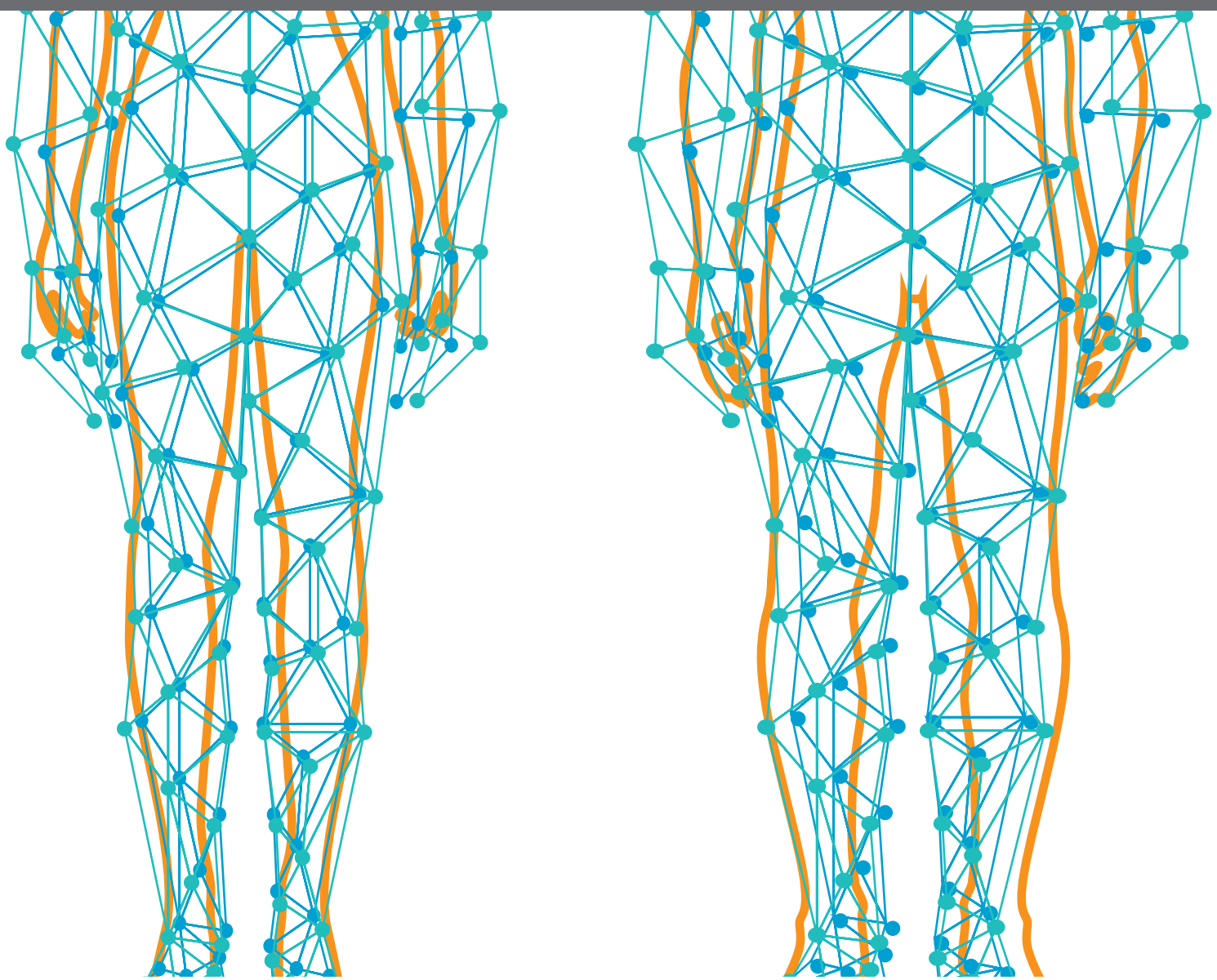

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INFECTIOUS DISEASE SURVEILLANCE: APPLYING COOPERATIVE RESEARCH TO RECENT OUTBREAKS INCLUDING COVID-19

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CASE REPORT
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Multisystem Inflammatory Syndrome With Complete Kawasaki Disease Features Associated With SARS-CoV-2 Infection in a Young Adult. A Case Report

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A severe multisystem inflammatory syndrome associated with Kawasaki disease manifestations (MIS-C) has been recently reported in children with signs of recent infection with SARS-CoV-2. We here reported the case of a young adult woman who presented the complete manifestations of Kawasaki disease associated with a severe myocarditis, acute respiratory distress syndrome and hemodynamic instability a few weeks after a transient anosmia. The detection of specific antibodies to SARS-CoV-2 in the absence of detection of the virus suggested that the syndrome was the result of a delayed immune response to a recent COVID-19 infection. A combined treatment with colchicine, tocilizumab, high dose immunoglobulins, and methylprednisolone allowed to control the inflammatory process and to limit the development of coronary aneurysm. The patient recovered without sequelae. This case emphasized the importance of SARS-CoV-2 serology for the diagnosis of delayed immune complications of COVID-19. Clinicians caring for adult patients must be aware that not only children but also young adults can be affected by a multisystem inflammatory syndrome with KD features associated with COVID-19.

Keywords: case report, Kawasaki disease, COVID-19, SARS-CoV-2, MIS-C, tocilizumab, serology

INTRODUCTION

Kawasaki's disease (KD) is a rare acute febrile disease affecting mostly children characterized by the association of conjunctivitis, erythema of the lips and oral mucosa, polymorphous exanthema, palmar-plantar erythema, and cervical lymphadenopathy. KD is a widespread vasculitis affecting small and medium sized arteries, with the possible occurrence of coronary aneurysms (1). Lung involvement is exceptional in KD.

Recently, Riphagen et al. (2) reported that previously healthy children presented a hyperinflammatory shock with Kawasaki disease-like features in association with infection with

severe acute respiratory syndrome coronavirus 2 (SAR-CoV-2). Shortly after, Verdoni et al. (3) confirmed these data, reporting that the incidence of KD was much higher in their area during the SARS-CoV-2 outbreak that in the same period in preceding years. This led Center of Disease Control (CDC) to publish an alert (HAN00432) on May 14, warning physicians on the occurrence of a multisystem Inflammatory Syndrome in Children (acronym "MIS-C") associated with SAR-CoV-2 (4). The case definition of MIS-C as defined by the CDC concerned an individual aged <21 years presenting fever >38°C for ≥24 h, laboratory evidence of inflammation and evidence of clinically severe illness requiring hospitalization, with multisystem (≥2) organ involvement (cardiac, renal, respiratory, hematologic, gastrointestinal, dermatologic, or neurological) and no alternative plausible diagnoses and positive for current or recent SARS-CoV-2 infection (4). Interestingly, even though CDC asked to report cases of patients younger than 21 years old, no case older than 15 years has yet been published.

We report the delayed occurrence of a multisystem inflammatory syndrome with complete Kawasaki disease features in a young adult patient recently infected by SARS-CoV-2.

CASE PRESENTATION

A 19.9 year-old woman of Caucasian origin without any significant personal or familial past history presented a sudden transient loss of smell on March 25, 2020 without any additional symptoms. She had participated without wearing a mask in a yoga session with several other people on March 12 and had been confined at home since March 13. Her parents were the only daily contacts. Between March 13 and March 21, her mother has been in close professional contact with a sick colleague which was finally diagnosed as severe Covid-19, but serologic tests performed end April in the mother and father were negative.

On April 14, she developed a febrile illness associated with cervical adenopathy, a morbilliform erythematous rash affecting the forearms, the hands and the buttocks, red and edematous lips and bilateral conjunctivitis with palpebral edema.

On April 17, she was admitted at the CHIREC hospital. On hospital admission, the heart rate was 137/min and the arterial blood pressure 129/73 mm Hg; she was not overweight (weight: 60 kg; BMI 24.7 kg/m^2); the throat was red, the cervical adenopathy was enlarged and painful; conjunctivitis and skin lesions were still present; lungs auscultation was clear and excepting marked tachycardia, heart exam was normal. Between April 17 and April 21, the patient remained febrile with a persistent inappropriate tachycardia. Symptoms time line are depicted on **Figure 1**. On admission the main blood laboratory results were as follows: white blood cells 11,100/μL, neutrophils 9,730/μL, lymphocytes 490/μL, eosinophils 350 /μL, platelets 147,000/μL, CRP 217 mg/L, fibrinogen 759 mg/dL, ferritin 285 μg/L, Na 131 mmol/L, creatinine 79,4 μmol/L, ASAT 52 U/L (n < 32). The evolution of principal blood parameters is depicted in **Figure 2** and hemodynamic and organ function variables in **Figure 3**.

A transthoracic echocardiography (TTE) demonstrated a severely decreased ejection fraction of the left ventricle (LVEF 15%), hyperechoic aspect of pericardium and small posterior pericardial effusion associated to a marked increased serum troponin T. A cardiac magnetic resonance imaging demonstrated myocardial edema typical of acute myocarditis. The mean arterial blood pressure dropped to 60 mm Hg with decreased oxygenation conditions and the patient was transferred to the ICU.

The first hemodynamic profile demonstrated a mean arterial pressure at 60 mmHg, a cardiac index at 2.1 l/min.M² and a central venous O_2 saturation ($ScvO_2$) at 47%. Dobutamine was initiated and an invasive hemodynamic monitoring device (PiCCO 5F catheter, Gettinge, Germany) was inserted. The invasive hemodynamic assessment under 5 mcg/kg min of dobutamine, reported a cardiac index at 3.7 L/min M² (normal 2.5–3.5), systemic vascular resistance at 685 dyne s cm^{-5} (normal 800–1,200), a global end diastolic volume at 932 ml/M² (normal 600–800), an extravascular lung water index at 17 ml/kg (normal <10) and a $ScvO_2$ at 63%, which suggests distributive shock with marked myocardial depression. Given this hemodynamic profile, inotropic, and vasopressor support was required for several days.

Dobutamine was infused from April 21 to April 28 (maximum dose 5 μg/kg/min) and synthetic human angiotensin 2 (Giapreza, maximum dose 20 mg/kg/min) from April 21 to April 25.

Multiple attempts of weaning these agents were performed daily. Despite hemodynamic stabilization, she rapidly developed ARDS according to Berlin criteria (5). She was mechanically ventilated and proned. Extensive workout was performed to rule out ongoing infections (including a bronchoalveolar lavage which disclosed an inflammatory pattern with predominance of neutrophils, without any detectable strain at direct examination, culture, as well as PCR for multiple respiratory pathogens). A gynecologic examination and multiple bacterial samplings were negative. There were no signs of macrophage activating syndrome (normal triglycerides—normal LDH- absence of very elevated ferritin). Serum IL-6 was 306 ng/mL (N < 20) and D-Dimer progressively increased from 3.9 μg/ml (N < 0.5) to 17.8 μg/ml.

The diagnosis of SARS-CoV-2 infection was considered. PCR tests were negative on two nasopharyngeal smears and on bronchoalveolar lavage but IgG and IgM against SARS-CoV-2 were detected on a blood sample taken on admission by the rapid test (Zhejiang Orient Gene Biotech Co., Ltd). A quantitative Ig G determination by chemiluminescence technology (DiaSorin, Italy) demonstrated an increase in specific IgG antibodies from 13.7 U on admission to 25 U after 7 days (negative <12 arbitrary units; positive >15 arbitrary units).

Given the consideration of SARS-CoV-2 related ARDS, myocarditis and distributive shock, tocilizumab (RoActemra, Roche), 480 mg was infused. PaO_2/FiO_2, CRP, and Troponin T rapidly improved (**Figures 2**, **3**). Impairment in myocardial function was resolved within 48 h.

Two days later, KD was considered based on clinical signs and significant eosinophilia; 1 g/kg intravenous immunoglobulins (Privigen, CSL Behring) were administered. A computed

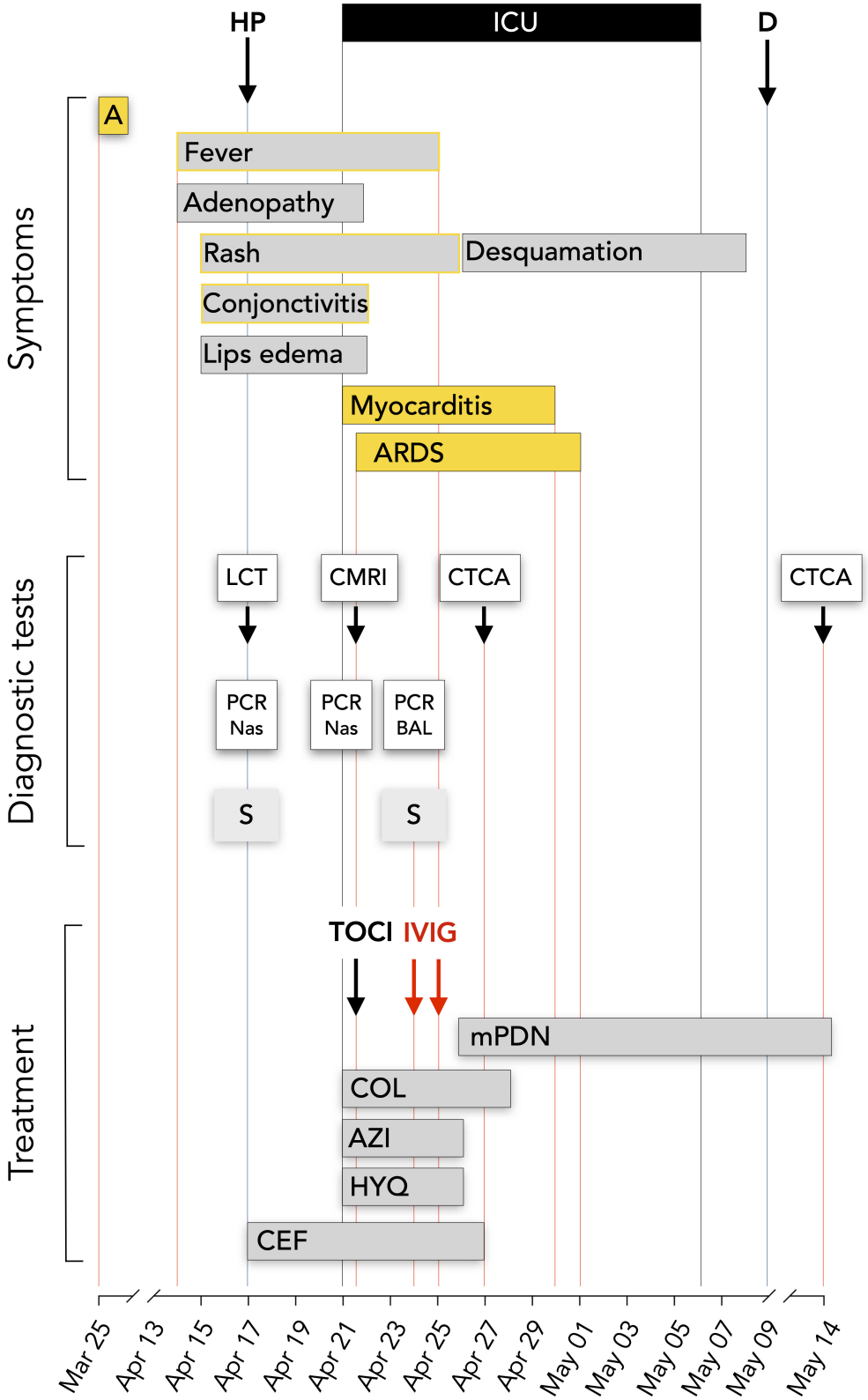

FIGURE 1 | Time line. Symptoms, diagnostic tests and treatment. HP, hospitalization; ICU, intensive care unit; D, discharged day; A, transient anosmia; LCT, lung computed tomography; CMRI, cardiac magnetic resonance imaging; CTCA, computed tomography coronary angiography; PCR Nas, SAR-CoV-2 PCR on nasopharyngeal smear; PCR BAL, SAR-CoV-2 PCR on bronchoalveolar lavage; S, SAR-CoV-2 serology; HYQ, Hydroxychloroquine 400 mg bid during 2 days and then 200 mg; AZI, Azithromycine 500 mg on day 1 than 250 mg/day; COL, Colchicine 0.5 mg bid; mPDN, methyprednisolone 60 mg IV bid initially, 48 mg oral dose at discharge; 24 mg at May 14; CEF, ceftriaxone 2 g/d; TOCI, tocilizumab IV 480 mg; IVIG, Privigen 60 g.

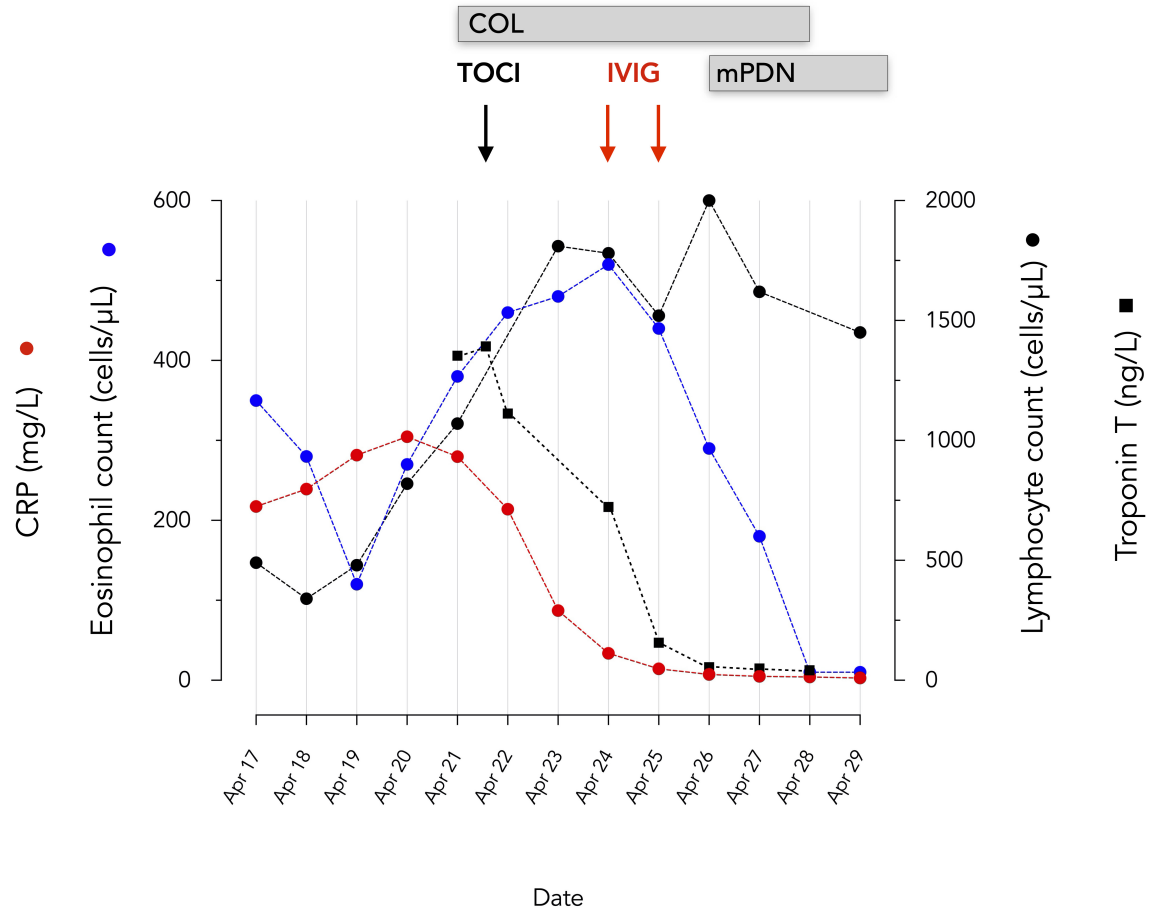

FIGURE 2 | Evolution of eosinophils, lymphocytes, serum CRP and troponin T. mPDN, methyprednisolone 60 mg IV bid initially, 48 mg oral dose at discharge; 24 mg at May 14; COL, Colchicine 0.5 mg bid; TOCI, tocilizumab IV 480 mg; IVIG, Privigen 60 g.

tomography coronary angiography (CTCA) demonstrated a coronary artery aneurism and high dose steroids were initiated, resulting in significative improvement 17 days later (**Figure 4**).

The patient fully recovered and was discharged on May 9.

DISCUSSION

This patient fulfilled all the criteria of typical KD (1) in addition to severe ARDS, distributive shock and severe myocarditis in the context of a recent SARS-CoV-2 infection suggested by the detection of IgG and IgM against SARS-CoV-2. She also fulfilled the criteria of the novel multisystem inflammatory syndrome reported in children being infected by SARS-CoV-2 (4).

The association of KD and multisystem inflammation with SARS-CoV-2 in children (MIS-C) has been reported in several European countries (2, 3, 6–10) and in US (11–13). The syndrome has been more recently requalified as pediatric inflammatory multisystem syndrome (PIMS) (6, 10, 14). As of the 15 of May 2020, in total, about 230 suspected cases have been identified in EU/EEA countries and the UK, including two fatalities (15). In France, respectively, 79 confirmed and 29 probable/possible PIMS related to SARS-CoV-2 (CoV-PIMS) have been collected between March 1 and May 17 (6). Of interest, the peak of the epidemic curve of PIMS occurred 4–5 weeks after the peak of the COVID-19 epidemic suggesting a delayed immune response to the virus. In Belgium, following the initial alerts for a Kawasaki-like disease mid-April 2020, a specific survey was set up by a pediatrician COVID-19 task force allowing centralized voluntary-based reporting of KD like/PIMS-TS by pediatricians across de country. Approximatively 25 cases of PIMS-TS have been reported through this survey up until June 23 (personal communication: Pr Stéphane Moniotte; Department of pediatrics, Cliniques universitaires Saint-Luc, UCLouvain, Brussels).

Of interest, in the largest published series, the median age was 8 years and 96% of the children were under the age of 16. No case older than 18 was reported yet (6).

There is no equivalent registry for adult patients and our case is the first to be reported so far. By the way it is important to acknowledge that the first report of KD or PIMS were published

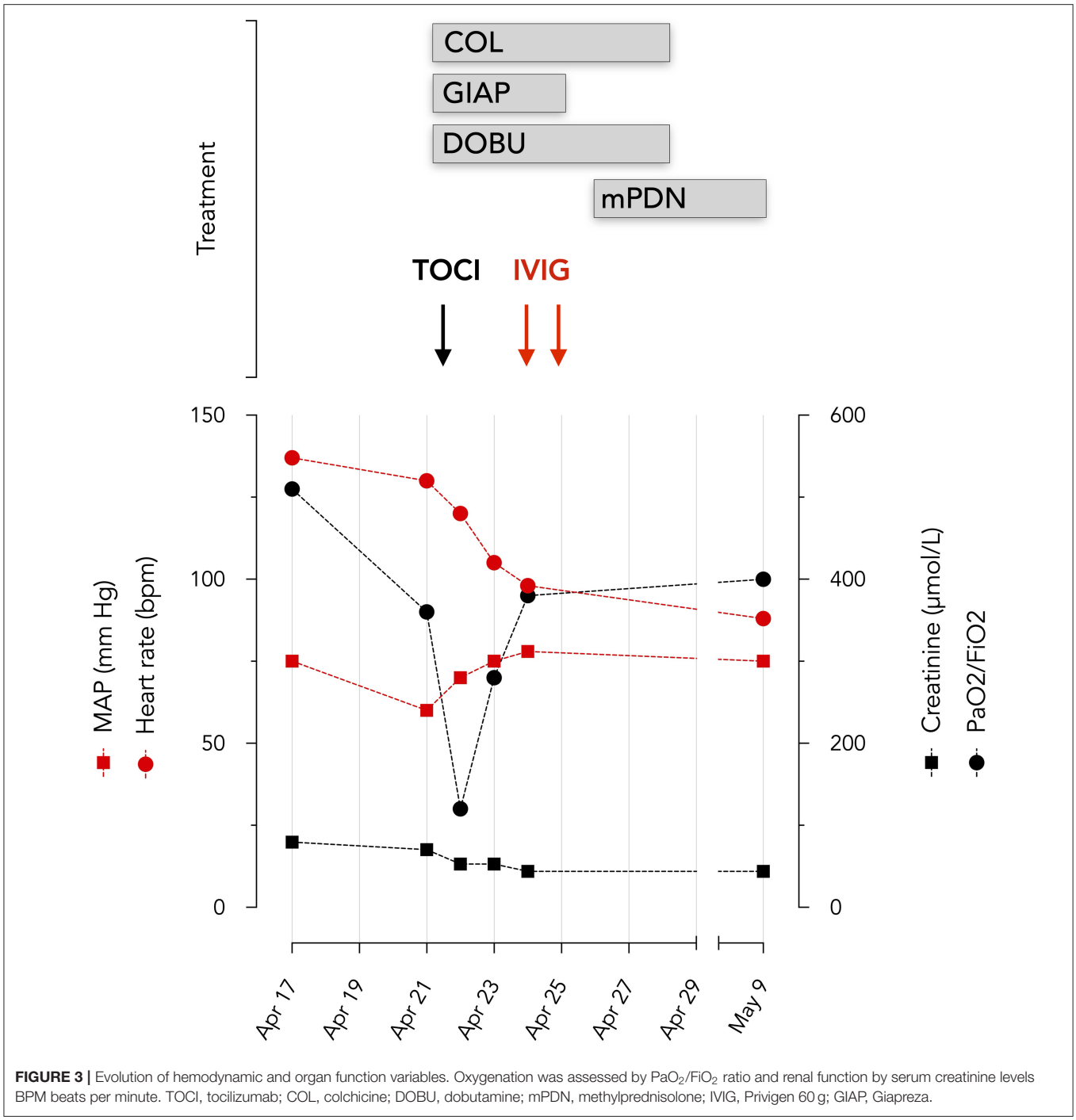

FIGURE 3 | Evolution of hemodynamic and organ function variables. Oxygenation was assessed by PaO_2/FiO_2 ratio and renal function by serum creatinine levels BPM beats per minute. TOCI, tocilizumab; COL, colchicine; DOBU, dobutamine; mPDN, methylprednisolone; IVIG, Privigen 60 g; GIAP, Giapreza.

online on April 27, several days after the onset of the disease in this patient.

Lung involvement and ARDS are extremely rare in classical KD unrelated to COVID-19 while these are common in SARS-CoV-2 infection as well as in CoV-PIMS. Indeed, critical care support was required in two third of CoV-PIMS cases, 43% requiring mechanical ventilation (6). On the other hand, infection with SAR-CoV-2 is associated with an uncontrolled inflammatory response and widespread endothelial cell dysfunction (16). As such, KD and SAR-CoV-2 share some similar pathophysiological mechanisms, which can lead to MIS-C/PIMS.

Admittedly, an important question is whether KD results from a late response to a recent infection or whether the infection was ongoing. On the one hand, the absence of detectable virus in the respiratory tract is in accordance of a late immune complication

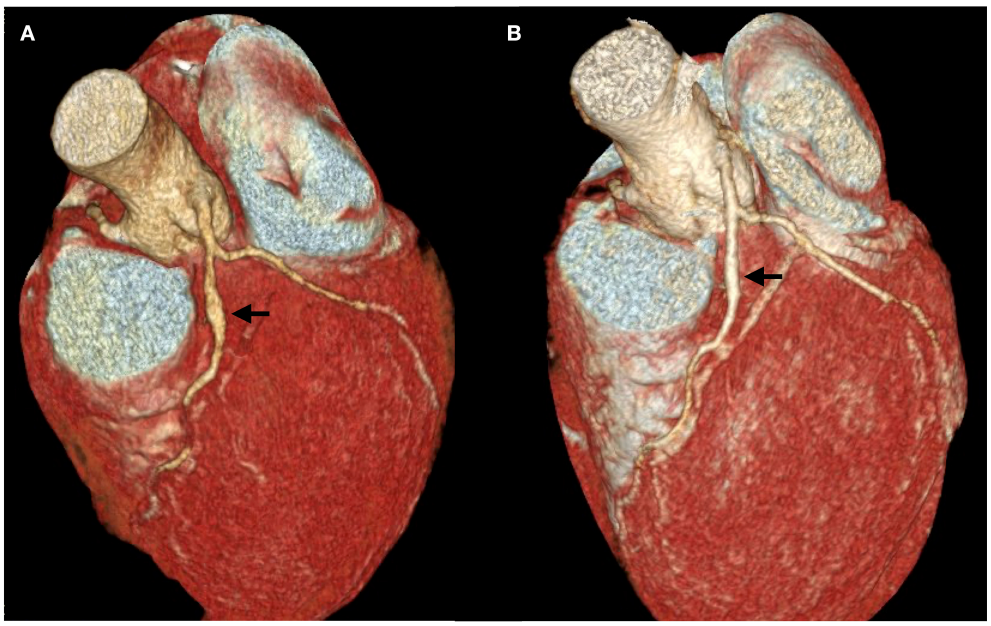

FIGURE 4 | Computed Tomography Coronary Angiography. **(A)** The first exam was performed on April 27, 13 days after the onset of the disease, 6 days after tocilizumab administration and 3 days after intravenous immunoglobulins. A 5 mm fusiform ectasia is present at the proximal part of the anterior interventricular coronary artery (arrow). The external diameter of the artery is 3 mm above and below the ectasia; dilation area 19,3 mm^2. **(B)** Second exam, performed on May 14, demonstrating a marked decrease in aneurism size. Four millimeter fusiform ectasia; dilation area 10,6 mm^2.

of viral infection in the absence of residual virus. The precise dates of contamination and of acute infection with SARS-Cov-2 remain difficult to determine with precision in our patient. The contamination could have occurred before March 13, the time of confinement. Alternatively, it is not excluded that our patient could have been contaminated by her mother during the confinement period but the negative serology performed in late April in the mother does not confirm this possibility. However, we cannot totally exclude that possibility since it appears that IgG antibodies against SARS-CoV-2 might disappear rapidly particularly in asymptomatic individuals (17). Finally, given the occurrence of an anosmia in our patient, even of a short duration, we considered the date of March 23 as that of a possible paucisymptomatic infection.

Such hypothesis in consistent with a 3 weeks delay between the infection and the onset of KD as reported in several other similar cases (6).

On the other hand, an ongoing infection may not be excluded given the rise in IgG during hospital admission and the lack of sensitivity of the PCR. Importantly, the pediatric literature has also reported that a minority of patients had positive RT-PCR nasal swabs while most were diagnosed positive for SARS-CoV-2 by serology. Hence, it is likely that SARS-CoV-2 infection triggers the massive cytokine storm which is responsible for the KD symptoms, ARDS and myocarditis. This immune response is likely to be delayed emphasizing the importance of performing SARS-CoV-2 serologic tests in these patients with diagnostic uncertainty.

We suggest that even in asymptomatic or paucisymptomatic individuals the SARS-CoV-2 infection hits the respiratory tract which became more sensitive to the immune consequences associated to KD. Old studies have suggested that the viral infection may play a role as a superantigen that drives an autoimmune response via clonal expansion of CD8 T cells (18). The theory of T cell activation by a superantigen that could be instrumental in KD was also suggested by Brogan et al. (19). More recently the discovery of the presence of IgA plasma cells together with an oligoclonal IgA response in arterial tissues from patients with KD suggest that the immune response is driven by entry of a pathogen at a mucosal site such as the respiratory tract (20). Ig A plasma cells infiltration was also identified in the proximal respiratory tract in acute KD (21). It is noteworthy that infection with SARS-CoV-2 is linked to the presence of the ACE2 and TMPRSS2 receptors in the same tissues (22).

Genetic susceptibility to abnormal immune responses to infectious agents play key roles in initiating KD. Th 17 expansion and Treg depletion could be the hallmarks of acute KD (23). Indeed, an imbalance between T helper 17 lymphocytes and regulatory T cells with very increased inflammatory cytokines in the acute phase of KD is suggested by some studies (24). Interestingly, the same kind of Th17 type response that contributes to the cytokine storm has been demonstrated to be involved in pulmonary viral infections including SARS-CoV-2 (25).

All these data are consistent with the hypothesis that KD is a consequence of an immune mediated endothelial cell damage likely triggered by an acute viral infection affecting the respiratory system. Otherwise, an association between another coronavirus (HCoV-NH) and KD has been previously reported

(26). Recent studies reported an increased incidence of KD associated to SARS-CoV-2 infection in children (2, 3). The hypothesis of a delayed onset of KD rather than an association with acute SARS-CoV-2 infection is suggested by the lack of detection of the virus in most affected children and the increased incidence of KD at time when the number of new COVID-19 cases decreases. However, in some other cases both diseases could be contemporary (3). In the present case, the occurrence of the inflammatory disorder 3 weeks after a possible pauci symptomatic SARS-CoV-2 infection (transient anosmia in the absence of other symptoms) favored the hypothesis of a delayed immune mechanisms induced by the viral infection as proposed by Belot et al. (6).

Tocilizumab, an anti-IL-6 receptor monoclonal antibody has been reported to be effective for patients with severe COVID-19 pneumonia and hyperinflammatory syndrome (27). It is also effective for the inflammatory syndrome associated to Kawasaki's disease in children (28). However, tocilizumab may have contributed to the formation of new-onset of coronary artery aneurysms in these children (28). As KD was not yet recognized at time of acumen of ARDS, myocardial dysfunction and distributive shock, we administered tocilizumab for its potential beneficial effects in this setting. We here noticed a very positive response to tocilizumab on ARDS and signs of cardiac involvement (troponin and cardiac function at echocardiography) which rapidly improved after tocilizumab administration. However, a cardiac aneurism was detected at CTCA 3 days later. While the development of cardiac aneurisms after tocilizumab therapy in KD is consistent with the previous observations of Nozawa et al. (28), it is important to notice that the onset of aneurism in the KD children treated with tocilizumab was quite late (several weeks), and it is thus possible that the aneurism pre-existed tocilizumab therapy in this patient. The regression of the coronary aneurysm after IVIG and methylprednisolone administration may suggest that these treatments are a mandatory part of the therapy when cardiac aneurisms are present, particularly for patients treated by tocilizumab. Apart from the administration of tocilizumab, the initiation of corticosteroid therapy was justified by the risk of non-response to IVIG. Indeed, the Kobayashi score had been evaluated at 6 points which constitutes a 50% risk of IVIG ineffectiveness in KD (29). The benefit of prednisolone administration is well-documented in these circumstances (30, 31).

It is interesting to notice that eosinophils, which have been associated with the development of more severe coronary vasculitis in KD (32) continued to increase despite tocilizumab even though troponin and CRP decreased. The eosinophilia detected in our patient with KD contrasts with the marked eosinopenia characteristic of patients with severe forms of COVID-19 (33, 34). Although KD is associated with a Th17 rather than a Th2 immune response, the pathogenic role of eosinophils in KD is underlined by their presence in the inflammatory infiltrates characterizing the coronary vasculitis of KD (20, 32). In addition, eosinophilia, sometimes marked, was present in all patients with KD developing coronary aneurysms (32). In the context of a COVID-19 epidemic, a blood eosinophilia could be a useful tool raising suspicion of KD.

Additionally, eosinophils may be an important indicator for requirement of additional glucocorticoid therapy in patients with multisystem inflammatory syndrome associated with SARS-CoV-2, especially when other markers of inflammation seem to be controlled.

There are several limitations to this report. First, KD was recognized relatively late in the course of the patient. It is important to acknowledge that the first report of KD or PIMS was published online on April 27, several days after the onset of the disease in this patient. This may have contributed to the late recognition of KD/PIMS in this patient. Also, several clinical features are common in KD and in acute SARS-CoV-2 disease: conjunctivitis, fever, rash, myocarditis. Accordingly, the initial diagnosis that was considered in this patient was SARS-CoV-2 ARDS and myocarditis, and KD was considered later in view of the continuous growth of eosinophils despite significant improvement of the other signs. Second, several drugs (hydroxychloroquine, azithromycin, colchicine) seem not indicated in KD. Again, these were indicated at time of consideration of SARS-CoV-2 ARDS, myocarditis and distributive shock. Colchicine was administered for myopericardial involvement. Of note colchicine has marked rheologic and anti-inflammatory properties, inhibits T-cell activation (35). It is a class IIa recommendation in pericarditis (36), and can even be used after acute myocardial infarction (37). Admittedly, its use in myocarditis has not been well-described, even though anti-inflammatory agents may have beneficial effects (38). Recently, Deftereos et al. (39) reported in a randomized study that colchicine improved the time to deterioration in patients hospitalized with SARS-CoV-2. Hydroxychloroquine and azithromycin were administered for SARS-CoV-2 ARDS. Ceftriaxone was administered in the context of ARDS and distributive shock, while waiting for the results of bacteriological sampling.

A final consideration regards the use of exogenous angiotensin 2 (Giapreza). After correction of the severe myocardial dysfunction with dobutamine, it became evident from the hemodynamic profile that the patient suffered from distributive shock. The choice of the vasopressor agent in the context of SARS-CoV-2 associated vasoplegia is still a matter of debate. Some theoretical considerations suggest that AT2 may have beneficial effects as exogenous AT2 administration is associated with internalization of AT2 receptors, a key receptor involved in the pathogenicity of SARS-CoV-2 (40). These theoretical considerations were supported by a case series by Zangrillo et al. (41) which demonstrated that AT2 administration was associated with a rapid improvement in gas exchanges and respiratory function.

CONCLUSION

Clinicians caring for adult patients must be aware that not only children but also young adults can be affected by a multisystem

inflammatory syndrome with KD features associated with COVID-19.

A careful clinical history is necessary to identify subtle symptoms (as loss of taste or smell) suggestive of SARS-CoV-2 infection in the preceding weeks as the symptoms of MIS associated with SARS-CoV-2 may mimic an acute onset of SARS-CoV-2.

As it appears to be a delayed immune reaction to SARS-CoV-2 infection, serology constitutes a mandatory diagnostic tool.

This case also suggests that co-administration of colchicine, tocilizumab, IVIG and corticosteroids had favorable effects on systemic inflammation and cardiac and pulmonary manifestations and may control the development of arterial coronary aneurysm.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s), and/or minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

EC and DD analyzed the data and wrote the paper. PF and GV take care of the patient. OC performed cardiac magnetic resonance imaging and CTCA. ND performed the serologic tests. All authors approved the final work.

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Global and Temporal COVID-19 Risk Evaluation

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The COVID-19 pandemic has caused unprecedented crisis across the world, with many countries struggling with the pandemic. In order to understand how each country is impacted by the virus and assess the risk on a global scale we present a regression based analysis using two pre-existing indexes, namely the Inform and Infectious Disease Vulnerability Index, in conjunction with the number of elderly living in the population. Further we introduce a temporal layer in our modeling by incorporating the stringency level employed by each country over a period of 6 time intervals. Our results show that the indexes and level of stringency are not ideally suited for explaining variation in COVID-19 risk, however the ratio of elderly in the population is a stand out indicator in terms of its predictive power for mortality risk. In conclusion, we discuss how such modeling approaches can assist public health policy.

Keywords: COVID-19, inform index, infectious disease vulnerability index, mortality risk evaluation, public health

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1. INTRODUCTION

At the end of 2019, a new respiratory tract infection emerged in Wuhan, China. Termed COVID-19, the virus has spread all over the globe, with the World Health Organization (WHO) designating it a pandemic. Highly contagious, the disease has severely impacted economies and elderly populations. Data scientists, epidemiologists and mathematicians are aiming to understand and project the spread of the virus or assess the risk in each country; specifically the risk of deaths is of deep and grave concern. It is widely established that a range of factors prevail, upon which the COVID-19 risk or vulnerability of an individual country depends (1, 2); extending from risk assessment of other viruses and pandemics (3). Therefore, amongst the large volume of work on the impact of COVID-19, a stream of research attempts to decipher the various baseline or constituent factors that could put a nation at risk to COVID-19 (4). These include the study of socio-demographic or economic factors as well as natural elements, such as climate or temperature (5). Typically, such factors are baseline in the sense that they cannot be altered or varied overnight. However, they can pre-empt contingency plans and action points for agencies and organizations enabling countries to be better prepared in response to the virus (6). A region or country at assumed high risk could take timely actions to prepare for and preempt the spread. Prior work has also in relation focused on how different countries compare on their level of risk (7). The wide variety of such indicators ultimately illustrates that selection of appropriate indicators requires clear justification. In prior work, we have also seen the usage of standardized risk indexes developed by large organizations. These indexes are an aggregate of many indicators and factors (8). Such indexes may also play an important role in the evaluation of COVID-19 risk. However, these indexes were in general derived before COVID-19 by considering other viruses and as such their charting of COVID-19 as a pandemic is not immediately clear or established. One may question if these indexes can be readily used to assess COVID-19 risk given that it is acknowledged that COVID-19 is

much more contagious than originally thought in comparison to other corona viruses (9). Further, computing the risk of COVID-19 is largely complicated (10)—leading to some efforts to deduce a customized index (11).

In parallel, we also have research focusing on the impact of factors which are more fluid and variable in nature and depend on government intervention and policy (12). These include social distancing measures, travel bans, lockdowns, economic shutdown and much more (13). These can in theory be implemented overnight and in conjunction with the baseline variables provide an effective response strategy. Timely interventions are hence what most government agencies aspire to. Evaluating their effectiveness should also be an integral component of risk modeling (14). Temporal evaluations at key checkpoints are essential to the successful implementation of a response plan keeping in mind non-pharmaceutical interventions, so that scalable strategies can be employed.

While some of the prior work aims to project the efficiency of future actions or predict the spread (15, 16), our research has attempted to evaluate the current or present situation in terms of identifying the mortality risk of COVID-19 on a *global* scale using a wide range of predictors or indicators. Most of the prior work attempts to assess the risk situation on a local scale (17). The aim of any intervention or introduction of a stringency level is to reduce the overall risk. Further, there should be regular and timely determinations to deduce if the interventions are contributing to a lowering of risk. Therefore, in this current paper, we present our analysis on modeling the current and temporal change in COVID-19 risk on a global scale through the incorporation and comparison of not only available and standardized risk indexes but also a temporal factor in terms of the prevailing stringency level. As mentioned prior, these risk indexes do not allow us to reassess the risk when the condition of a country changes (such as when government interventions or lockdowns are activated or softened). Nevertheless, a number of such pre-made indexes are available and it would be worthwhile to compare them in terms of their ability to explain the risk of COVID-19. Therefore, in our research, we compare two standardized indexes in terms of their efficacy to assess the risk and vulnerability of each country toward COVID-19 and also introduce an additional factor in terms of the stringency level of each country.We believe our analysis is a contribution to literature as previous temporal risk assessments for COVID-19 are for specific countries, such as China (18) or South Africa (19).

2. METHOD

Our aim was to discern the risk of COVID-19 on a global yet temporal scale.In order to compute the risk, we wanted to benchmark each country against its prevailing conditions and disease vulnerability as measured by various indicators and predictors.

2.1. Index Selection

In Gilbert et al. (8), a range of indexes and their applicability to assess COVID-19 risk are discussed. Two indicators namely the Inform global risk index (20) and the Infectious Disease index (21) were deemed relevant due to their ability to "account" for not only demographic, socio-economic, environmental and political factors but also transmission risk, infrastructure, vulnerability and coping capacity. Hence, we selected the indicators comprised as part of the two indexes as the primary factors of our risk assessment, given that they nicely complimented each other. Their constituent indicators are summarized in a bullet list below. We also recorded the overall value or composite score of the indexes themselves. Data related to these indexes was obtained from their available official documentation (20, 21); including both the constituent indicators and the composite index score. For the Inform global risk index the composite score was available in the official documentation as "Enhanced Inform 2019." For the Infectious Disease Vulnerability index, the composite score was available in the official documentation as "Overall Score Normed."

Each of the indicators were normalized on a range of 0–1. We did however notice, that there was no mention of the ratio of elderly population amongst the list of indicators. Proportion of children was included and this may have been due to the focus of the indexes on prior epidemics which were different in their risk demographic. Therefore, we also included an additional static indicator which illustrated the ratio of elderly in the population (above 65 years old), as provided from World Bank. This index was termed as "A65abp" in our data.

- Inform Index
  1. Natural
  2. Human
  3. Hazard and Exposure
  4. Social-Economics Vulnerability
  5. Vulnerable Groups
  6. Vulnerability
  7. Institutional
  8. Infrastructure
  9. Lack of Coping Capacity.
- Infectious Disease Vulnerability Index
  1. Demographic Domain
  2. Health Care Domain
  3. Public Health Domain
  4. Disease Dynamics
  5. Political Domestic
  6. Political International
  7. Economic Domain.

2.2. Stringency Level

Each country's response to the emerging threat of COVID-19 has been fluid, dynamic and unique. There is no one size fits all approach. Therefore, representation of the prevailing stringency is important to model in any risk assessment. With the application of stringent measures the risk of future spread should reduce. In order to capture a temporal assessment of variation of risk we utilized the stringency index proposed as the Oxford COVID-19 Government Response Tracker (OxCGRT) (22). This is defined as "a policy stringency index

(calculated) by combining 13 policy indicators, including school and workplace closures, travel bans, as well as fiscal policy measures." To allow for the measures to take effect, and to give increased importance for measures taken earlier rather than later, in our modeling we considered a weighted average stringency level for any day based on a formula provided in literature (4).

2.3. Modeling

Our model considered COVID-19 and stringency data between 22-01-20 and 11-05-20 on 2 weeks intervals, namely (02-03-20, 16-03-20, 30-03-20, 13-04-20, 27-04-20, and 11-05-20), such that the model was run for each date in this list for a temporal risk assessment across all countries data ($N = 156$). The data associated with the virus was extracted from the John Hopkins Repository (23). The number of confirmed cases and mortality were normalized per capita based on the population, per one million people and not on the basis of confirmed cases; due to the inaccuracies and irregularities in testing (24).

We ran eight multiple regression models for each of the six dates mentioned. We had two dependent variables: normalized confirmed cases and normalized mortality. Each dependent variable was modeled four times by using the two sets of indicator independent variables in both split and composite form. The four sets of independent variables were the indicators in the Inform Index (9 in total) and those in the Infectious Disease Vulnerability Index (7 in total) as well each of the indexes in their composite form (a single score each). There was no intermixing of indexes as independent variables across each other or in their split or composite form. All regression models further included A65abp (ratio of elderly in the population) and weighted stringency level on that particular date as additional independent variables. The list of 8 regression model types is summarized below.

For each date in our window of six identified dates

1. DV = normalized confirmed cases; lm (IV = 9 constituent factors of the Inform Index, A65abp, stringency level)
2. DV = normalized confirmed cases; lm (Enhanced Inform 2019, A65abp, stringency level)
3. DV = normalized mortality; lm (IV = 9 constituent factors of the Inform Index, A65abp, stringency level)
4. DV = normalized mortality; lm (Enhanced Inform 2019, A65abp, stringency level)
5. DV = normalized confirmed cases; lm (IV = 7 constituent factors of the Infectious Disease Vulnerability Index, A65abp, stringency level)
6. DV = normalized confirmed cases; lm (Overall Score Normed, A65abp, stringency level)
7. DV = normalized mortality; lm (IV = 7 constituent factors of the Infectious Disease Vulnerability Index, A65abp, stringency level)
8. DV = normalized mortality; lm (Overall Score Normed, A65abp, stringency level).

Afterwards, the regression predictors were then assessed for relative importance or assigning of weights using the relaimpo package (25). All modeling was carried out in R.

3. RESULTS

Initially, we tested for various assumptions of linear regression models. Using residual plots we checked for normality and only minor deviations from normality were observed. None of the predictor variables were considered to be dropped across the four types of regression models; however, we did check for multicollinearity using the measure variance inflation factor (VIF). We realized the importance of this step in particular when we considered the indexes in their split form, as it could be expected that the predictors may possibly be correlated with each other. VIF scores for all 9 predictor variables within the Inform Index were high and beyond tolerance (>5) for both confirmed cases and mortality. A65abp and the stringency level were within an acceptable range (<5). When we used the composite score of the Inform index alongside A65abp and the stringency level, there were no issues whatsoever with respect to multicollinearity. VIF scores for the predictors within the Infectious Disease Vulnerability Index were beyond tolerance for three of them (>5); namely economic domain, political domestic and health care. A65abp and the stringency level were within an acceptable range (<5). When we used the composite score of the Infectious Disease Vulnerability index alongside A65abp and the stringency level, there were no issues whatsoever with respect to multicollinearity. As with the Inform Index, VIF scores mirrored each other across mortality and confirmed cases for the Infectious Disease Vulnerability Index.

Summary of our multiple linear regression models are presented in the form of tables (see **Tables 1**–**4**). **Tables 1**, **2** tend to illustrate that the Inform index had slightly higher predictive power for the risk of COVID-19 confirmed cases. Particularly, **Table 1** shows that both A65abp and the composite value of the Inform Index have significant predicting power for the risk of COVID-19 confirmed cases. None of the predictor indicators within the Inform index were significant for either of mortality or confirmed cases. Their high multicollinearity also enforces us to focus on the results from the regression model of the composite Inform Index.

Tables 3, **4** highlight that the Infectious Disease Vulnerability Index has lower predictive power than the Inform Index for the risk of both confirmed cases and mortality for COVID-19. **Table 4** also highlights the weakness of the Infectious Disease Vulnerability Index to explain COVID-19 mortality risk as none of its constituent predictors or the index itself in its composite form emerged as significant. Extending from our checks of multicollinearity, we re-executed our linear regression model using the split form of the Infectious Disease Vulnerability Index; but dropped the three predictors having high VIF mentioned earlier. In these new models (six each for normalized cases and normalized mortality; six representing the six dates chosen in our temporal analysis), there were no drastic changes in R^2, if anything it further deteriorated. Our R code and all output generated is presented in a documented **Supplementary File**.

3.1. Discussion

In general the response variable variation or R^2 was low for both indexes and for both response variables, where most of the

TABLE 1 | Regression Results for risk of confirmed cases as predicted by Inform Index in both split and composite form; where for *p*-values "****" represents $p < 0.001$ "***" represents $p < 0.01$ and "*" represents $p < 0.05$.

	Split form			Composite form		
Date (all dates are 2020)	R^2	**Significant *p*-values**	**Top weights**	R^2	**Significant *p*-values**	**Top weights**
March 2	0.17	Stringency***	Stringency (0.64)	0.16	Stringency***	Stringency (0.84)
March 16	0.30	None	Social economics vulnerability (0.14) Lack of coping capacity (0.13)	0.21	Inform index*, A65abp*	Inform index (0.48), A65abp (0.44)
March 30	0.33	None	Institutional (0.17) Lack of coping capacity (0.15)	0.23	Inform index*, A65abp**	Inform index (0.46), A65abp (0.54)
April 13	0.40	None	Institutional (0.16) Lack of coping capacity (0.16)	0.28	Inform index**, A65abp***	Inform index (0.46), A65abp (0.54)
April 27	0.43	None	Institutional (0.16) Lack of coping capacity (0.17)	0.30	Inform index***, A65abp*	Inform index (0.56), A65abp (0.43)
May 11	0.40	None	Infrastructure (0.13) Lack of coping capacity (0.17)	0.27	Inform index***	Inform index (0.69), A65abp (0.29)

TABLE 2 | Regression Results for risk of mortality as predicted by Inform Index in both split and composite form; where for *p*-values "****" represents $p < 0.001$ "***" represents $p < 0.01$ and "*" represents $p < 0.05$.

	Split form			Composite form		
Date (all dates are 2020)	R^2	**Significant *p*-values**	**Top weights**	R^2	**Significant *p*-values**	**Top weights**
March 2	0.18	Stringency***	Stringency (0.63), natural (0.15)	0.15	Stringency***	Stringency (0.89)
March 16	0.07	None	A65abp (0.42)	0.06	A65abp**	Inform index (0.12), A65abp (0.87)
March 30	0.16	A65abp*	A65abp (0.39), lack of coping capacity (0.12)	0.14	A65abp***	Inform index (0.17), A65abp (0.82)
April 13	0.28	A65abp**	A65abp (0.30), lack of coping capacity (0.12), institutional (0.13)	0.22	A65abp***	Inform index (0.20), A65abp (0.82)
April 27	0.31	A65abp**	A65abp (0.27), lack of coping capacity (0.14), institutional (0.14)	0.24	A65abp***	Inform index (0.21), A65abp (0.78)
May 11	0.32	A65abp**	A65abp (0.26), lack of coping capacity (0.15), institutional (0.14)	0.24	A65abp***	Inform index (0.21), A65abp (0.77)

indicators were not integral to the predictive power of the model. The trend of both indexes was similar, as slight improvements in predictive power of the model occurred over time. The inform index did not have any significant constituent indicators within its ranks.

Indicators from within the Infectious Disease Vulnerability Index, such as public health domain were at different times able to explain the variation in risk of COVID-19 confirmed cases across the countries as shown by its significant predictive power. The inform index in its composite form was a stronger predictor for the risk of COVID-19 confirmed cases, whereas the Infectious Disease Vulnerability Index was a significant predictor of the risk of COVID-19 confirmed cases on May-11. This clearly highlights that these indexes if anything, the indexes were slightly more effective in explaining the variation in confirmed cases on a global scale as compared to mortality. The seemingly overall weak results highlight the need of a customized and tailored composite index for risk assessment related to COVID-19, particularly for mortality. Prior literature also identifies the importance of considering COVID-19 as very different from infectious diseases of the past [such as SARS (26)]. Our identified indexes in this paper were conceptualized in the pre-COVID era and hence are finding it difficult to map and predict the risk of COVID-19. Nevertheless, the ratio of elderly (A65abp) as a self-introduced indicator was an important predictor for mortality risk as evidenced by its weight and significance across the models. The mortality risk associated with COVID-19 and the elderly is widely recognized (27) and this association also emerged in our results.

TABLE 3 | Regression Results for risk of confirmed cases as predicted by Infectious Disease Vulnerability Index in both split and composite form; where for *p*-values "***" represents $p < 0.001$ "**" represents $p < 0.01$ and "*" represents $p < 0.05$.

	Split form			Composite form		
Date (all dates are 2020)	R^2	**Significant *p*-values**	**Top weights**	R^2	**Significant *p*-values**	**Top weights**
March 2	0.09	Stringency*	Stringency (0.38), health care domain (0.16), A65abp (0.18)	0.07	Stringency*	Stringency (0.52), A65abp (0.37)
March 16	0.09	None	Health care domain (0.19), A65abp (0.27)	0.07	A65abp*	Infectious Disease Vulnerability Index (0.19), A65abp (0.66)
March 30	0.24	Public health domain**	Public health domain (0.18), A65abp (0.18), political domestic (0.17)	0.16	A65abp**	Infectious Disease Vulnerability Index (0.30), A65abp (0.66)
April 13	0.29	Public health domain**	Health care domain (0.17), A65abp (0.17), political domestic (0.18)	0.19	A65abp***	Infectious Disease Vulnerability Index (0.36), A65abp (0.63)
April 27	0.29	Public health domain**, health care domain*	Health care domain (0.18), economic domain (0.22), political domestic (0.16)	0.18	A65abp**	Infectious Disease Vulnerability Index (0.43), A65abp (0.56)
May 11	0.30	Public health domain**, health care domain*, economic domain**	Health care domain (0.20), economic domain (0.3)	0.17	Infectious Disease Vulnerability Index*	Infectious Disease Vulnerability Index (0.55), A65abp (0.45)

TABLE 4 | Regression Results for risk of mortality as predicted by Infectious Disease Vulnerability Index in both split and composite form; where for *p*-values "***" represents $p < 0.001$ "**" represents $p < 0.01$ and "*" represents $p < 0.05$.

	Split form			Composite form		
Date (all dates are 2020)	R^2	**Significant *p*-values**	**Top weights**	R^2	**Significant *p*-values**	**Top weights**
March 2	0.22	Stringency***, political domestic domain**	Stringency (0.56), political domestic domain (0.15)	0.14	Stringency***	Stringency (0.93)
March 16	0.06	None	Health care domain (0.19), A65abp (0.36)	0.05	A65abp*	Infectious Disease Vulnerability Index (0.18), A65abp (0.71)
March 30	0.10	A65abp*	Health care domain (0.16), A65abp (0.39)	0.08	A65abp**	Infectious Disease Vulnerability Index (0.18), A65abp (0.80)
April 13	0.19	A65abp*	Health care domain (0.14), A65abp (0.36), political international (0.14)	0.15	A65abp***	Infectious Disease Vulnerability Index (0.22), A65abp (0.77)
April 27	0.23	A65abp*	Political international (0.15), political domestic (0.14), A65abp (0.34)	0.19	A65abp***	Infectious Disease Vulnerability Index (0.26), A65abp (0.74)
May 11	0.25	A65abp**	Political international (0.16), political domestic (0.15), A65abp (0.33)	0.21	A65abp***	Infectious Disease Vulnerability Index (0.28), A65abp (0.72)

A key incremental novelty of our modeling approach to assess COVID-19 risk was the introduction of a temporal independent variable in the form of the stringency level of each country. Stringency appeared to have predictive power till early March, which is when there was most variation in stringency data. Once the World Health Organization declared COVID-19 as a pandemic around mid March (28), most countries scaled up their stringency levels and it lost any association that it had in modeling the mortality risk of COVID-19. This is exemplified by the standard deviation of weighted stringency level for the entire data set, which went from 11.3 on 02-03-2020 to 12.4 on 16-03-2020. This could also explain the drop or no change in R^2 for our date of 16-03-2020 (for 6 of the 8 scenarios). Prior research has shown stronger outcomes in temporal assessments of COVID-19 toward the start of the outbreak (29). Related research (30) further informs us that controlling the spread and risk of the COVID-19 virus relies more on personal and individual measures, such as social distancing rather than only emphasizing large scale governmental interventions.

4. CONCLUSION

In our analysis, we have attempted to use existing indexes to assess prevailing COVID-19 spread and mortality risk. Our results show that due to the inherent differences primarily related to transmission between COVID-19 and other pandemics of the past, future effort is to be dedicated to design customized indexes once the impact of COVID-19 is understood. Further, the level of stringency that a country had imposed was unable to explain the variation across countries when it came to COVID-19 risk. We discuss how this may have been a by-product of COVID-19 being declared a pandemic around mid March 2020 when most countries increased their stringency levels significantly. Our analysis also confirmed the significant association between the ratio of elderly (or above 65 years old) living in a population and COVID-19 mortality risk as well as between the local prevailing demographics and risk of COVID-19 spread. In addition, future analysis of the like can also focus on regional assessments of risk rather than global determinations which shrink countries to single homogeneous index based indicators. Such analysis can contribute toward a better understanding of public health policy on a regional level, where there are more subtle nuances in the available data. As our results have shown global indexes although meant to discern countries at a world level; the complexities of COVID-19 ultimately create challenges in mapping and projecting its outlook on a global scale.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analysed in this study. This data can be found here: https://data.worldbank.org/; https://covidtrackerbsg.ox.ac.uk/; https://drmkc.jrc.ec.europa.eu/inform-index; https://www.rand.org/content/dam/rand/pubs/research_reports/RR1600/RR1605/RAND_RR1605.pdf.

AUTHOR CONTRIBUTIONS

MA conducted the analysis in R. MA and OM were involved in the initial drafting, conceptualization, identification of indexes, and design of the regression modeling. OM drafted and wrote the paper. In sum, MA and OM have contributed equally to the final product. All authors conceived the study, brainstorming discussions, ideas listing and contributed to the drafting of the final document as well as subsequent revisions during the review process.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://cloudstor.aarnet.edu.au/plus/s/PfujZPSMZOi4nPQ.

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Knowledge, Attitude, and Practice of the Lebanese Community Toward COVID-19

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Objectives: Distinct measures were adopted in Lebanon to prohibit the spreading of SARS-CoV-2. These actions provide positive results only if the population chooses to be compliant.

Aim: Accordingly, this study aimed to reflect the Lebanese population adherence by determining their level of knowledge and practice during this pandemic.

Method: A cross-sectional online survey was performed in April 2020. It involved 410 volunteers from the main 5 Lebanese districts. The questionnaire was divided into 3 parts; sociodemographic, knowledge, and practice. A score was calculated out of 18 points to evaluate the knowledge of the respondents. The last 8 questions reflected the participants' precautionary methods during the pandemic. Descriptive statistics and one-way analysis of variance tests were conducted using SPSS version 20.

Results: The overall correct rate of the knowledge questionnaire was 75%. Survey completers of extreme age groups (under 18 and >44), elementary education level, and medical occupation displayed the least level of knowledge compared to other groups ($p < 0.05$). Most of the participants showed proactive practices to protect themselves against COVID-19. They covered their mouths (81.2%), threw the used tissues (93.7%), and washed their hands (66.6%) after sneezing or coughing. Moreover, they wore face masks if they were sick (59%) or in a crowded place (79.3%). Concerning Hydroxychloroquine, 10% claimed that they would take it if they have COVID-19 symptoms.

Conclusion: This survey sheds the light on the fact that one mandatory measure does not fit all the population; there must be a specialized method of prevention for each profession, age group, and area of the country to prevent the outbreak of COVID-19.

Keywords: COVID-19, knowledge, attitude, pandemic, Lebanese

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INTRODUCTION

The Human coronavirus, which derives from the family *Coronaviradae,* includes a group of positive sensed, single-stranded RNA. These RNA viruses have the largest number of genomes ranging from 26 to 32 kilo-bases in comparison to single-stranded viruses (1). Accordingly, coronavirus has evolved by gene recombination and mutation to be subdivided into 4 categories; alpha, beta, gamma and delta (1, 2). Moreover, this virus has shown mutations with higher antigenicity, or infection potential, as a consequence of the host's development of humoral and cell-mediated

immunity (3). It is one of the major pathogens that initially targets the human respiratory system (4). In fact, in December 2019, 40 cases of pneumonia were reported in Wuhan, China which etiology was soon established to be the new virus called novel coronavirus (3, 5). The severe acute respiratory syndrome coronavirus (SARS-CoV), which was the causative agent of an outbreak in 2003, and SARS-CoV-2 (the novel coronavirus), are highly similar because they have high nucleotide homology of around 77%. SARS-CoV-2 is less pathogenic, but more transmissible compared to SARS-CoV (1). SARS-CoV-2 is transmitted from one person to another by direct contact or droplets from sneezing or coughing (3).

Until the 22nd of April 2020, this outbreak resulted in 2,503,412 cases around the world whereby 171,809 lost the battle and passed away (6). The first coronavirus case in Lebanon was identified on February 21st. Eight days later, schools were locked down followed by bars, restaurants, and finally the airport. By March 15, the entire country had been on lockdown by the government due to increased case numbers (7). Even though experts at this point assumed high numbers of infected persons and mortalities, until the 25th of April, 696 cases were tested positive for coronavirus disease of 2019 (COVID-19), and 21 patients passed away (6).

As a consequence of the actions taken to prevent the spread of this outbreak, an economic crisis arose. More than 100 countries declared flight restrictions and boarder closure, causing a major drop in the number of flights per day from 150,000 to 200,000 to <100,000. The Chinese industrial production fell by 13.5% in the first 2 months of the year. According to an article published by BBC on the impact of this outbreak, 6.6 million claimed to be unemployed in the USA during April 2020 (8).

Even though everyone is at risk of developing COVID-19, yet some conditions make the patient more susceptible to the disease. Being above 65, living in a nursing home, having lung diseases, cardiovascular conditions, diabetes, liver or kidney disease, or being immune-compromised not only increases the chance of acquiring the novel coronavirus but also the complications (9).

The World Health Organization (WHO) and the Center for Disease Control and Prevention (CDC) declared that the most common signs and symptoms of this virus are fever, dry cough, and shortness of breath (9, 10). Two to 14 days and 2–10 days are the incubation periods of coronavirus which are identified by the CDC and WHO, respectively. Nevertheless, two cases were reported after being incubated for 19 and 27 days, respectively (6). WHO reported in a press conference on February 10, that a long incubation period could reflect a double exposure (6).

Up till now the golden standard of COVID-19 diagnosis is nucleic acid detection, which is obtained from throat or nose swab sampling by real-time reverse transcriptase polymerase chain reaction (11). Rapid diagnostic test (RDT) is a small and portable qualitative test that detects the presence of coronavirus antibodies; immunoglobulin G and M. The results need between 10 and 30 min to be obtained. This test was approved by food and drug administration (FDA), after the announcement of an emergency use of authorization (EUA) as a diagnostic tool (12). The WHO states that the results of this diagnostic tool can be influenced by many factors which include the time from onset of illness, the concentration of the virus in the specimen, the quality of the specimen collected, and the formulation of the reagents in the test kits. Therefore, the sensitivity of this test may vary between 34 and 80%. Moreover, this serology test doesn't indicate if the patient has an active infection. It rather indicates the presence of antibodies (13).

Unfortunately, up till today, there is no specific treatment or vaccine against the novel coronavirus. Proper symptomatic treatment along with oxygen supplementation are the major intervention for patients with severe symptoms. IDSA recommends against the use of corticosteroids in patients having pneumonia due to coronavirus. However, it supports its use when there are acute respiratory symptoms (ARDS) (14). There are several potential drug candidates including nucleoside analogs lopinavir/ritonavir, neuraminidase inhibitors, remdesivir, umifenovir, DNA synthesis inhibitors (tenofovir), tocilizumab, anti-malarial chloroquine, dexamethasone, and Chinese traditional medicine (15).

Chloroquine and Remdesivir were highly effective in the control of this virus according to a study by Lai et al. (16). A study done in France on 30 male patients showed that the antimalarial drug (hydroxychloroquine) combined with azithromycin reduced the SARS-CoV-2 load (17). The precise knowledge of hydroxychloroquine's side effects helps prevent some irreversible damages. In fact, hydroxychloroquine is not only responsible for minor side effects such as anorexia, diarrhea, and nausea, but it may also cause severe ones. Hyperpigmentation and photodynamic reaction may develop. Retinopathy will be the result of the accumulative deposition of hydroxychloroquine in the cornea (18). Concerning the heart, chloroquine, hydroxychloroquine, and azithromycin prolong the QT interval leading to a risk of arrhythmic death, especially when concomitant used (19). Consequently, the Lebanese ministry of Public health banned all pharmacies from dispensing hydroxychloroquine or chloroquine without a prescription from a specialist. Moreover, the pharmacist has to keep this prescription for tracking purposes (20). Concerning Remdesivir, an antiviral drug previously tested against Ebola virus, it did shorten the hospital stay but did not affect the mortality rate (21).

According to the RECOVERY trial, launched in March in the United Kingdom, dexamethasone decreased mortality by 20% in severe cases of COVID-19 on oxygen or ventilation. Nevertheless, dexamethasone did not affect mild infections (22).

More than 90 vaccines are being developed across the world. They work by blocking or killing the virus through exposing the body to an antigen. They rely on the virus itself, the viral vector, the nucleic acid, or the protein subunits. Other vaccines being tested are the existing ones against poliovirus or tuberculosis. Out of these vaccines, around 8 reached the safety trials while others are still being tested on animals (23).

The WHO and other health organizations declared some preventive measures. Avoid close contact, frequent handwashing with soap, and water, always carrying an alcohol-based hand sanitizer, and application of strict hygiene measures in emergency departments and hospitals. All tissues used to cover a sneeze or cough should be tossed away immediately. Finally, health care providers should utilize contact and airborne

precautions. They should wear face masks, gloves, gowns, and eye protection (15).

The lack of proper awareness caused the death of 27 people in Iran not because of the virus itself but after drinking industrial alcohol believing that it is a preventive measure (24). A man and his wife died, under critical care in Arizona, after taking chloroquine in an attempt to self-medicate against SARS-CoV-2 (24). In Lebanon, the ministry of public health, in collaboration with the WHO, has issued several awareness campaigns on social media to prevent the spreading of the disease and prevent inadequate measures. Nevertheless, no study reflected the awareness of the population toward COVID-19 and its prevention. Consequently, this study was designed to evaluate the knowledge and attitude of the COVID-19 among Lebanese residents.

MATERIALS AND METHODS

Study Design and Population

A cross-sectional anonymous survey was designed in April, 2020 targeting people living all over Lebanon. Due to quarantine, this study was conducted via a link shared on social networking platforms to limit the spreading of the disease.

Study Tool

The survey questionnaire was designed in English and then translated to Arabic, the native language in Lebanon. Both surveys were available and the participants had the freedom to choose between the two versions.

Pilot Study

A preliminary phase was conducted to assess the validity and reliability of the questionnaire before its use. Two experts were asked to review the questionnaire in order to make sure that it reflects the knowledge and attitude of the Lebanese population on COVID-19. Accordingly, the questionnaire was modified to meet the aim required. To check for clarity of the questionnaire, a pilot study was conducted which included 10 participants that took the survey in either language. Further modifications were done after feedback retrieval from the participants.

Data Collection

An online open-access google form survey was created and participants from all areas of Lebanon were invited via social networking platforms to participate. Beirut, North, South, Bekaa, and Mount Lebanon were the 5 focal points. The survey link was sent to different socioeconomic levels, via WhatsApp, who were asked to spread it to their relatives and friends to overcome some limitations of the online data gathering.

Sampling

The sample size was calculated using the online sample size "Raosoft®" calculator, assuming the Lebanese population to account for 6.825 million. The results showed that a total of 384 participants and above provides a representative sample with a 5% margin error and a 95% confidence level. The spreading of the survey link started on the 22nd of April, and this link was closed on Saturday April 25th when the number of participants exceeded the calculated representative number.

Questionnaire

The online survey was divided into four parts that included 24 mandatory questions. The first one requiring the sociodemographic information of the participant. The second one, having 6 knowledge questions requiring multiple answers. Each right answer was given one point, and each wrong or uncertain answer was given a zero. A score out of 18 was made. The third and last part included 8 questions reflecting the attitude of the respondents.

Statistical Analysis

The results were analyzed using Statistical Package for the Social Science (SPSS®) software version 20 (IBM, New York-USA). Categorical data were expressed as frequencies (percentages) while continuous data as means ± standard deviation (SD). The ANOVA test was used to compare means (after ensuring normality and variance homogeneity). All results were considered "statistically significant" when the *P*-value was <0.05 with a confidence interval (CI) of 95%.

Ethical Consideration

The study was an observational one that respects the participant's confidentiality and autonomy. The participant had the choice to defer from submitting the filled form. This survey also didn't require neither names nor emails, and thus there were no traceability of the participant. Accordingly, Beirut Arab University Institution Review Board waived the approval for this study.

RESULTS

A total of 410 participants were included in the study. From the total participants, the age group between 25 and 44 years, female, Lebanese, and single accounted for 53.2, 58, 95.9, and 62%, respectively. Fifty-eight point five percent of the respondents live in Beirut, the capital of Lebanon. Around 80% hold a Bachelor's degree, and 32.2% work in the medical field. Concerning the participants' income per month, 31.5% reported to acquire <750,000 Lebanese pounds per month. As for the past medical history, 57.6% were in good health while 7.6%, and 4.6% had hypertension and lung diseases, respectively. Thirty-four point one percent of the participants were smokers (**Table 1**).

Most of the participants had a good knowledge of COVID-19 with a mean score of 13.51 ± 2.56 over 18. Risk factors for acquiring the novel coronavirus, as reported by the participants, were older age, cardiovascular disease, respiratory disease, diabetes, cancer, and smoking with percentages of 75.6, 73.7, 66.1, 50.5, 64.1, and 48.3%, respectively. SARS-CoV-2 was identified as a virus by 96.8% of the participants. Two to 14 days was the incubation period as acknowledged by 89% of the respondent. COVID-19 was recognized as a contagious condition by 98.1% of the participants. According to the route of transmission, 98.6 and 92.4% were positive that it can be passed via droplets and from contacting infected surfaces, respectively. On the other hand,

TABLE 1 | Demographic data of the participants.

Demographics	% (frequency)
Age	
Under 18	10% (41)
18–24	25.9% (106)
25–44	53.2% (218)
45–54	8.3% (34)
55–64	1% (4)
>65	1.6% (7)
Gender	
Male	42% (172)
Female	58% (238)
Nationality	
Lebanese	95.9% (393)
Non-Lebanese	4.1% (7)
Marital Status	
Single	62% (254)
Married	36.1% (148)
Divorced	1.2% (5)
Widowed	0.7% (3)
Region	
Beirut	58.5% (240)
Mount Lebanon	29.3% (120)
South	6.1% (25)
North	5.4% (22)
Bekaa	0.7% (3)
Highest level of education	
None	0.2% (1)
Elementary	1.2% (5)
Secondary	15.1% (62)
University	79.8% (327)
Others	3.7% (15)
Occupation	
Medical	32.2% (132)
Non-medical	48.4% (182)
Unemployed	23.4% (96)
Income/month L.L.	
<750,000	31.5% (129)
750,000–1,500,000	18.5% (76)
1,500,000–2,250,000	18.8% (77)
2,250,000–3,000,000	11.2% (46)
3,000,000–3,750,000	5.1% (21)
>3,750,000	14.9% (61)
Comorbidities	
Hypertension	7.6% (31)
Diabetes	2.4% (10)
Lung disease	4.6% (18)
Others	30% (123)
None	57.6% (236)
Smoking status	
Cigarette	13.4% (55)
Hubble-bubble	17.5% (72)
Other	3.2% (13)
None	65.9% (270)

TABLE 2 | Knowledge on COVID-19.

Statement or question	Percentage (frequency)
PART 1	
What are the risk factors for acquiring COVID-19?	
Being an elderly patient	75.6% (310)
Being a pediatric	15.4% (63)
Having cardiovascular disease (high blood pressure, high cholesterol)	73.7% (302)
Having a respiratory disease (Asthma, COPD...)	66.1% (271)
Having diabetes	50.5% (207)
Having cancer	64.1% (263)
Being a smoker	48.3% (198)
None	1.2% (5)
I don't know	0.7% (3)
Others	0.2% (1)
COVID-19 is caused by:	
Virus	96.8% (397)
Bacteria	2.4% (10)
Others	0.7% (3)
Incubation period of COVID-19 is: %(n)	
2–14 days	89% (365)
Up to 1 month	2.4% (10)
Up to 3 months	1.0% (4)
I don't know	2.4% (10)
Is COVID-19 a contagious disease?	
Agree/strongly agree	98.1% (402)
Uncertain	1.5% (6)
Disagree/strongly disagree	0.5% (2)
How is covid-19 transmitted?	
Through air	
Agree/strongly agree	29% (119)
Uncertain	40% (164)
Disagree/strongly disagree	31% (127)
Through droplets transmission from sneezing or coughing	
Agree/strongly agree	98.6% (404)
Uncertain	0.7% (3)
Disagree/strongly disagree	0.7% (3)
Through contacting infected surfaces	
Agree/strongly agree	92.4% (379)
Uncertain	5.6% (23)
Disagree/strongly disagree	2% (8)
Through sexual intercourse	
Agree/strongly agree	29.5% (121)
Uncertain	34.6% (142)
Disagree/strongly agree	35.9% (147)
PART 2	
The most common sign and symptoms of COVID-19 include:	
Fever	
Agree/strongly agree	97.6% (400)
Uncertain	1.7% (7)
Disagree/strongly agree	0.7% (3)

(Continued)

TABLE 2 | Continued

Statement or question	Percentage (frequency)
Fatigue	
Agree/strongly agree	91.5% (375)
Uncertain	7.3% (30)
Disagree/strongly agree	1.2% (5)
Shortness of breath	
Agree/strongly agree	97.8% (401)
Uncertain	1.5% (6)
Disagree/strongly agree	0.7% (3)
Nose bleed	
Agree/strongly agree	21.2% (87)
Uncertain	40.2% (165)
Disagree/strongly agree	38.6% (158)
Rash	
Agree/strongly agree	10.5% (43)
Uncertain	37.3% (153)
Disagree/strongly agree	52.2 (214)

35.9% disagree that it is transmitted through sexual intercourse (**Table 2**—Part 1). Fever (97.6%), fatigue (91.5%), and shortness of breath (97.8%) were the highly acknowledged symptoms of COVID-19 (**Table 2**—Part 2). The participants who were under 18 years old had a mean knowledge score significantly lower than ages between 18 and 44 years. Moreover, Beirut citizens had a significantly lower score than Mount Lebanon residents but similar score with the North, Bekaa, and South areas. Concerning the level of education, the participants who only reached elementary level scored significantly the least (9.5 ± 0.22) ($p < 0.05$). Controversially, medical field workers had a significantly lower score than non-medical field workers and unemployed. On the other hand, other demographic data did not influence the knowledge of the participants ($p > 0.05$; **Table 3**).

Furthermore, regarding precautions taking toward COVID-19 spreading, the overall attitude was acceptable. After sneezing or coughing, 81.2% of the respondents cover their mouths, 93.7% throw the used tissue, 93.9% turn their faces from other people, and 66.6% wash their hands. Face mask was worn by 59 and 79.3% of the participants, in case they are sick or if they are in a crowded place, respectively. Finally, 75.6% replace their face mask after a single use (**Table 4**). Moreover, as a measure to boost their immunity, the participants increased their fruit and vegetable (75.6%), and vitamin C (59.5%) intakes. More than half of the participants (56.1%) started conducting light exercises. Other measures taken were avoiding take away food (47.6%), consuming ginger (22.9%), Echinacea (3.2%), and zinc (15.9%) as supplements (**Figure 1**).

Referring to studies on hydroxychloroquine, only 10% agreed to take hydroxychloroquine if signs and symptoms of COVID-19 were present. The most serious side effects of the anti-malarial drug recognized by the participants were electrical disturbances of the heart (38%) and eye damage (18.8%). On the other hand, 50% did not recognize any side effect of hydroxychloroquine (**Tables 5**, **6**).

For medical inquiry, the survey participants prefer to seek the advice of a health care provider (60%) or the Ministry of Public Health hotline (50%). Nevertheless, 20% seek medical information from social media platforms (**Figure 2**). In the presence of COVID-19 symptoms, 52.7% of the participants

TABLE 3 | Demographic factors associated with the knowledge.

Demographic data	Mean knowledge score ± SD/18	*P*-value
Age		
Under 18	12.43 ± 2.72	–
18–24	13.84 ± 2.39	0.017
24–44	13.57 ± 2.64	0.048
45–54	13.91 ± 1.96	0.084
55–64	13.60 ± 2.07	0.923
>65	12.50 ± 2.65	1
Gender		
Female	13.76 ± 2.53	0.164
Male	13.16 ± 2.56	0.195
Nationality		
Lebanese	13.51 ± 2.55	0.129
Non-Lebanese	13.59 ± 2.83	0.687
Marital status		
Single	13.42 ± 2.64	–
Married	13.69 ± 2.43	0.736
Divorced	13.20 ± 1.64	0.997
Widow	12.33 ± 3.21	0.883
Region		
Beirut	13.13 ± 2.49	–
Mount Lebanon	14.03 ± 2.65	0.014
South	14.04 ± 2.30	0.426
North	14.32 ± 2.46	0.217
Bekaa	13.33 ± 2.52	1.000
Education		
Elementary	9.50 ± 0.22	–
Secondary	12.19 ± 0.29	0.051
University	13.79 ± 0.14	0.000
Others	14.47 ± 0.48	0.000
Occupation		
Medical	12.65 ± 2.88	–
Non-medical	13.73 ± 2.37	0.000
None	14.30 ± 2.05	0.000
Income		
<750,000	13.14 ± 2.73	–
750,000–1500,000	13.26 ± 2.52	0.999
1,500,000–2,250,000	13.95 ± 2.60	0.239
2,250,000–3,000,000	13.96 ± 2.53	0.425
3,000,000–3,750,000	13.71 ± 2.35	0.931
>3,750,000	13.66 ± 2.19	0.783

The mean score was calculated from all knowledge questions.
ANOVA test was done followed by tukey post-hoc test.
P < 0.05 was considered statistically significant.

TABLE 4 | Attitude toward COVID-19.

	Always % (*n*)	Sometimes % (*n*)	Never % (*n*)
After sneezing/coughing do you			
Cover your mouth	81.2% (333)	17.1% (70)	1.7% (7)
Throw the used tissue	93.7% (384)	4.9% (20)	1.5% (6)
Turn your face	93.9% (385)	3.9% (16)	2.2% (9)
Wash your hands	66.6% (273)	30.5% (125)	2.9% (12)
Do you wear a face mask if you are			
Sick	59% (242)	27.3(112)	13.7% (56)
In a crowded place	79.3% (325)	14.6% (60)	6.1% (25)
Do you replace the face mask after			
Single use	75.6% (310)	19% (78)	5.4% (22)

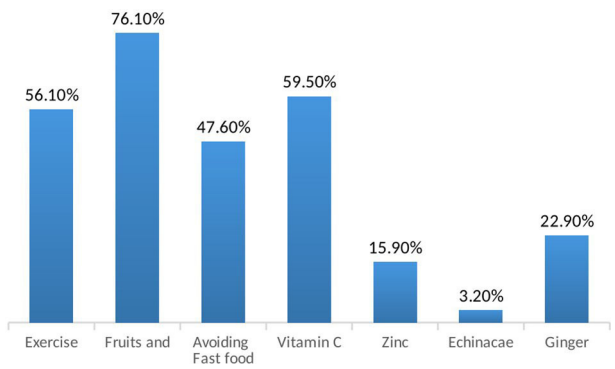

FIGURE 1 | Preventive Non-pharmacological measures followed against COVID-19.

TABLE 5 | Attitude of the participants toward Hydroxychloroquine use in COVID-19 infection.

In case of having COVID-19 symptoms, would you take Hydroxychloroquine if it was available at home?	% (frequency)
Yes	10% (41)
No	70.5% (289)
Maybe	19.5% (80)

TABLE 6 | Knowledge of the participants on the side effects of Hydroxychloroquine.

Most serious side effects of Hydroxychloroquine	% (frequency)
Eye damage	18.8% (77)
Electric Disturbances of the heart	38% (156)
Headache	11.7% (48)
Nausea	12.7% (52)
Abdominal pain	10.7% (44)
I don't know	50% (205)
Others	1.2% (5)

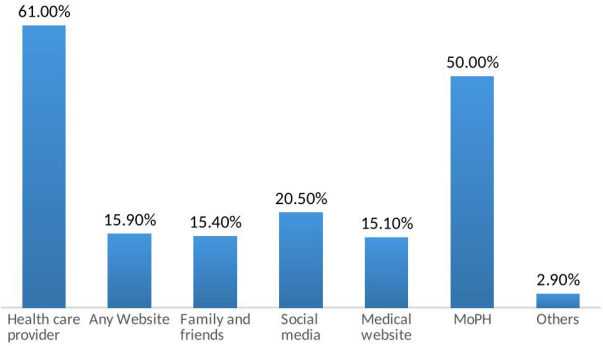

FIGURE 2 | Medical resources for inquiries on COVID-19.

TABLE 7 | Attitude of the participants in the presence of COVID-19 symptoms.

In case I have COVID-19 symptoms I would	% (frequency)
Seek emergency at any hospital	25.6% (105)
Call the Lebanese Red Cross to be taken to COVID-19 assigned hospitals	52.7% (216)
Seek health care providers (pharmacist, physician...)	10.5% (43)
Don't refer to any and self-medicate	11.2% (46)

consider calling the Lebanese Red Cross to be transferred to the assigned hospitals, 25% visit any hospital, 10.5% seek help from their health care provider (physician, pharmacist...), and 11.2% self-medicate themselves without seeking medical advice (**Table 7**).

DISCUSSION

COVID-19 represents a global health threat that boosts all local and international organizations to take preventive measures. In general, measures should encompass the source of the infection, its transmission route, and the susceptible population. Accordingly, knowledge of these vital elements is a necessity (25, 26). In the current study, the respondents displayed a decent knowledge regarding risk factors, etiology, and route of transmission, incubation period, and signs and symptoms of COVID 19. The mean knowledge score was 13.51 ± 2.56 over 18 which can be attributed to the fact that nearly 80% of the participants had a university degree. Moreover, the main primary sources of COVID-19 information for the survey participants were health care providers (61%), and the Lebanese Ministry of Public Health (50%). In fact, the Lebanese authorities have released a new website for all information regarding the COVID-19. Moreover, television channels have broadcasted preventive measures (27). Nevertheless, despite all the effort taken by the Ministry of Health through schools, under 18 were the least informed on this condition followed by the elderly. In fact, age groups >50 years had similar results to Chinese and Egyptian residents (28–30). An alarming finding showed

that Lebanese health care providers scored lower than non-healthcare providers. According to a survey on the perception and knowledge of health care workers, this discrepancy can be explained by the fact that some are experts in other domains than infectious diseases (31). This finding imposes that awareness should target all categories of citizens. Two distinct websites should be created; one for the health care providers and one for the non-healthcare providers. Television channels should broadcast animated recommendations targeting the children. Areas of low knowledge should be targeted by posting on the road billboards and phone message notifications.

Knowledge is a requirement for establishing prevention beliefs, developing positive attitudes, and encouraging positive behaviors toward the disease (32). This was reflected in the practice of most of the participants in the survey. Nevertheless, even though 98.6% of the participants agreed that SARS-CoV-2 is transmitted through sneezing or coughing droplets, 17.1 and 1.7% sometimes and never cover their mouth after sneezing or coughing in public places, respectively. Moreover, 14.6% sometimes wear a mask in crowded places and 6.1% never did. Being sick also did not trigger wearing a mask in 13.7% of the respondents. Consequently, the virus can be transmitted easily by this minority since it is highly contagious.

Even though the U.S. National Institutes of Health does not recommend the use of any agent as prophylaxis against COVID-19 (33), 76.1% increased their consumptions of fruits and vegetables, 59.5% consumed vitamin C supplements, 56.1% did exercise on a daily basis, and 47.6% avoided fast food in an attempt to increase their immunity and consequently decrease the risk of acquiring the virus. These lifestyle modifications are a necessity to avoid most diseases including diabetes and cardiovascular diseases.

According to the Ministry of Health, patients with symptoms should contact the ministry through the hotline or call the Red Cross to be transferred to the assigned hospital. Moreover, the Lebanese Order of Pharmacists stated that anyone with fever, shortness of breath, or cough, or had been in contact with COVID-19 patients, or had been outside the country is not allowed to enter the pharmacies and has to refer to the assigned hospitals by the Ministry of Health. With all these restrictions, 10.5% still would seek pharmacists or physician clinics in case of having COVID-19 symptoms which puts the health care provider along with staff, waiting patients, and possible pediatrics at risk of developing the disease. Furthermore, 11.2% will choose to self-medicate instead of medical referral. This is alarming, especially that 10% will have a tendency to take hydroxychloroquine, if it is available at home. The National Institutes of Health stated that there isn't enough data to recommend or ban the use of hydroxychloroquine or chloroquine for the treatment of COVID-19 (33). In addition, hydroxychloroquine may cause life-threatening side effects, such as fatal cardiac arrhythmias, which is known by only 38% of the questioned participants.

CONCLUSION AND RECOMMENDATION

This study showed that there is a fair knowledge and positive attitude toward COVID-19. However, more awareness campaigns should be conducted as new cases were reported. The personnel in charge should develop a plan in a way that limits the transmission of this disease once quarantine is lifted. Face masks, should not be put by choice, enforcing the mandatory wearing of a face mask in public should be a must. Enforcing laws, including the allowance of a limited number of personnel in a supermarket and shops based on its area, could also provide the required social distancing and thus limits the spread of the virus. Finally, proper awareness should not only be restricted to social media platforms nor Televisions; but also the Ministry of Public Health should come up with a focus group to target both educated and uneducated, extreme age groups, and poor sanitary areas of the country.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because it contains some confidential information. Requests to access the datasets should be directed to t.domyati@bau.edu.lb.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The idea of the research was suggested by GI. This study design was elaborated by all authors. SD did the data analysis. All authors were involved in the writing and revising processes.

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Analysis of the Risk Factors for Mortality in Adult COVID-19 Patients in Wuhan: A Multicenter Study

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Objectives: An outbreak of coronavirus disease (COVID-19) in 2019 in Wuhan, China, has spread quickly worldwide. However, the risk factors associated with COVID-19-related mortality remain controversial.

Methods: A total of 245 adult patients with laboratory-confirmed COVID-19 from two centers were analyzed. Chi-square, Fisher's exact, and the Mann-Whitney *U*-tests were used to compare the clinical characteristics between the survivors and non-survivors. To explore the risk factors associated with in-hospital death, univariable and multivariable cox regression analyses were used.

Results: Of the 245 patients included in this study, 23 (9.4%) died in the hospital. The multivariate regression analysis showed increased odds of in-hospital deaths associated with age, D-dimer levels >1,000 ng/L, platelet count < 125, and higher serum creatinine levels.

Conclusions: We identified risk factors that show significant association with mortality in adult COVID-19 patients, and our findings provide valuable references for clinicians to identify high-risk patients with COVID-19 at an early stage.

Keywords: COVID-19, mortality, risk factors, SARS-CoV-2, Wuhan

INTRODUCTION

In December 2019, infectious pneumonia broke out in Wuhan, Hubei province. It was caused by a new coronavirus, which was named "SARS-CoV-2" by the World Health Organization (WHO) on February 13, 2020 (1). Meanwhile, the infectious disease caused by SARS-CoV-2 was named "COVID-19." In China, according to the National Health Commission (2), a total of 82,341 cases were diagnosed, of which 77,892 patients were discharged, and 3,342 died as of April 15. Since the early stages of the outbreak, the disease has spread to most countries in the world. As of April 15, 2020, more than 1,900,000 people have been infected and over 120,000 people have died worldwide (3). The situation is likely to worsen with the rapid escalation and global spread of infection (4).

The symptoms for COVID-19 vary from cough and fever to dyspnea, which can be difficult to identify. Approximately 20% of the patients are estimated to develop severe illness, with overall mortality around 2.3% (5). Given that there are no drugs that have been proven to be clinically effective in targeting the SARS-CoV-2 directly, it is particularly important to identify the risk factors associated with disease progression and mortality (6, 7).

In this study, we explored the potential host risk factors associated with death in a retrospective cohort of 245 laboratory-confirmed COVID-19 patients admitted to the two appointed hospitals in Wuhan. We present a detailed review of the medical information of each patient to clarify the clinical manifestations, laboratory test results, and outcomes to better understand the disease progression and prognosis.

MATERIALS AND METHODS

Study Design and Patients

This multiple center retrospective study was approved by the Institutional Ethics Board of the Zhongnan Hospital of Wuhan University (NO. 2020015) and the Central Hospital of Wuhan (NO. 2020072). Oral informed consent was obtained from patients before data collection with their privacy rights protected. According to the Seventh Interim Guidance of Diagnosis and Treatment of COVID-19 published by the Chinese National Health Commission on March 3, 2020 (8), patients with radiological characteristics of viral pneumonia were recognized as suspected cases. Meanwhile, patients who tested positive with the real-time reverse-transcription–polymerase-chain-reaction (RT-PCR) test for SARS-CoV-2 or showed high-level homology with SARS-CoV-2 detected through gene sequencing were diagnosed as confirmed cases. Therefore, patients who were negative on repeated RT-PCR test and those without clear outcomes within the observation cut-off time were excluded from the study. Ultimately, we enrolled a total of 245 patients who were admitted to Zhongnan Hospital of Wuhan University and the Central Hospital of Wuhan between December 26, 2019, and February 15, 2020. All the patients were followed up until March 7, 2020.

Data Collection

We collected data on the demographic, clinical, laboratory, and radiological characteristics, as well as treatment and clinical outcomes of the included patients. All the information was acquired from their electronic records and independently checked by three participants (SW, SC, and YH) to verify data accuracy.

The demographic features included gender (male or female), age at diagnosis, comorbidities, symptoms, and time from onset to admission. Laboratory tests included routine blood tests, blood coagulation tests, and tests for renal and liver functions, creatine kinase, infection-related biomarkers, brain natriuretic peptide (BNP), and RT-PCR analysis for SARS-CoV-2. The radiographic examination included chest CT or X-ray. Clinical management and outcomes data included information on therapies, and clinical outcomes until the end of the follow-up period, time from onset to a negative RT-PCR result, and changes in chest x-rays and CT scans.

Statistical Analysis

The baseline characteristics of survivors and non-survivors were compared. Categorical and continuous variables were presented as n (%) and median (IQR), respectively. The Chi-square test or Fisher's exact test were performed for categorical variables, and the Mann-Whitney *U*-test was used for continuous variables. Univariable and multivariable cox regression analyses were applied to investigate the risk factors associated with in-hospital deaths. Candidate factors for the multivariable analysis were chosen on the basis of the previous findings and findings of the univariable analysis. For laboratory results, we considered the normal ranges used in the Zhongnan Hospital of Wuhan University as the reference. All data analyses were performed using SPSS (version 26.0).

RESULTS

Demographics and Clinical Characteristics of Patients With COVID-19

The 245 laboratory-confirmed COVID-19 patients were divided into survivor ($n = 222$) and non-survivor ($n = 23$) groups. The demographic characteristics and symptoms of all the patients are shown in **Table 1**. The median age of all patients was 54 [interquartile range (IQR): 37–64] years, while that of patients in the survivor group was 52 years. The median age of the non-survivor group was 76 years. The proportion of men (48.2%) and women (51.8%) was comparable across the whole cohort. Female patients accounted for a higher percentage (55.0%) of the survivor group, while male patients accounted for the majority (78.3%) of the non-survivor group. About half the patients ($n = 122$) had underlying diseases including hypertension, diabetes, chronic obstructive lung disease, cardiovascular disease (CVD), carcinoma, nervous system disease, and hepatic disease. Hypertension was the most common comorbidity in the non-survivor group, followed by CVD. The most common symptoms on admission were fever and cough, followed by fatigue and expectoration (**Table 1**). The median time from the onset to admission was 6 (IQR: 3–8.5) days.

Laboratory Results and Treatments

Table 2 summarizes the laboratory results of the study population. On admission, 168 (73.0%) patients had lymphocyte counts below the normal range, while 60 (26.0%) of them had it within the normal range. The non-survivor group had higher counts of white blood cells (median value: 6.88 vs. 4.19) and neutrophils (5.83 vs. 2.60) compared to the survivor group while the latter had lower counts of lymphocytes (0.85 vs. 0.91) and platelets (157.00 vs. 166.50). Compared to the survivor group, the non-survivor group had three times higher levels of D-dimer as well as higher baseline levels of alanine aminotransferase (ALT), aspartate transaminase (AST), total bilirubin, serum creatine, all infection-related biomarkers and brain natriuretic peptide. Based on chest x-rays and CT manifestations, 91.9% (159/173) of the patients

TABLE 1 | Demographics and clinical characteristics of 245 patients with COVID-19.

	Total ($n = 245$)	Non-survivor ($n = 23$)	Survivor ($n = 222$)	*P*-value
Age, median (IQR)	54.0 (37.0–64.0)	76.0 (64.5–81.0)	52.0 (36.0–62.0)	<0.001
Gender				0.002
Female	127 (51.8%)	5 (21.7%)	122 (55.0%)	
Male	118 (48.2%)	18 (78.3%)	100 (45.0%)	
Any comorbidity	122 (49.8%)	19 (82.6%)	103 (46.4%)	0.001
Hypertension	72 (29.4%)	14 (60.9%)	58 (26.1%)	<0.001
Diabetes	31 (12.7%)	6 (26.1%)	25 (11.3%)	0.088
Chronic obstructive lung disease	7 (2.9%)	2 (8.7%)	5 (2.3%)	0.133
Cardiovascular disease	29 (11.8%)	9 (39.1%)	20 (9.0%)	<0.001
Carcinoma	11 (4.5%)	3 (13.0%)	8 (3.6%)	0.121
Nervous system disease	13 (5.3%)	5 (21.7%)	8 (3.6%)	0.001
Hepatic disease	19 (7.8%)	3 (13.0%)	16 (7.2%)	0.557
Respiratory rate>24 breaths per min	39 (15.9%)	5 (21.7%)	34 (15.3%)	0.616
Initial symptoms				
Fever	201 (82.0%)	16 (69.6%)	185 (83.3%)	0.176
Fatigue	132 (53.9%)	10 (43.5%)	122 (55.0%)	0.293
Cough	142 (58.0%)	11 (47.8%)	131 (59.0%)	0.301
Expectoration	70 (28.6%)	5 (21.7%)	65 (29.3%)	0.446
Dyspnea	70 (28.6%)	8 (34.8%)	62 (27.9%)	0.489
Muscle ache	56 (22.9%)	3 (13.0%)	53 (23.9%)	0.239
Headache	19 (7.8%)	2 (8.7%)	17 (7.7%)	1.000
Sore throat	18 (7.3%)	1 (4.3%)	17 (7.7%)	0.873
Diarrhea	27 (11.0%)	1 (4.3%)	26 (11.7%)	0.469
Rhinorrhea	7 (2.9%)	2 (8.7%)	5 (2.3%)	0.133
Onset to admission (IQR)	6.00 (3.00–8.50)	4.00 (1.00–7.50)	6.00 (3.75–9.00)	0.025

IQR, interquartile range.

had pneumonia performance. Among them, 136 patients had bilateral pneumonia and 23 patients had unilateral pneumonia. **Table 3** shows information on treatment, medication, and clinical outcomes. In all the cases, more than 50% of the patients received antiviral treatment, 73.9% of the patients were given synthetic corticosteroids (methylprednisolone/dexamethasone), and 9% (22/245) of the patients had respiratory failure. Among the 80 cases with ongoing viral nucleic acid testing data, 95% (76/80) of them tested negative within 14 days from onset (continuous bilateral detection) and there was one death case. In all the other non-survivors, the virus was detectable until death. The imaging results showed that in 16% (18/112) of the patients, the disease was in the progressive state. Of these, 87.5 and 10.6% were in the non-survivor and survivor groups, respectively.

The Risk of Death

The univariable analysis found higher odds of in-hospital death in patients with chronic obstructive lung disease, and CVD (**Table 4**). Age, sex, lymphocyte counts, prothrombin time, levels of albumin, ALT, sodium, myoglobin, C-reactive protein, and ribavirin were also significantly associated with death ($p < 0.05$) (**Table 4**). The multivariate regression analysis showed (**Table 5**) that the risk of hospitalization death increased with age [hazard ratio (HR): 1.165, 95% CI (confidence interval): 1.051–1.292, $p = 0.004$], D-dimer >1,000 ng/L (HR: 11.516, 95% CI: 1.157–114.658, $p = 0.037$), platelets $< 125 \times 10^9$/L (HR: 7.731, 95% CI: 1.303–45.674, $p = 0.024$), and higher serum creatinine levels (HR: 1.004, 95% CI: 1.002–1.007, $p = 0.002$). However, cardiovascular disease and lymphocyte counts showed no significant association with death based on the multivariate analysis.

DISCUSSION

Though the epidemiology of patients with COVID-19 is widely studied and reported, the death-related risk factors and detailed clinical characteristics of the disease have not been well-described. In this study, we summarized the clinical characteristics of 245 patients diagnosed with COVID-19 at two centers in Wuhan. We also identified risk factors associated with COVID-19-related deaths. These included older age, D-dimer > 1,000 ng/L, platelets $< 125 \times 10^9$/L, and higher serum creatinine levels.

In our study, 9.4% of the patients died of COVID-19. While the average age of the total cohort was 54 years, that of the non-survivor group was 76 years. In another retrospective, multicenter study on COVID-19 cases from Wuhan (9), 54 (28.3%) of the patients died in hospital, the median age of

TABLE 2 | Laboratory results of 245 patients with COVID-19.

		Median (IQR)/number (%)			
	Normal range	**Total**	**Non-survivor**	**Survivor**	***P*-value**
Blood routine					
Leucocytes ($\times 10^9$/L)	3.5–9.5	4.30 (3.01–6.00)	6.88 (3.13–10.98)	4.19 (3.01–5.55)	0.015
<3.5		80/229 (34.9%)	6/20 (30.0%)	74/209 (35.4%)	0.106
3.5–9.5		127/229 (55.5%)	8/20 (40.0%)	119/209 (56.9%)	
>9.5		22/229 (9.6%)	6/20 (30.0%)	16/209 (7.7%)	
Neutrophils ($\times 10^9$/L)	1.8–6.3	2.91 (1.88–4.95)	5.83 (3.65–9.57)	2.60 (1.82–4.29)	0.001
Lymphocytes ($\times 10^9$/L)	1.1–3.2	0.91 (0.66–1.11)	0.85 (0.56–1.07)	0.91 (0.66–1.11)	0.647
<1.1		168/230 (73.0%)	17/22 (77.3%)	151/208 (72.6%)	0.827
1.1–3.2		60/230 (26.0%)	3/22 (13.6%)	57/208 (27.4%)	
>3.2		2/230 (1.0%)	2/22 (9.1%)	0	
Platelets ($\times 10^9$/L)	125.0–350.0	166.00 (124.75–200.00)	157.00 (112.25–187.75)	166.50 (126.00–202.50)	0.327
<125.0		55/220 (25.0%)	7/20 (35.0%)	48/200 (24.0%)	0.450
125.0–350.0		162/220 (74.0%)	12/20 (60.0%)	150/200 (75.0%)	
>350.0		3/220 (1.0%)	1/20 (5.0%)	2/200 (1.0%)	
Hemoglobin (g/L)	130.0–175.0	131.00 (118.00-141.00)	127.50 (93.95–136.48)	131.00 (119.00–142.00)	0.111
Coagulation function					
Prothrombin time (s)	9.4–12.5	12.15 (11.28–13.10)	12.10 (11.40–13.10)	12.20 (11.25–13.05)	0.640
Activated partial thromboplastin time (s)	25.1–36.5	30.20 (28.00–33.00)	28.85 (26.50–34.50)	30.25 (28.00–32.70)	0.853
D-dimer (ng/L)	0–500	240.00 (108.00–633.00)	762.00 (10.18–1574.75)	230.00 (108.50–505.00)	0.079
Blood biochemistry					
Albumin (g/L)	40–55	38.20 (34.90–41.48)	34.55 (32.73–38.48)	38.60 (35.38–41.60)	0.004
Alanine aminotransferase (U/L)	9–50	25.00 (15.45–42.23)	30.20 (18.90–55.00)	25.00 (15.30–39.00)	0.267
Aspartate aminotransferase (U/L)	15–40	27.55 (19.30–42.78)	34.00 (25.00–54.00)	27.00 (19.30–40.00)	0.179
Total bilirubin (μmol/L)	5.0–21.0	9.40 (7.60–13.22)	15.50 (7.90–22.05)	9.30 (7.60–12.50)	0.058
Potassium (mmol/L)	3.5–5.3	4.02 (3.61–4.36)	4.13 (3.91–4.94)	4.01 (3.60–4.35)	0.116
Sodium (mmol/L)	137.0–147.0	139.40 (135.90–141.60)	139.30 (132.00–141.00)	139.45 (136.00–141.60)	0.598
Serum creatinine (μmol/L)	64.0–104.0	67.90 (54.40–84.40)	96.60 (72.90–133.25)	67.15 (53.30–81.55)	0.001
Myocardial enzyme					
Myoglobin (ng/mL)	<140.1	50.20 (31.20–106.30)	158.60 (84.08–245.95)	48.30 (25.75–72.50)	0.001
Infection-related biomarkers					
Procalcitonin (ng/mL)	<0.05	0.09 (0.06–0.22)	0.23 (0.13–1.07)	0.08 (0.06–0.15)	<0.001
<0.05		118/220 (53.6%)	3/22 (13.6%)	115/198 (58.1%)	<0.001
≥0.05		102/220 (46.4%)	19/22 (86.4%)	83/198 (41.9%)	
Interleukin-6 (pg/mL)	0–7.0	22.81 (6.94–58.66)	68.59 (32.64–223.23)	18.47 (5.68–45.03)	0.001
<7.0		32/118 (27.1%)	2/16 (12.5%)	30/102 (29.4%)	0.159
≥7.0		86/118 (72.9%)	14/16 (87.5%)	72/102 (70.6%)	
Erythrocyte sedimentation rate (mm/h)	0–20	25.00 (13.25–53.00)	80.00 (48.00–96.00)	24.00 (13.00–43.00)	<0.001
<20		57/141 (40.4%)	2/13 (15.4%)	55/128 (43.0%)	0.054
≥20		84/141 (59.6%)	11/13 (84.6%)	73/128 (57.0%)	
C-reactive protein (mg/L)	0–10	5.90 (1.82–25.05)	9.46 (3.27–68.94)	5.35 (1.80–24.32)	0.106
<10		120/201 (59.7%)	10/19 (52.6%)	110/182 (60.4%)	0.510
≥10		81/201 (40.3%)	9/19 (47.4%)	72/182 (39.6%)	
Brain natriuretic peptide (pg/mL)	0–1,800.0	29.70 (10.00–189.85)	283.80 (10.20–363.00)	24.23 (10.00–107.55)	0.013
Chest x-ray and CT manifestations					
Bilateral pneumonia		136/173 (78.6%)	14 (87.5%)	122/157 (77.7%)	0.235
Unilateral pneumonia		23/173 (13.3%)	2 (12.5%)	21/157 (13.4%)	
No abnormality		14/173 (8.1%)	0	14/157 (8.9%)	

191 patients was 56 (IQR 46–67) years, and that of the non-survivors was 69 (63–76) years, which was comparable to our findings. In a study by Zhong et al. (10), which enrolled 1,099 laboratory confirmed COVID-19 cases from 552 hospitals across China, the median age of the study population was 47 years and 1.4% of the patients died of the disease. The fatality rate

TABLE 3 | Clinical treatments and outcomes of patients with COVID-19.

Project	Total	Non-survivor	Survivor	*P*-value
	(*n* = 245)	(*n* = 23)	(*n* = 222)	
Antibiotic therapy	238 (97.1%)	22 (95.7%)	216 (97.3%)	0.503
Antiviral therapy				
Oseltamivir	131 (53.5%)	14 (60.9%)	117 (52.7%)	0.455
Ribavirin	43 (17.6%)	5 (21.7%)	38 (17.1%)	0.79
Abidol	64 (26.1%)	4 (17.4%)	60 (27.0%)	0.317
Alpha-interferon	19 (7.8%)	1 (4.3%)	18 (8.1%)	0.490
Corticosteroid treatment	181 (73.9%)	20 (87.0%)	161 (72.5%)	0.134
Respiratory failure	22 (9.0%)	9 (39.1%)	13 (5.9%)	<0.001
Onset to RT-PCR turning negative (d)				
≤14	76/80 (95.0%)	1	75 (94.9%)	0.819
>14	4/80 (5.0%)	0	4 (5.1%)	
Imaging changes				
Improvement	88/112 (78.6%)	0	88 (84.6%)	<0.001
Progression	18/112 (16.0%)	7/8 (87.5%)	11 (10.6%)	
No change	6/112 (5.4%)	1 (12.5%)	5 (4.8%)	

in Wuhan is significantly higher than that in other regions. It is probably because of the concentration of a large number of cases.

It has been reported that older patients have a worse prognosis of the Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS) (11, 12). Previous studies have found that older age is related to stronger host innate responses to virus infection and stronger activation of the pro-inflammatory pathways, with a marked decline in cell-mediated as well as humoral immune functions (13, 14).

In addition to age factors, we also found D-dimer levels >1,000 ng/L, and platelet counts $< 125 \times 10^9$/L on admission were associated with fatal outcomes in COVID-19 patients. High levels of D-dimer have been verified to be a significant prognostic factor in patients with suspected infection and sepsis (15). Increased D-dimer is distinctly associated to disseminated intravascular coagulation, which is considered as an early stage of diffuse pulmonary intravascular coagulopathy (16). Extensive thrombosis in small vessels and the microvasculature has been confirmed histologically as one of the important basic pathological changes of lung in hospitalized COVID-19 patients (17). Platelets also play an equally important role as a consumable substance in the clotting process. Therefore, the reduction of platelets may also affect the survival status of patients through the same way. As we all know, COVID-19 has many similarities to SARS. In a retrospective study, 20–45% of SARS patients had thrombocytopenia (18). There are 7(35%) of non-survivor patients had platelets counts below the normal range on admission. Moreover, severe infections could lead to secondary thrombocytopenia characterized by a rapid decline in platelet count (19). These conditions are associated with damages to the hematopoietic system and the lungs, in which mature megakaryocytes release platelets (20). However, Qu et al. (21) have reported that while COVID-19 patients with significantly elevated platelet count during treatment were on an average hospitalized longer, the platelet count at admission was lower in severely ill patients (169.67 ± 48.95) compared to those who were not severely ill (192.26 ± 58.12). The initial increase in platelet count, followed by a decrease in severely ill patients, may be associated with the progression of COVID-19.

Basal CVD did not show a significant association with COVID-19-related mortality. Coronary heart disease has been correlated with acute cardiac incidents and adverse outcomes in respiratory viral infections (22–24). However, consistent with our findings, previous studies (9) have also verified that CVD did not play an independent role in multivariate regression analysis of COVID-19-related mortality.

This study has some limitations. First, laboratory tests such as hypersensitive troponin, creatine kinase, and serum ferritin were missing because the study was retrospective. At the same time, because many cases were hospitalized in the early stage of the epidemic, there are also many missing data of viral nucleic acid test. Second, the outcomes were evaluated at the end of the follow-up period instead of at a fixed time point during the course of the disease. Finally, the sample size was small. Hence, the interpretation of our results might be limited. However, by including patients from two appointed hospitals for COVID-19, we believe the object of our study is representative of the total patient population.

Although Wuhan achieved victory over COVID-19 through hard work, this new coronary pneumonia is now a worldwide disease. The virus can be eliminated only if all countries take appropriate measures. The medical workers in Wuhan have accumulated experience fighting this epidemic, and have no reservations in providing advice to colleagues in need. We are also committed to passing on our knowledge and expertise to other countries currently fighting COVID-19.

TABLE 4 | Univariate cox regression analysis of clinical parameters in patients with COVID-19.

	Univariable OR (95% CI)	*P*-value
Demographics and clinical characteristics		
Age, years	1.089 (1.049–1.132)	<0.001*
Male sex (vs. female)	3.024 (1.110–8.237)	0.030*
Comorbidity present (vs. not present)	2.667 (0.891–7.982)	0.079
Chronic obstructive lung disease	5.050 (1.115–22.867)	0.036*
Hypertension	1.966 (0.829–4.664)	0.125
Diabetes	1.404 (0.535–3.683)	0.490
Cardiovascular disease	2.637 (1.093–6.360)	0.031*
Carcinoma	3.252 (0.941–11.240)	0.062
Nervous system disease	1.668 (0.571–4.878)	0.350
Hepatic disease	1.478 (0.435–5.027)	0.531
Blood routine		
Leucocytes (× 10^9/L)		
< 3.5	1.315 (0.453–3.820)	0.614
3.5–9.5	Ref	
> 9.5	2.493 (0.833–7.457)	0.102
Neutrophils (× 10^9/L)	1.116 (0.985–1.264)	0.085
Lymphocytes (× 10^9/L)		
<1.1	2.432 (0.692–8.543)	0.166
1.1–3.2	Ref	
>3.2	22.499 (3.434–147.387)	0.001*
Platelets (× 10^9/L)		
<125.0	1.863 (0.720–4.821)	0.199
125.0–350.0	Ref	
>350.0	2.337 (0.291–18.755)	0.424
Hemoglobin (g/L)		
Coagulation function		
Prothrombin time (s)	1.791 (1.348–2.379)	<0.001*
Activated partial thromboplastin time (s)	1.008 (0.998–1.019)	0.115
D-dimer (ng/L)	1.000 (1.000–1.000)	0.130
Blood biochemistry		
Albumin (g/L)	0.924 (0.858–0.996)	0.038*
Alanine aminotransferase (U/L)	1.002 (1.000–1.003)	0.009*
Aspartate aminotransferase (U/L)	1.001 (0.998–1.004)	0.580
Total bilirubin (μmol/L)	0.999 (0.963–1.037)	0.976
Potassium (mmol/L)	1.454 (0.813–2.602)	0.207
Sodium (mmol/L)	0.971 (0.943–0.999)	0.045*
Serum creatinine (μmol/L)	1.001 (1.000–1.002)	0.089
Myocardial enzyme		
Myoglobin (ng/mL)	1.005 (1.002–1.007)	<0.001*
Infection-related biomarkers		
Procalcitonin (ng/mL)		
<0.05	Ref	
≥0.05	6.090 (0.806–46.016)	0.080
Interleukin-6 (pg/mL)		
<7.0	Ref	
≥7.0	3.505 (0.778–15.787)	0.102
Erythrocyte sedimentation rate (mm/h)		
<20	Ref	
≥20	3.666 (0.461–29.140)	0.219
C-reactive protein (mg/L)		
<10	Ref	
≥10	4.140 (1.583–10.829)	0.004*
Brain natriuretic peptide (pg/mL)		
Clinical treatments		
Oseltamivir	1.867 (0.791–4.405)	0.154
Ribavirin	0.275 (0.088–0.862)	0.027*
Abidol	0.364 (0.107–1.245)	0.107
Corticosteroid treatment	0.426 (0.111–1.641)	0.215
Alpha-interferon	0.434 (0.054–3.497)	0.433
Multicenter		
Zhongnan hospital	Ref	
Central hospital	0.369 (0.151–0.901)	0.029*

(Continued)

TABLE 4 | Continued

**P < 0.05.*

TABLE 5 | Multivariate cox regression analysis of clinical parameters in patients with COVID-19.

	Multivariable OR (95% CI)	*P*-value
Age	1.165 (1.051–1.292)	0.004*
Cardiovascular disease	0.312 (0.066–1.483)	0.143
Lymphocytes (× 10^9/L)		
<1.1	5.387 (0.810–35.848)	0.082
1.1–3.2	Ref	
Platelets (× 10^9/L)		
<125	7.731 (1.303–45.674)	0.024*
≥125	Ref	
D-dimer (ng/L)		
<500	Ref	
500–1,000	3.601 (0.308–42.089)	0.307
>1,000	11.516 (1.157–114.658)	0.037*
Serum creatinine	1.004 (1.002–1.007)	0.002*
Total bilirubin	1.037 (0.980–1.098)	0.209
Respiratory failure	2.456 (0.645–9.361)	0.188

**P < 0.05.*

CONCLUSIONS

In conclusion, almost 9.4% of our study population died of COVID-19. Older age, elevated D-dimer levels, and thrombocytopenia on admission were independent risk factors for death. More studies on the risk factors associated with COVID-19-related death are needed to control disease progression and to improve its treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Zhongnan Hospital of Wuhan University (NO. 2020015) and the Central Hospital of Wuhan (NO. 2020072). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

LG, ZL, and BC: conceptualization. SW, YH, WL, SC, ML, WZh, DC, LZ, and MWa: data collection and processing. BC, MT, and HX: interpretation. All authors: wrote-review and editing.

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Epidemiological and Genomic Analysis of SARS-CoV-2 in 10 Patients From a Mid-Sized City Outside of Hubei, China in the Early Phase of the COVID-19 Outbreak

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A novel coronavirus known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of the ongoing Coronavirus Disease 2019 (COVID-19) pandemic. In this study, we performed a comprehensive epidemiological and genomic analysis of SARS-CoV-2 genomes from 10 patients in Shaoxing (Zhejiang Province), a mid-sized city outside of the epicenter Hubei province, China, during the early stage of the outbreak (late January to early February, 2020). We obtained viral genomes with >99% coverage and a mean depth of 296X demonstrating that viral genomic analysis is feasible via metagenomics sequencing directly on nasopharyngeal samples with SARS-CoV-2 Real-time PCR C_t values <28. We found that a cluster of four patients with travel history to Hubei shared the exact same virus with patients from Wuhan, Taiwan, Belgium, and Australia, highlighting how quickly this virus spread to the globe. The virus from another cluster of two family members living together without travel history but with a sick contact of a confirmed case from another city outside of Hubei accumulated significantly more mutations (9 SNPs vs. average 4 SNPs), suggesting a complex and dynamic nature of this outbreak. Our findings add to the growing knowledge of the epidemiological and genomic characteristics of SARS-CoV-2 and offers a glimpse into the early phase of this viral infection outside of Hubei, China.

Keywords: 2019-nCoV, COVID-19, genotype, metagenomic sequencing, mutation rate, transmission, genomic epidemiology, SARS-CoV-2

INTRODUCTION

Coronaviruses (CoVs) are a large family of single-stranded RNA viruses that can be isolated from a variety of animals including camels, rats, birds, and bats (1). These coronaviruses can cause a range of disease states in animals including respiratory, enteric, hepatic, and neurological disease (2). Before late 2019, there were six known CoVs capable of infecting humans (Hu-CoVs). The first four Hu-CoVs that cause mild disease are HKU1, NL63, OC43, and 229E and are known to circulate in

the human population (3). The other two Hu-CoVs, known as severe acute respiratory syndrome-CoV (SARS-CoV) and middle east respiratory syndrome-CoV (MERS-CoV), caused two previous epidemics in 2003 (4) and 2012 (5), respectively. Both SARS-CoV and MERS-CoV were the results of recent spillover events from animals. These two epidemics highlighted how easy it is for spillover events in CoVs to occur and cause outbreaks in humans.

In December 2019, another spillover event occurred and a seventh Hu-CoV appeared known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), previously named 2019-nCoV (6). SARS-CoV-2 has been spreading rapidly across the world since it was first reported in Wuhan, Hubei province, China (6, 7). The advances and accessibility of sequencing technologies have allowed researchers all over the world to quickly sequence the genome of SARS-CoV-2 (8, 9). Zhou et al. (9) showed that SARS-CoV-2 shared 79.6% sequence identity to SARS-CoV and 96% sequence identity to a bat CoV further supporting the theory of another spillover event.

Genomic analysis of SARS-CoV-2 genomes suggested that there were two major genotypes in the early phase of the outbreak, known as L type and S type, based on almost complete linkage between two SNPs (10). Tang et al. (10) proposed that the S type was more ancient while the L type evolved later and may be more aggressive in replication rates and spreads more quickly. Recent reclassifications of SARS-CoV-2 proposed the use of clade nomenclature and divided all viral genomes into 7 major clades including O, S, L, V, G, GR, and GH, with the S clade corresponding to the S type in the Tang study (11–13). Here we present a comprehensive epidemiological and genomic analysis of SARS-CoV-2 genomes from 10 patients in Shaoxing (Zhejiang Province), a mid-sized city about 500 miles away from Wuhan at the early stages of the outbreak.

MATERIALS AND METHODS

Study Design and Ethics

Ten remnant nasopharyngeal swab samples collected between 1/27/2020 and 2/7/2020, and tested positive by a SARS-CoV-2 real-time PCR assay with cycle threshold (C_t) values of <28, were included in this study. The samples were de-identified except the associated epidemiological data were retained. Since the patient identification was removed and the samples used in this study were remnant and otherwise would be discarded, the Shaoxing Center for Disease Control and Prevention had determined that the institutional review boards (IRB) approval was waived for this project, and the informed consent form was not required.

SARS-CoV-2 PCR and RNA Sequencing

Total nucleic acid was extracted from the nasopharyngeal swabs using the Total Nucleic Acid Extraction Kit (IngeniGen XMK Biotechnologies, Inc., Zhejiang, China). Real-time PCR was performed by using the IngeniGen XMKbio 2019-nCoV (SARS-CoV-2) RNA Detection kit, which targets the highly specific sequences in the *ORF1ab* and *N* genes of the virus, on the ABI 7500 system (ThermoFisher Scientific, Inc., MA, USA). The RNA libraries were constructed using the Ingenigen XMKbio RNA-seq Library Prep Kit (IngeniGen XMK Biotechnologies, Inc., Zhejiang, China). Briefly, DNase was used to remove residual human DNA and the RNA was fragmented, followed by double-strand cDNA synthesis, end-repair, dA-tailing, and adapter ligation. Sequencing was performed by using the 2 × 75 bp protocol on the Nextseq 550 system (Illumina, Inc., CA, USA). Sequencing data have been deposited to NCBI SRA under BioProject PRJNA638211, and to GISAID with accessions EPI_ISL_463889 and EPI_ISL_463894 to 463902.

Data Analysis

Quality control and trimming of paired-end reads was performed using custom Python scripts as follows: (1) trim 3′ adapters; (2) trim reads at ambiguous bases; (3) filter reads shorter than 40 bp; (4) filter reads with average quality score <20. Host-derived reads were removed by alignment against the GRCh38.p13 genome reference using bowtie2 (v2.3.4.3) (14) with default parameters. The retained reads were then mapped to 163 published SARS-CoV-2 reference genomes obtained from GISAID (https://www.gisaid.org/CoV2020/, accessed March 2, 2020) by bowtie2 (v2.3.4.3) with default parameters. Snippy (v4.5.0) was used for variant and indel calling, and core SNP alignment against the Wuhan-Hu-1 (NC_045512.2) reference (6), FastTree (v2.1.3) which infers approximately-maximum-likelihood phylogenetic trees (15) was used for tree construction using default parameters, and Figtree (v1.4.4) (16) was used to visualize the resulting phylogenetic tree. HISAT2 (v2.2.0) and StringTie (v2.1.3) were used for RNA-Seq alignment and transcript assembly to identify novel isoforms (17, 18). Additional statistical analyses and visualizations were performed using the "ggplot2" package in the R statistical environment (v3.6). The clade

TABLE 1 | Epidemiological history of the 10 Shaoxing patients.

ID	Age range	History of travel or sick contact	Date of symptom onset	Date of sample collection
Shaoxing-01	30–39	Family members traveled together to Hubei province (1/15–1/24)	1/24/20	1/27/20
Shaoxing-02	70–79		1/29/20	1/30/20
Shaoxing-03	60–66		1/28/20	1/30/20
Shaoxing-04	50–59		1/29/20	1/31/20
Shaoxing-05	50–59	Traveled to Hubei (1/16–1/23)	1/29/20	1/31/20
Shaoxing-06	30–39	Resident of Wuhan; Traveled to Shaoxing on 1/17	1/29/20	1/31/20
Shaoxing-07	<10	Traveled to Hubei (1/11–1/24); Two family members were confirmed cases	1/30/20	1/30/20
Shaoxing-08	50–59	Unknown	1/31/20	2/7/20
Shaoxing-09	30–39	Family members living together no travel history; contact with a confirmed case from Ningbo, Zhejiang on 1/27	2/2/20	2/5/20
Shaoxing-10	30–39		2/5/20	2/6/20

and lineage nomenclature was determined by using pipeline pangolin (https://github.com/hCoV-2019/pangolin) as described previously (13).

RESULTS

Epidemiology of Shaoxing Patients

All 10 patients presented with symptoms (fever and cough) consistent with COVID-19 in late January and early February of 2020. The patients can be categorized into two epidemiologic groups with either a travel history to the Hubei province or sick contact with a confirmed case (**Table 1**). There was one case where we were unable to obtain a travel or exposure history (Shaoxing-8).

There are two apparent clusters in these 10 patients. The first cluster involves four patients who are relatives and traveled together to Hubei province for a wedding in late January. The first patient in this cluster had symptom onset on their last day in Hubei province while the other three patients had symptom onset 4–5 days after coming back to Shaoxing (**Table 1**). The second cluster involves two patients who are family members that live together and did not travel to Hubei province. One of the family members (Shaoxing-09) had a sick contact with a confirmed case who visited her but lived in Ningbo, a more populated city in Zhejiang province (**Table 1**).

Metagenomic Sequencing

The patients were confirmed to have SARS-CoV-2 infection by a commercial Real-time PCR assay. The average C_t values for the 10 patient samples were 23.17 for *ORF1ab* and 24.54 for *N* (**Table 2**). Metagenomic sequencing was performed to recover the full viral genome. The total number of sequence reads per samples ranged from 10.4 to 27.5 million with an average of 17.1 million. A small percentage of these reads mapped to SARS-CoV-2 RNA genome and we did not identify any novel transcripts or fusion events, nor did we detect

TABLE 2 | Summary of sequencing results of 10 Shaoxing patient samples.

ID	Ct value (ORF1ab)	Ct value (*N*)	Total reads (PE 75)	2019-nCoV RNA (raw reads)	2019-nCoV RNA (log value)	Genome coverage (%)	Mean depth (X)
Shaoxing-01	21.57	23.62	17,158,277	40,057	4.60	99.4	219
Shaoxing-02	18.86	20.93	13,602,710	149,682	5.18	99.9	929
Shaoxing-03	20.09	22.25	24,769,343	163,158	5.21	99.8	1,024
Shaoxing-04	24.02	24.68	21,509,477	15,424	4.19	100.0	81
Shaoxing-05	21.81	23.81	14,043,326	99,521	5.00	99.9	591
Shaoxing-06	25.88	27.08	18,909,299	3,535	3.55	99.9	18
Shaoxing-07	23.11	23.87	10,480,051	5,063	3.70	99.8	26
Shaoxing-08	23.34	24.53	11,506,909	2,413	3.38	99.9	12
Shaoxing-09	26.81	27.85	27,517,291	8,897	3.95	99.9	47
Shaoxing-10	26.24	26.8	11,071,595	2,911	3.46	99.7	15
Min	18.86	20.93	10,480,051	2,413	3.38	99.4	12
Max	26.81	27.85	27,517,291	163,158	5.21	100.0	1,024
Mean	23.17	24.54	17,056,828	49,066	4.22	99.8	296

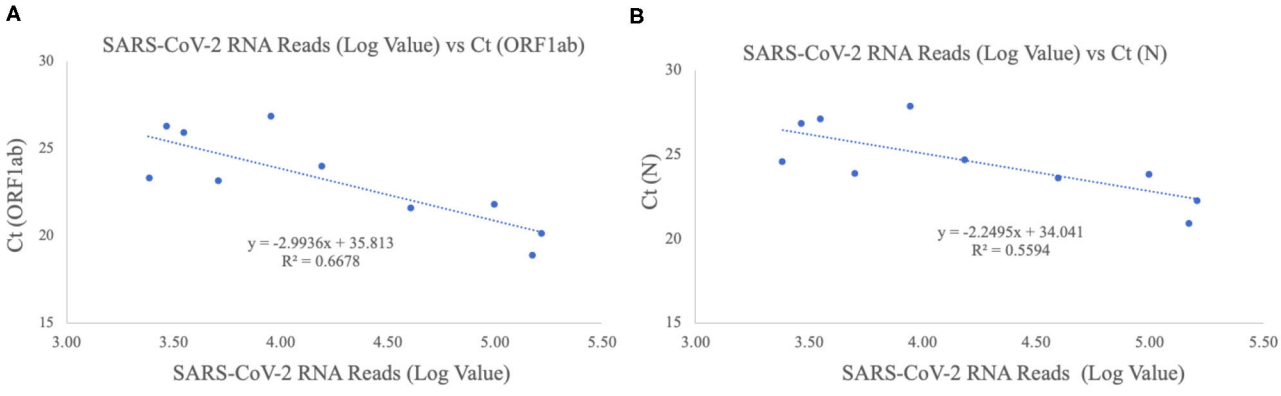

FIGURE 1 | Correlation of C_t values and SARS-CoV-2 RNA reads. **(A)** *ORF1ab* gene target. The X-axis plots the log value of the SARS-CoV-2 RNA reads while the Y-axis plots the C_t values for the *ORF1ab* gene for the 10 Shaoxing patients. **(B)** *N* gene target. The X-axis plots the log value of the SARS-CoV-2 RNA reads while the Y-axis plots the C_t values for the *N* gene for the 10 Shaoxing patients.

any insertion or deletion (**Table 2**). The range of sequence reads that mapped to SARS-CoV-2 RNA was 2,413–163,158 with an average of 49,066. We observed a clear negative correlation between the C_t values of each gene (*ORF1ab* and *N*) and the log value of SARS-CoV-2 RNA reads (**Figure 1**). However, the linearity is not significant ($R^2 = 0.6628$, 0.5595 for *ORF1ab* and *N*, respectively), indicating that the number of RNA reads measured by metagenomics sequencing are only semi-quantitative and cannot be interpreted directly as viral loads.

With a large variation in the SARS-CoV-2 RNA mapped reads, we were still able to obtain excellent coverage and depth when each genome was mapped to the first SARS-CoV-2 genome, Wuhan-Hu-1 [(6); **Figure 2A**]. The coverage for all genomes was above 99% and the mean depth for the genomes ranged from 12X to 1024X (**Table 2**, **Figure 2B**). Genomes sequenced to a relatively low mean depth (12X to 47X) were still able to be genotyped successfully but our results suggest that SARS-CoV-2 read counts of at least 15,000 yield sufficiently high depth to characterize even low prevalence or rare mutations.

Estimation of Mutation Rate

To determine the single nucleotide polymorphisms (SNPs) of SARS-CoV-2 in these 10 patients, we mapped each genome to the original Wuhan-Hu-1 reference which was collected on December 31, 2019 (6). The genomes contained a fairly moderate number of SNPs (mean of 4 SNPs, range 1-9) (**Table 3**), consistent with previous reports of relatively low mutation rates (19). The genomes with the largest number of SNPs came from individuals who had contact with a confirmed case from Ningbo, Zhejiang and no travel history to the Hubei province (**Table 3**, Shaoxing-9 and 10).

TABLE 3 | Summary of genomic descriptions for the Shaoxing SARS-CoV-2 genomes.

ID	Clade	No. of SNP[a]	No. of Days[b]	Mutation rate (#SNP/day)	Mutation rate (#SNP/day/nt)	Mutation rate (#SNP/yr/nt)
Shaoxing-01	S	2	27	0.07	2.48E-06	9.04E-04
Shaoxing-02	S	2	30	0.07	2.23E-06	8.14E-04
Shaoxing-03	S	2	30	0.07	2.23E-06	8.14E-04
Shaoxing-04	S	2	31	0.06	2.16E-06	7.87E-04
Shaoxing-05	L	2	31	0.06	2.16E-06	7.87E-04
Shaoxing-06	S	3	31	0.1	3.24E-06	1.18E-03
Shaoxing-07	L	5	30	0.17	5.57E-06	2.03E-03
Shaoxing-08	L	1	38	0.03	8.80E-07	3.21E-04
Shaoxing-09	S	9	36	0.25	8.36E-06	3.05E-03
Shaoxing-10	S	9	37	0.24	8.13E-06	2.97E-03
Min		1	27	0.03	8.80E-07	3.21E-04
Max		9	38	0.25	8.36E-06	3.05E-03
Mean		4	32	0.11	3.74E-06	1.37E-03

[a] *SNP calculated by mapping each genome to the genome of Wuhan-Hu-1 (NC_045512.2) (6).*

[b] *Number of days between the date that the sample was collected and the date the Wuhan-Hu-1 sample was collected (12/31/2019).*

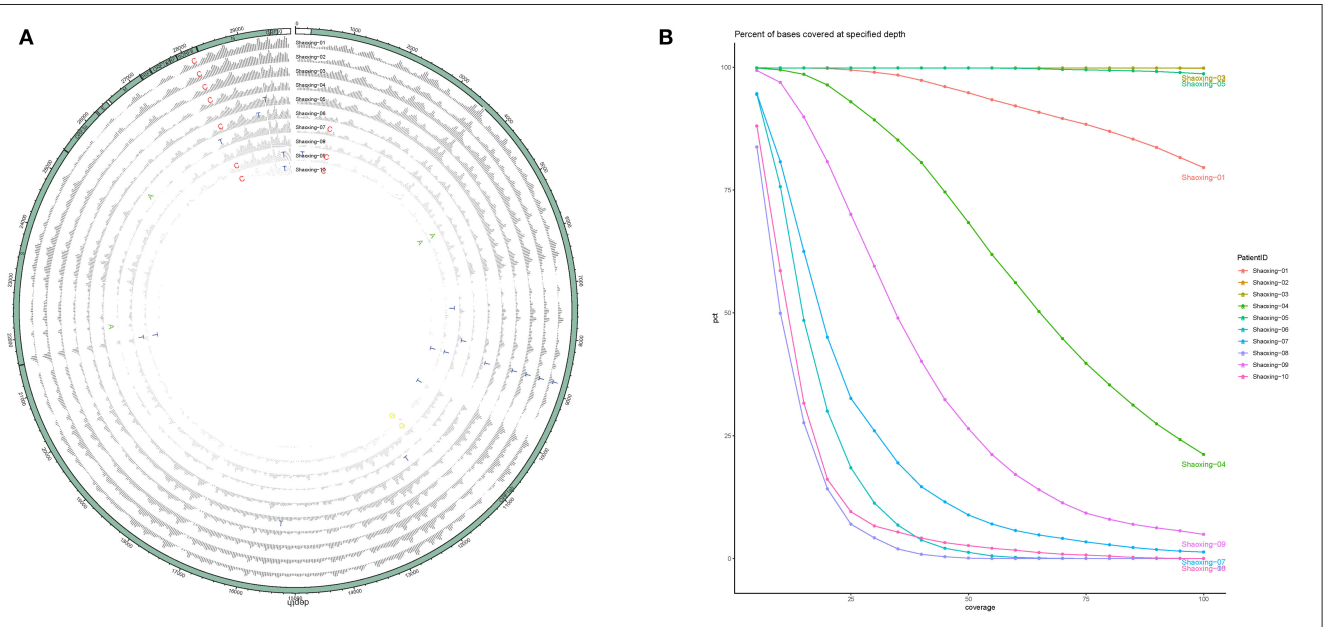

FIGURE 2 | Coverage and depth. **(A)** Coverage and depth map. Normalized depth at each position along the genome are shown for each sample, with SNPs marked by the alternate allele. **(B)** Depth ratio. The X-axis plots the log value of the depth for each genome while the Y-axis plots the cumulative percentage of bases covered to the specified depth.

TABLE 4A | Summary of SNPs in the 10 SARS-CoV-2 genomes.

SNP#	Position	Gene	Reference nt	MutationString	MutationString2	Shaoxing-01	Shaoxing-02	Shaoxing-03	Shaoxing-04	Shaoxing-05	Shaoxing-06	Shaoxing-07	Shaoxing-08	Shaoxing-09	Shaoxing-10
1	207	Non-coding	C	NA	NA									T (non-coding)	
2	889	orf1ab	T	A208A	orf1ab:A208A							C (A)			
3	946	orf1ab	T	G227G	orf1ab:G227G									C (G)	C (G)
4	5099	orf1ab	T	S1612T	orf1ab:S1612T									**A (S->T)**	**A (S->T)**
5	7420	orf1ab	C	I2385I	orf1ab:I2385I									T (I)	T (I)
6	8344	orf1ab	C	D2693D	orf1ab:D2693D								T (D)		
7	8782*	orf1ab	C	S2839S	orf1ab:S2839S	T (S)*	T (S)*	T (S)*	T (S)*		T (S)*			T (S)*	T (S)*
8	9962	orf1ab	C	H3233Y	orf1ab:H3233Y										**T (H->Y)**
9	11430	orf1ab	A	Y3722C	orf1ab:Y3722C									**G (Y->C)**	**G (Y->C)**
10	11916	orf1ab	C	S3884L	orf1ab:S3884L							**T (S->L)**			
11	15324	orf1ab	C	N5020N	orf1ab:N5020N					T (N)					
12	21676	S	C	Y38Y	S:Y38Y									T (Y)	T (Y)
13	22081	S	G	Q173Q	S:Q173Q							A (Q)			
14	25672	ORF3a	C	L94I	ORF3a:L94I							**A (L->I)**			
15	28000	ORF8	C	P36L	ORF8:P36L							**T (P->L)**			
16	28144*	ORF8	T	L84S	ORF8:L84S	C (L->S)*	C (L->S)*	C (L->S)*	C (L->S)*		C (L->S)*			C (L->S)*	C (L->S)*
17	29095	N	C	F274F	N:F274F						T (F)				
18	29303	N	C	P344S	N:P344S					**T (P->S)**					
19	29625	ORF10	C	S23F	ORF10:S23F									**T (S->F)**	**T (S->F)**
									Non-Synonymous	Synonymous					

SNP analysis was based on NC_045512.2 (Wuhan-Hu-1) as the reference genome (6). Variants are denoted as nucleotides vs. the reference base. Amino acid changes listed in parentheses with synonymous mutations listed as a single residue. The non-synonymous mutations are bolded.

**Mutations of the S clade.*

TABLE 4B | Depth of SNP in the 10 SARS-CoV-2 genomes.

Position	MutationString	Shaoxing-01	Shaoxing-02	Shaoxing-03	Shaoxing-04	Shaoxing-05	Shaoxing-06	Shaoxing-07	Shaoxing-08	Shaoxing-09	Shaoxing-10
207	NA	147	421	643	65	380	11	20	5	17	7
889	A208A	363	1,091	1,666	177	1,018	21	44	19	74	21
946	G227G	166	453	746	80	434	12	24	5	45	17
5099	S1612T	192	617	784	60	575	8	23	7	49	10
7420	I2385I	97	384	403	34	211	5	9	6	10	5
8344	D2693D	161	492	598	63	350	20	4	12	22	11
8782	S2839S	319	948	1,102	108	697	18	29	9	43	20
9962	H3233Y	285	973	1,085	116	796	23	33	23	60	13
11430	Y3722C	180	642	642	78	336	12	18	16	39	17
11916	S3884L	170	698	761	80	444	15	12	21	28	13
15324	N5020N	232	840	877	92	528	22	14	15	35	14
21676	Y38Y	246	1,010	941	105	543	30	36	12	62	14
22081	Q173Q	157	841	694	56	347	15	7	17	41	8
25672	L94I	124	557	476	41	328	7	15	7	37	11
28000	P36L	313	1,112	1,090	113	814	33	57	25	106	44
28144	L84S	189	815	728	79	495	29	44	21	53	19
29095	F274F	386	1,271	1,331	106	798	29	81	25	105	34
29303	P344S	497	1,438	1,452	168	1,067	43	128	36	173	67
29625	S23F	296	1,053	1,004	108	618	34	62	26	100	30

Orange highlighted cells indicate detected mutations and the unhighlighted cells indicate wild-type.

Using the SNP analysis, we calculated the various mutation rates using the number of days between the date that the sample was collected and the date the Wuhan-Hu-1 sample was collected. The mutation rate (SNP per day) ranged from 0.03 to 0.25 (**Table 3**). We used this mutation rate to calculate the nucleotide substitution per site per day and the nucleotide substitution per site per year. We saw an average mutation rate of 3.74×10^{-6} nucleotide substitution per site per day and an average mutation rate of 1.37×10^{-3} nucleotide substitution per site per year (**Table 3**).

We investigated each SNP to determine if there were any non-synonymous mutations in genes important to the virus lifecycle. Several non-synonymous mutations were found in the following genes: *orf1ab, orf3, N, orf8,* and *orf10* (**Table 4A**). These mutations were identified with sufficient confidence as at least 5X depth was achieved in all the SNPs (**Table 4B**, highlighted in orange), and at least 4X depth was achieved at the positions of these SNPs in all samples (**Table 4B**, unhighlighted). In 7 samples, we identified C8782T and T28144C mutations which are the landmark events of the S clade (**Table 4A**). We did not identify other GISAID clade defining mutations outlined by Mercatelli and Giorgi (12). No non-synonymous mutations were found in the S gene, which encodes the spike protein that's critical for viral binding to human receptor ACE2 (9). Notably in the cluster of the two family members (Shaoxing-9 and−10), the two viruses are closely related but not identical. Shaoxing-9 was infected first and then transmitted to Shaoxing-10, whose virus gained a non-synonymous mutation C9962T in the ORF1ab gene (**Table 4**). This could be explained by the sequential transmission, however, we could not rule out a possibility of intra-host viral heterogeneity in the two patients.

SARS-CoV-2 Genotype and Phylogenetic Characteristics

Previous reports demonstrate that SARS-CoV-2 has two genotypes known as L type and S type in the early phase of the outbreak (10); however, recent classification has divided the SARS-CoV-2 genomes into 7 different clades (O, S, L, V, G, GR, GH) (12). We decided to compare our 10 SARS-CoV-2 genomes to 163 other SARS-CoV-2 genomes obtained from GISAID (8) published by mid-March. Although the majority of the SARS-CoV-2 genomes obtained from GISAID in the early phase of the pandemic belonged to the L clade (**Figure 3**, green), the 10 Shaoxing SARS-CoV-2 genomes (**Figure 3**, black boxes) were distributed throughout these genomes with more of them classified in the S clade (**Figure 3**, orange). The more detailed phylogenetic tree with bootstrap values is shown in **Supplementary Figure 1**.

Interestingly, four of the Shaoxing SARS-CoV-2 genomes (Shaoxing−1 to−4) were identical to six other GISAID SARS-CoV-2 genomes (**Figure 3**, Cluster 1). These six other genomes were isolated from patients all over the world: two from Wuhan, two from Taiwan, one from Belgium, and one from Australia (**Figure 3**, Cluster 1). Shaoxing-6 is identical to five other genomes isolated in Shenzhen, Guangdong Province in Southern China (**Figure 3**, Cluster 2). Notably, in all 10 Shaoxing patients, we found no virus with D614G Spike gene mutation, which was shown to start spreading in Europe in early February, and

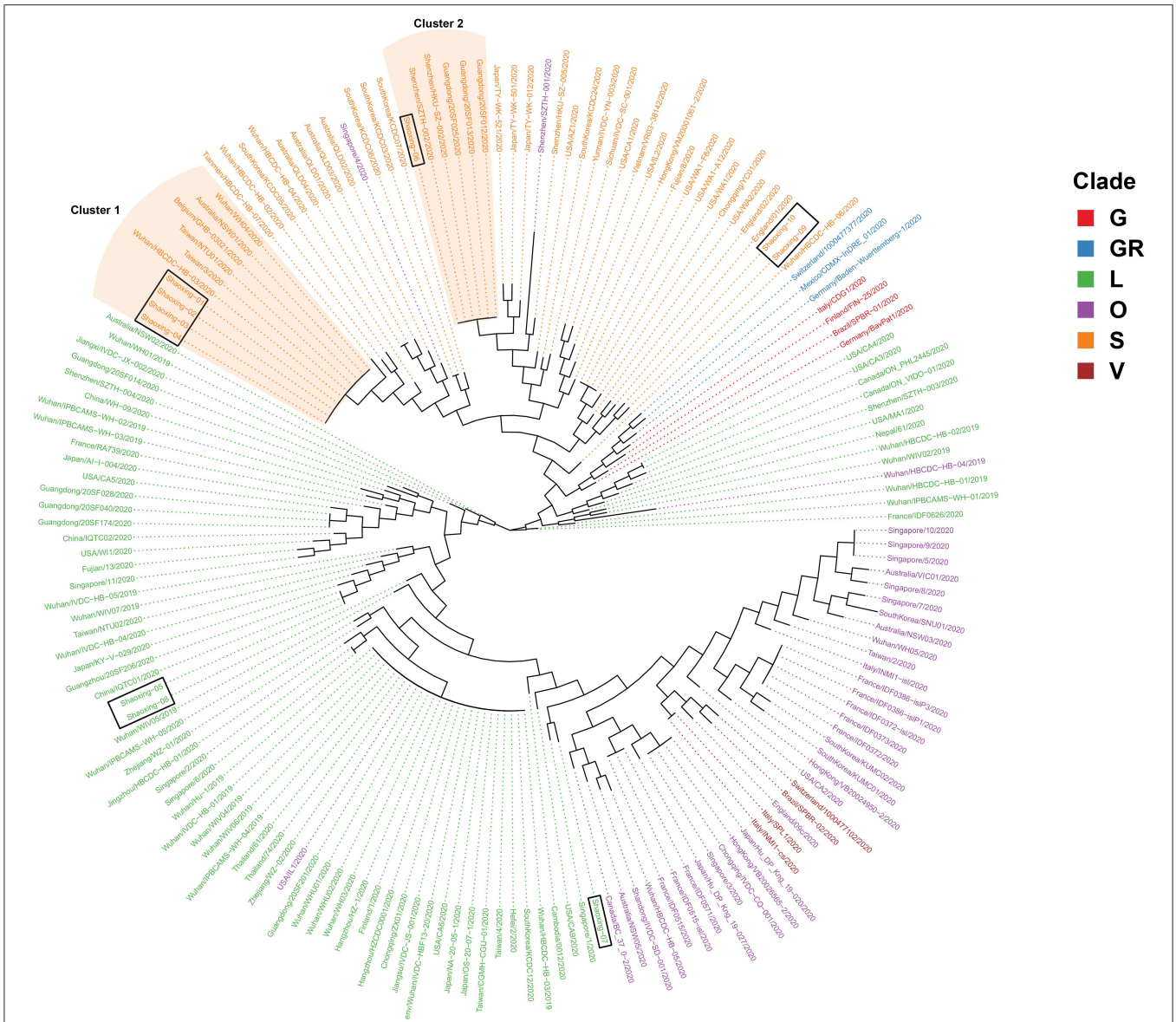

FIGURE 3 | Phylogenetic comparison of SARS-CoV-2 genomes. Phylogenetic comparison of 163 published genomes from GISAID (8) and the 10 Shaoxing genomes (boxed out). Genomes are color-coded based on their clade.

rapidly become the dominant form in the rest of the world out of China (20).

DISCUSSION

In this study, we sequenced the SARS-CoV-2 genome from 10 patient samples from Shaoxing, Zhejiang, China. Using metagenomic sequencing, we were able to obtain above 99% coverage and an average depth of 296X for all 10 SARS-CoV-2 genomes. Although not statistically significant, there does appear to be a clear negative correlation between the C_t values of both gene targets and the log count of SARS-CoV-2 RNA sequence reads acquired by metagenomics sequencing. This suggests that the log value of RNA sequence reads by metagenomics sequencing may be used as a semi-quantitative measurement for SARS-CoV-2 viral loads.

The rapid spread of this virus is highlighted by the fact that four SARS-CoV-2 genomes from Shaoxing individuals were identical to six other SARS-CoV-2 genomes from patients all over the world. Our data support recent publications that the virus had spread rapidly around the world especially in Europe before the United States (21–23).

Overall, we did not see a large number of SNPs in these SARS-CoV-2 genomes. The greatest number of SNPs

seen was 9 and these two SARS-CoV-2 genomes were from individuals with no travel history to Hubei province (**Table 3**, Shaoxing-9 and 10). Instead, Shaoxing-9 and 10 had contact with a confirmed case from Ningbo, another city outside of Hubei. We can use these data to infer that the virus accumulated more mutations when it was spread to another city outside of Hubei first before coming to Shaoxing, compared to the virus from people traveled to Shaoxing directly from Hubei.

We combined epidemiologic data with the SNP analysis to estimate the mutation rate of the SARS-CoV-2 from these 10 patients. We saw an average mutation rate of 1.37×10^{-3} nucleotide substitution per site per year for SARS-CoV-2, which is consistent with other reports on the mutation rate of SARS-CoV-2(19, 24) and SARS-CoV-1 with a reported mutation rate of $0.80–2.38 \times 10^{-3}$ nucleotide substitution per site per year (25). These data demonstrate that SARS-CoV-2 is similar in the mutation rate as other coronaviruses.

The major limitation of this study is that we only had 10 samples analyzed due to the requirement of sufficient SARS-CoV-2 RNA from a metagenomic sample. However, with the development of SARS-CoV-2 probe enrichment or multiplex PCR protocols, this type of viral sequencing analysis may be applied to samples with lower viral loads, thereby enabling more complete molecular epidemiological surveillance. In addition, the C_t value cut-off of 28 established in this study may not be directly applicable to other real-time PCR assays due to the technical differences. Inevitably, exclusion of samples with lower viral load could introduce bias in the genomic surveillance of SARS-CoV-2 and potentially lead to missed identification of important genotypes. Last, we did not analyze or predict the potential biological changes that may be caused by the identified mutations, such as alterations in RNA secondary structure, protein stability, interaction with host proteins, and codon usage, etc.

In summary, we demonstrated that a full viral genomic analysis is feasible via metagenomics sequencing directly on nasopharyngeal samples, which allows retrospective molecular surveillance on SARS-CoV-2 to understand the dynamics of the outbreak in the early phase. The identical virus found in patients in Shaoxing, a mid-sized city outside of Hubei, China, and patients in Europe and Australia was striking. Our analysis added to the growing body of evidence that SARS-CoV-2 spread extremely quickly around the globe as early as January. Although only 10 patients were included in this study, we found numerous mutations (both synonymous and non-synonymous) across the entire viral genome. Our study contributed to the understanding of the SARS-CoV-2 evolution in the early phase of the COVID-19 pandemic.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The raw FASTQ files have been deposited to NCBI SRA under BioProject PRJNA638211, and the snippy-based consensus genome sequences have been deposited to GISAID. They can be found with accessions EPI_ISL_463889 and EPI_ISL_463894 to EPI_ISL_463902.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JC, EH, FL, ZL, JT, and SY conceived and planned the experiments. JC, ZJ, QZ, JW, and YW carried out the experiments. EH, HW, and FL performed the data analysis. JC and EH took the lead in writing the manuscript. ZL, JT, and SY supervised the research project. All authors provided critical feedback and helped shape the research, analysis and manuscript.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.567621/full#supplementary-material

Supplementary Figure 1 | Phylogenetic tree of SARS-CoV-2 genomes with bootstrap values.

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Conflict of Interest: HW and YW were employed by the company Shaoxing IngeniGen XMK Biotechnologies. FL was the Chief Executive Officer of the company Three Coin Analytics. The authors declare that this study received funding from Shaoxing IngeniGen XMK Biotechnologies. The funder had the following involvement with this study: providing sequencing data and preliminary bioinformatics analysis.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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An Asymptomatic SARS-CoV-2-Infected Infant With Persistent Fecal Viral RNA Shedding in a Family Cluster: A Rare Case Report

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An outbreak of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become pandemic worldwide. A better understanding of asymptomatic infections is crucial to prevent and control this epidemic. Here, we report the epidemiological and clinical characteristics of a family cluster with SARS-CoV-2 infection. In the family cluster, a 32-year-old male (case 1) and a 53-year-old female (case 2, the mother-in-law of case 1) exhibited clinical symptoms of COVID-19, while case 1's 32-year-old wife (case 3) and their 11-month-old daughter (case 4) were both asymptomatic. Notably, case 4's nasopharyngeal swab samples was negative for nearly 80 days, and her immune system has been boosted for at least 57 days, but the fecal samples have tested positive for 100 days (May 13, 2020), suggesting SARS-CoV-2 may invade enterocytes and may exist in individuals with low antiviral immunity for a long term. This report highlights that asymptomatic infections should be managed with caution and vigilance. For SARS-CoV-2 testing of asymptomatic cases, besides the normally used nasopharyngeal swab, fecal sample testing is also needed.

Keywords: SARS-CoV-2, COVID-19, asymptomatic, fecal viral RNA shedding, family cluster

INTRODUCTION

An outbreak of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become pandemic worldwide. Although most individuals with SARS-CoV-2 infection exhibit clinical symptoms, a few [1.2% in China (1)] cases do not. Mounting evidence indicates asymptomatic carriers may still be infectious (2–4). Given that asymptomatic cases are not easily identified from the population, they are often neglected by infection prevention and control protocols, and they therefore have a greater potential transmission risk. A better understanding of asymptomatic infections is crucial to

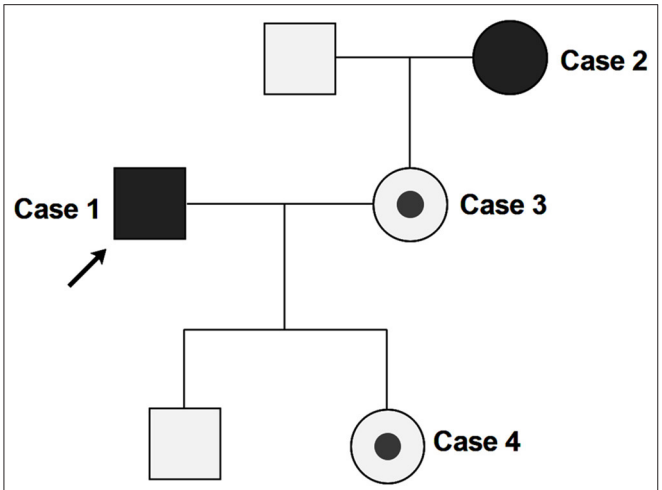

FIGURE 1 | Pedigree of the clustered SARS-Cov-2 infection cases. Arrow indicates the proband (case 1). Case 2, 3, and 4 are close contacts of case 1. Full black fills indicate the individuals with COVID-19 symptoms (case 1 and 2), while partial black fills indicate asymptomatic carriers of SARS-CoV-2 (case 3 and 4).

prevent and control of this epidemic. Here, we report the epidemiological and clinical characteristics of a family cluster with SARS-CoV-2 infection, notably including an 11-month-old infant with persistent fecal viral RNA shedding for 100 days until now (May 13, 2020).

CASE PRESENTATION

In this family cluster, a 32-year-old male (case 1) and a 53-year-old female (case 2, the mother-in-law of case 1) exhibited clinical symptoms, while case 1's 32-year-old wife (case 3) and their 11-month-old daughter (case 4) were both asymptomatic (**Figure 1**).

On Jan 19, 2020, case 1 (index patient) visited Wuhan (Hubei, China) for a meeting and then took a train from Wuhan on Jan 21 and arrived at Liaocheng (Shandong, China) on Jan 22 (**Figure 2**). He had a fever of 37.8°C and a productive cough after 4 days (**Figure 2** and **Table 1**). On Jan 28, he was taken to Dong'e People's Hospital (Liaocheng, Shandong, China) by ambulance. After pre-screening and triage, he was admitted to the Infectious Disease Unit for isolation as a suspected case. His chest computed tomogram (CT) scans showed multiple mottling and ground-glass opacities in the bilateral lung (**Figure 3A**), suggesting this patient has developed to pneumonia. SARS-CoV-2 nucleic acid tests (ORF1ab and N genes) of the nasopharyngeal swab samples were positive by qRT-PCR (BioGerm, Shanghai, China). According to the instruction, the kit covers 100% of known COVID-19 sequences and has no cross-reactivity with other pathogens, and the limit of detection of the kit is 1,000 copies/mL. His clinical classification was moderate. Remission was achieved after treatment with antiviral and traditional Chinese medicine.

Case 2 was a close contact of case 1 from Jan 22 to 26, and afterwards she developed to a fever of 38.9°C and cough (**Figure 2**). On Feb 1, she visited the hospital for diagnosis and treatment. Her chest CT showed multiple inflammatory changes and bronchitis in the bilateral lung (**Figure 3B**). Her nasopharyngeal swab sample was detected with positive SARS-CoV-2 RNA. She was classified as a moderate case. After antiviral treatment and supplement of albumin and human immunoglobulin, her symptoms were gradually relieved. After two consecutive negative SARS-CoV-2 nucleic acid tests of nasopharyngeal swabs with 24 h interval, case 1 and 2 were discharged on Feb 15 (**Figure 2**).

As close contacts of case 1 and 2, case 3, and 4 visited the Fever Clinic for further examination on Feb 4 (**Figure 2**). Both individuals did not show any clinical symptoms, such as fever, chills, cough, sore throat, rhinorrhea, or diarrhea, and no signs of pneumonia was observed on chest CT scans (**Table 1** and **Figures 3C,D**). However, their nasopharyngeal swab and fecal samples were both tested positive for SARS-CoV-2. On the same day, the two cases were admitted to the Infectious Disease Unit for isolation as asymptomatic cases. After 36 days of isolation and observation, case 3's nasopharyngeal swab and fecal samples turned negative for SARS-CoV-2 (**Figure 2**).

For case 4, her nasopharyngeal swab samples turned negative after 22 days of isolation, but the fecal samples have tested positive until now (100 days by May 13; **Figure 2**). Repeated blood routine examination showed decreased neutrophils and elevated lymphocytes (**Supplementary Table 1**), indicating a persistent viral infection. Flow cytometry showed that the counts of NK cells, B cells, and T-cell subtypes were all out of the reference ranges (**Supplementary Table 1**), indicating activated innate and adaptive immunity responses. The SARS-CoV-2 specific IgG was detectable on Mar 18 and Apr 12, indicating an active humoral immune reaction against the virus (5, 6).

DISCUSSION

The transmission concealment, symptom subjectivity and detection limitations increase the transmission risk of asymptomatic infections. In this report, case 2 and case 1 appeared symptoms of infection almost at the same time, suggesting that the infection seems to be transmitted during the incubation period of the index patient. To the best of our knowledge, case 4 represents the longest SARS-CoV-2 virus RNA shedding (100 days) in asymptomatic carriers. Although RT-PCR-based SARS-CoV-2 tests of the nasopharyngeal swab samples have turned negative for nearly 80 days, and the infant's immune system has been boosted for nearly 60 days, her fecal samples have still been SARS-CoV-2 positive.

Currently, case 4 has no respiratory symptoms; considering the negative SARS-CoV-2 of nasopharyngeal swab samples, the viruses might have been eliminated from the respiratory system. Since RNA fragments' half-life is very short (7), we speculate that SARS-CoV-2 viruses, rather than the RNA fragments, still

Abbreviations: COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CT, computed tomogram; ACE2, angiotensin-converting enzyme 2.

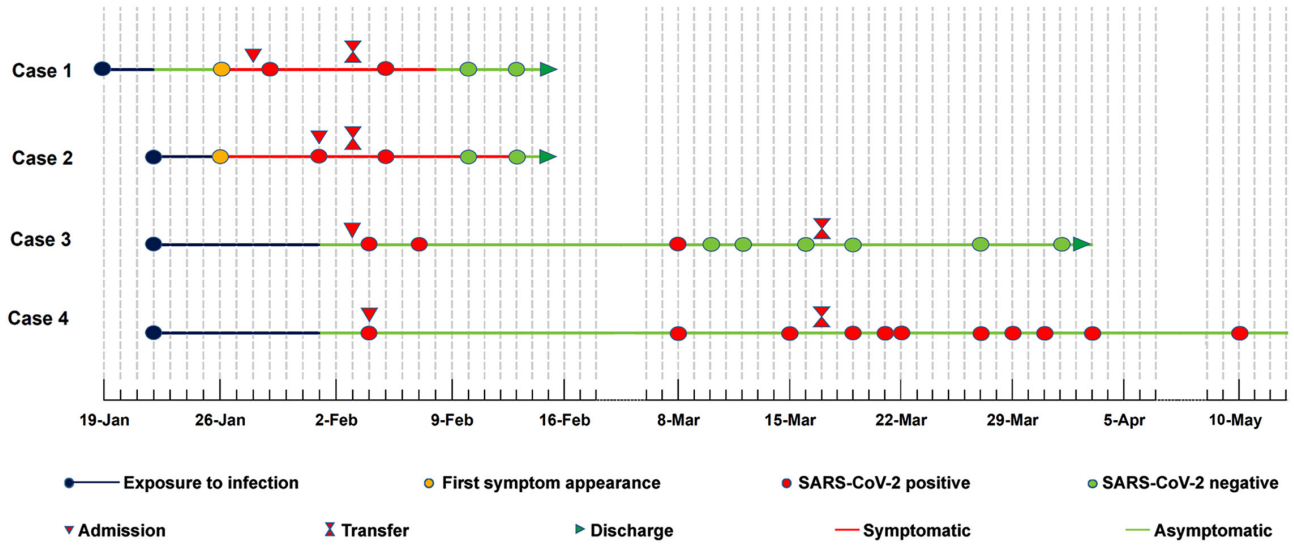

FIGURE 2 | Timeline of the cases exposed to SARS-CoV-2.

TABLE 1 | Epidemiological and clinical characteristics of the family-clustered cases with SARS-CoV-2 infection.

Characteristics	Case 1	Case 2	Case 3	Case 4
Age	32 years	53 years	32 years	11 months
Gender	Male	Female	Female	Female
Symptoms at admission				
Fever	√	√	×	×
Chills	×	×	×	×
Cough	√	√	×	×
Sore throat	×	×	×	×
Rhinorrhea	×	×	×	×
Diarrhea	×	×	×	×
Chest CT scan				
Mottling and ground-glass opacity	√	√	×	×
Blood analysis				
Leukocytes (× 10^9/L; normal range 5–12)	4.85	6.46	7.00	11.35
Neutrophils (× 10^9/L; normal range 1.8–6.3)	3.07	4.74	4.56	1.33
Lymphocytes (× 10^9/L; normal range 1.1–3.2)	1.37	1.30	1.88	9.27
CRP (mg/L; normal range 0–10)	10.90	22.70	0.50	3.00
Antibody of respiratory pathogen test				
Influenza A	×	×	×	×
Influenza B	×	×	×	×
Parainfluenza	×	×	×	×
Respiratory syncytial virus	×	×	×	×
Adenovirus	×	×	×	×
Mycoplasma pneumoniae	×	×	×	×
Chlamydia pneumoniae	×	×	×	×
Legionella pneumophila	×	×	×	×

√, with; ×, without; CRP, C-reactive protein.

exist, which is supported by the reduced neutrophils and elevated lymphocytes. As fecal samples have been constantly positive, we suspect that the viruses may be present in the gastrointestinal tract. Previous studies have shown that angiotensin-converting enzyme 2 (ACE2), a critical SARS-CoV-2 receptor (8), is not only expressed in the lung cells but also in the enterocytes (9).

Even though the enterocytes of case 4 may have viruses, she did not show any gastrointestinal symptoms, such as diarrhea, abdominal pain, nausea, or vomiting. Despite the production of IgG, the immune function of infants under 3-year-old has not been fully developed (10), which could be the reason for incomplete elimination of SARS-CoV-2 viruses in this case.

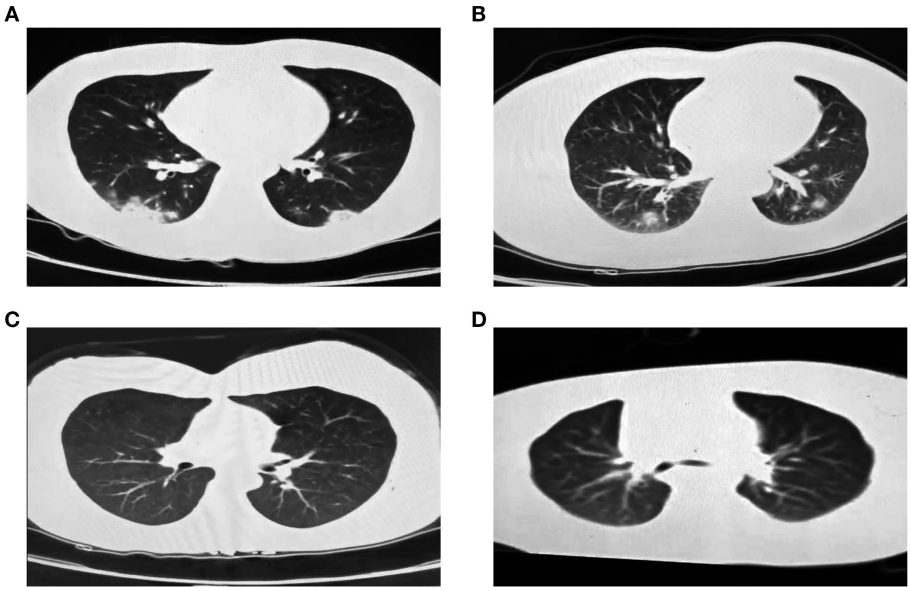

FIGURE 3 | Chest CT images of the four family-clustered cases. **(A)** Case 1. **(B)** Case 2. **(C)** Case 3. **(D)** Case 4.

Although carrying viruses, the body might be in an immune-tolerance stage and as such may not present related symptoms. It is still unpredictable whether and when the SARS-CoV-2 infection would turn negative in this case. It is still unknown whether SARS-CoV-2, like hepatitis B, could exist in individuals with low anti-viral immunity for a long time and who may become long-term carriers.

The infectivity, transmissibility, and epidemiology of asymptomatic carriers have not been clearly illustrated, and further knowledge is urgently needed. Nevertheless, before thorough understanding, asymptomatic infections still should be managed with caution and vigilance. According to the clinical experience management for asymptomatic carriers, here are some suggestions: (1) pay close attention to asymptomatic infections with intensive monitor and detection; (2) similar to the confirmed COVID-19 patients, asymptomatic carriers should also be isolated and observed until the nucleic acid test turns negative; (3) close contacts of asymptomatic carriers should also be screened for SARS-CoV-2 infection; and (4) for the SARS-CoV-2 nucleic acid test, besides the normally used nasopharyngeal swab, fecal sample-testing is also needed.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

SheC, JS, WT, LP, MA, HZ, SX, and KW collected the samples and contributed to data acquisition. BF, ShuC, WZ, and WL designed the study. SheC, AZ, and BF wrote and edited the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Effectiveness of Surgical Face Masks in Reducing Acute Respiratory Infections in Non-Healthcare Settings: A Systematic Review and Meta-Analysis

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Background: Acute respiratory illnesses (ARIs) are the most common respiratory infectious diseases among humans globally. Surgical mask (SM) wearing has been shown to be effective in reducing ARI among healthcare workers. However, the effectiveness of SM in reducing ARI in the non-healthcare settings remains unclear. This review aims to summarize and assess the association between SM wearing and ARI incidence, from existing interventional and observational studies conducted in non-healthcare settings.

Methods: Systematic literature searches conducted in PubMed, Cochrane Library, and Embase databases identified 503 unique studies. After screening, 15 studies (5 randomized controlled trials and 10 observational studies) were assessed for reporting and methodological qualities. Proportions of ARI episodes in each group and adjusted summary statistics with their relevant 95% CIs were extracted. Data from 10 observational studies were pooled using the generic inverse variance method.

Results: A total of 23,892 participants between 7 and 89 years old involved across 15 studies from 11 countries were involved. Key settings identified were Hajj, schools, and in-flight settings. A modest but non-significant protective effect of SM on ARI incidence was observed (pooled OR 0.96, 95% CI 0.8–1.15). Subgroup analysis according to age group, outcome ascertainment and different non-healthcare settings also revealed no significant associations between SM use and ARI incidence.

Conclusion: Surgical mask wearing among individuals in non-healthcare settings is not significantly associated with reduction in ARI incidence in this meta-review.

Keywords: surgical mask, systematic review, acute respiratory infection, non-healthcare settings, prevention

INTRODUCTION

Acute respiratory infections (ARIs) have resulted in significant morbidity and mortality globally. Many respiratory viruses attribute to ARI. These include influenza viruses, rhinoviruses, and coronaviruses. Coronaviruses, namely, human coronavirus NL63, 229E, OC43, and HKU1, attributed to a significant proportion of ARI (1, 2). Similarly, SARS-CoV (2003) (3), MERS-CoV

(2012) (4), and the recent SARS-CoV-2 (5) are transmitted via droplet/aerosols and close contacts and resulted in significant fatality. At the time of writing, the global toll of COVID-19 stands at 2,145,512 cases, including 143,308 deaths (6).

In the absence of pharmaceutical interventions such as vaccine and anti-virals for most respiratory viruses including coronavirus disease 2019 (COVID-19) (7), non-pharmaceutical interventions such as personal protection equipment are crucial to curb community spread (7). However, there are inconsistent policies and recommendations on the use of surgical masks (SM) in the community in the early stage of the COVID-19 pandemic. WHO (8), Centers for Disease Control and Prevention (CDC), and national authorities have advocated the usage of SM, as opposed to N95 respirators, only among symptomatic individuals. Otherwise, one is to practice good personal and hand hygiene as the key mitigation measure.

WHO only conditionally recommends SM wearing by asymptomatic individuals in the community in situations of epidemic and pandemic (9). However, as community transmission becomes more rampant in many countries at the early phase of the pandemic, mask wearing has become a norm, as asymptomatic transmission remains a possibility with limited evidence to show otherwise (10, 11). With an increase in SM usage worldwide, a global shortage which is detrimental to the healthcare setting and pandemic control ensues.

The efficacy of SM usage to prevent transmission of influenza-like illness (ILI) and laboratory-confirmed influenza have been shown in a number of studies among symptomatic patients (12–14). However, the protective effect of SMs among healthy individuals in a community setting remains unclear. Existing systematic reviews and meta-analyses consistently found SMs ineffective at preventing ILI or influenza episodes when worn by an uninfected individual (15–17). However, a study that examined the protective effect of SM use against secondary influenza episode in a household setting, found a 70% reduction in reported episodes when participants were compliant in SM use (18).

Conflicting stance regarding the usage of SMs among healthy individuals to reduce the risk of respiratory infections remains even with the publication of a systematic review assessing the efficacy/effectiveness of SM against respiratory infections in 2011 (16). The review found face mask to be the best performing non-pharmaceutical intervention across seven included studies. However, all included studies primarily assessed SARS incidence only, and were predominantly hospital based (85.7%) or only involved healthcare workers (71.4%). The remaining non-hospital-based study involved SM usage in households with healthcare workers as the index case, and another included non-healthcare workers who were hospitalized. In hospital settings and/or among healthcare workers, occupational requirements, and increased knowledge on personal protection increase compliance to SM usage. However, SM usage may differ significantly in non-healthcare-related settings or workers. Thus, the review's findings may not extend to a community setting and/or a non-healthcare setting. With the limited supply of surgical mask for the healthcare workers globally to manage the large influx of COVID-19 patients, there is a pressing need to investigate the efficacy or effectiveness of SM use in the non-healthcare settings so as to guide policyholder on the usage of SM in the community. Thus, the study aims to perform a systematic review and meta-analysis to assess the effectiveness or efficacy of SM usage in decreasing the incidence of respiratory infectious disease and non-influenza respiratory infection in the community.

MATERIALS AND METHODS

Search Identification and Selection

Systematic literature searches were conducted in PubMed, Cochrane Library, and Embase databases. Eligible studies were assessed for the reporting and methodological quality. Proportions of populations reporting ARI episodes in each group and adjusted summary statistics with their relevant 95% CIs were extracted when reported. A pooled odds ratio was estimated using the generic inverse variance method and heterogeneity was assessed. Relevant peer-reviewed literature that assessed the effectiveness/efficacy of surgical face masks (SM) in preventing community-acquired acute respiratory infections (ARIs) were identified and extracted from PubMed, EMBASE, and Cochrane databases on February 25, 2020. Specific search terms defined by the Population, Intervention/Exposure, Comparator, and Study design (PICOS/PEOS; **Supplementary Table 1**) utilized for each database are provided in (**Supplementary Table 2**). In all databases, a filter to identify studies published from 2010 was applied to capture more recent published studies that are more representative of the current social, behavioral, educational, and economic status of the general population, which may be attribute to the risk of ARI and compliance to SM usage. Reference lists of relevant reviews were also hand-searched to identify additional studies. This study was conducted in accordance to Cochrane's Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

Identified publications were screened according to criteria in the following hierarchy by three authors, and any disagreement was reviewed by the fourth author to reach a final consensus, and included in the review if they fulfilled all criteria:

- Type of intervention/exposure: Surgical face mask usage in comparison with a comparable control group (no surgical face mask usage or use of hand hygiene practices only).
- Type of study: Peer-reviewed publications on interventional (randomized controlled trials) and observational (cohort studies, cross-sectional studies, and case-controlled studies) studies.
- Type of participants: Participants are individuals living in a general community setting, not healthcare workers or patients in clinical and medical setting.
- Types of outcomes: Incidence or episodes of (i) acute respiratory infectious disease and (ii) non-influenza respiratory infections in a community setting.

This review defines ARI as any acute respiratory infectious disease, including influenza-like illnesses and non-influenza respiratory infections, regardless whether the illness was clinically diagnosed, laboratory confirmed, or self-reported as defined by

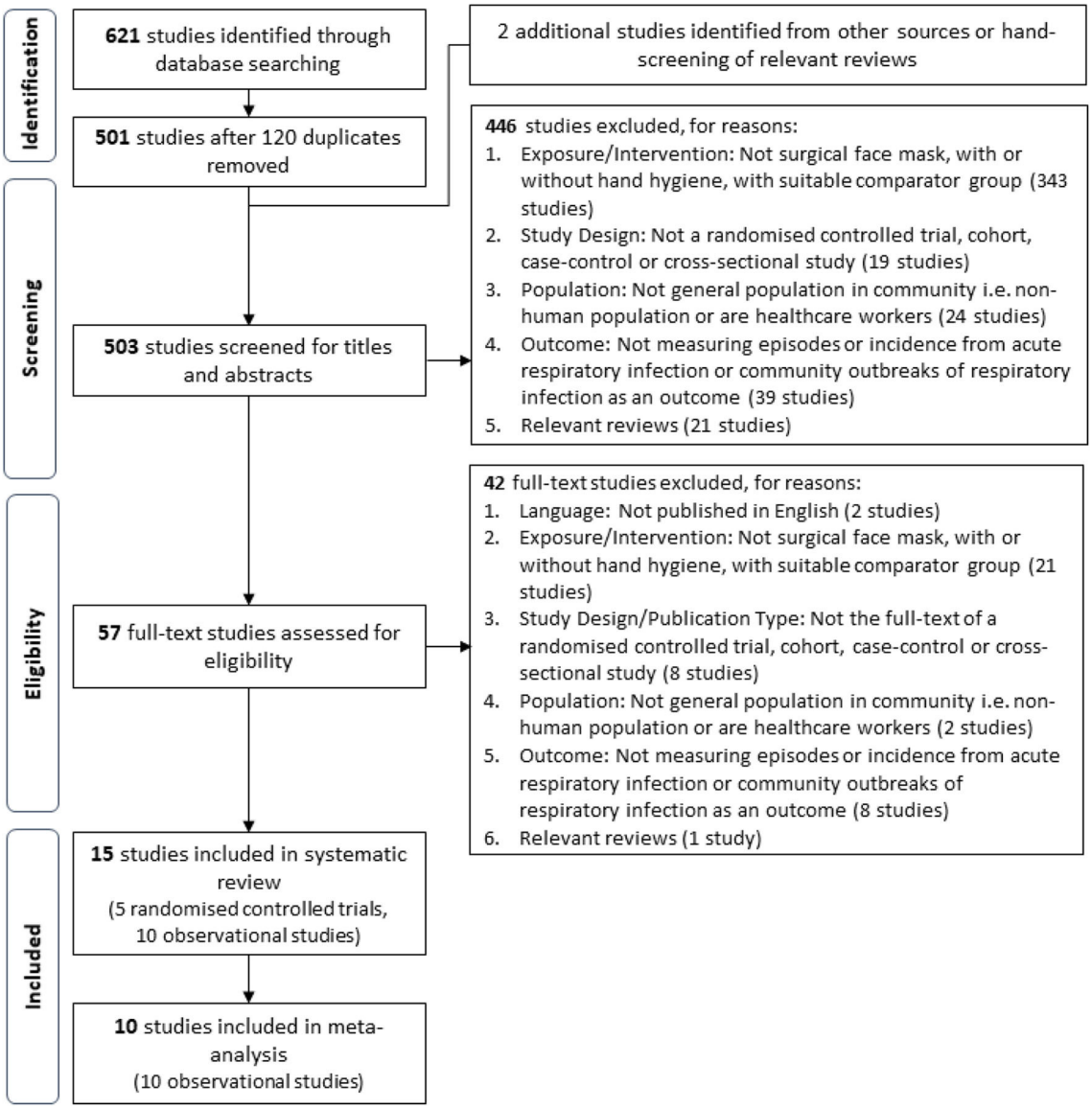

FIGURE 1 | Flowchart of the process of screening and study selection.

the study. Studies utilizing variations of facial protective gear (e.g., respirators and goggles) as an intervention/exposure or were conducted in settings outside of the general community were excluded in alignment with the goal to assess the recommendation of SM usage in a community setting. Relevance of the extracted studies was first assessed with titles and abstracts, before full texts of relevant studies were retrieved for further screening and validation based on the aforementioned criteria. A PRISMA flow diagram of the study selection process is shown in **Figure 1**.

Data Extraction

Data extracted from included studies were consolidated with Microsoft Excel 2016, and presented in **Tables 1**, **2**. Corresponding authors of included studies were contacted when clarification or more information were required. The following data were extracted from each study: authors, year of publication, study and population characteristics, description of the measures implemented in intervention and control groups, and outcomes. Study designs of included studies were also assessed based on their design features as recommended by the Cochrane Handbook for Systematic Reviews and Meta-analyses (33), in addition to extracting their reported study design. Outcome measures extracted for the intervention and control groups, when available, include (1) number of ARI episodes, (2) summary statistic for ARI incidence [relative risk (RR), odds ratio (OR), or hazard ratio (HR)] and their corresponding 95% confidence interval (95% CI), and (3) any other key findings.

In addition, the review authors noticed some of the selected studies had an additional intervention group which

TABLE 1 | Characteristics of included studies.

References	Study design	Country of study; study duration	Population size (% men); percentage with recent flu vaccine			Age group, mean age (SD) in years; description of population health status	Description of measures implemented		Overall quality	
			Overall	Control	Intervention		Control	Intervention	Reporting	Methods
Randomized controlled trials										
Aiello et al. (19)	Cluster randomized trial	USA; 6 weeks	930 (26.7%); 13.8%	552 (18.1%); 14.7%	378 (39.2%); 12.4%	Adult; 18.7 (0.8); Participants are seemingly healthy college students from University of Michigan during the 2006–2007 influenza season	Basic hand hygiene education	Wearing surgical facemask as much as possible in residence hall during intervention period	High	High
Simmerman et al. (20)	Cluster randomized trial	Thailand; 1 week	583 (40.5%); 0%	292 (40.1%); 0%	291 (40.9%); 0%	Adult; 34 (24–42)*; Household contacts of a pediatric index case with influenza-like illness, health status of participants not reported	Hand washing education and kit	Mask wearing in index patients and all household contacts from any point in time within 7 days from randomization, and hand washing education and kit	High	High
Aiello (2012) (21)	Cluster randomized trial	USA; 6 weeks	762 (43.0%); 16.3%	369 (43.9%); 17.6%	391; (42.5%); 15.1%	Adult; 18.95 (0.9); Participants are seemingly healthy college students from University of Michigan during the 2007–2008 influenza season	Basic education on proper hand hygiene and use of standard surgical face masks	Wearing surgical facemask for ≥6 h in residence hall during intervention period	High	High
Suess et al. (18)	Cluster randomized trial	Germany; 8 days	151 (48.3%); 9.3%	82 (47.6%); 7.3%	69 (49.3); 11.6%	Adult and child Control*: 2009: 35 (18–40) 2010: 38 (12–43) Intervention*: 2009:37 (12–43) 2010: 35 (17–42) Household members of laboratory-confirmed index patients during 2 consecutive influenza seasons (Nov 2009–Jan 2010 and Jan–Apr 2011) in Berlin. Chronic illness was present in household members in the following proportions: 19.8% of the control group and 15.4% of the intervention group	Provision of general information on infection control to household	Healthy household members to wear masks at all times when in one room with the index patient and/or any other household member with respiratory symptoms	High	High

(Continued)

TABLE 1 | Continued

References	Study design	Country of study; study duration	Population size (% men); percentage with recent flu vaccine			Age group, mean age (SD) in years; description of population health status	Description of measures implemented		Overall quality	
			Overall	Control	Intervention		Control	Intervention	Reporting	Methods
Barasheed et al. (22)	Cluster randomized trial	Australia; 1 week	89 (NR); NR	53 (NR); NR	36 (NR); NR	Adult and child Control: 41.6 (17–72)* Intervention: 48 (19–80)* Australians attending Hajj in 2011, health status of the pilgrims were not reported but 36 of them were aged 65 and above or had chronic disease	No face masks provided; only general information on hygiene was provided	Provision of face masks, and advice and instructions on mask usage through participants' stay in Mina	Low	High
Observational studies										
Deris et al. (23)	Cross-sectional	Malaysia; entire hajj duration	387 (56.6%); 72.9%	105 (NR); NR	282 (NR); NR	Adult; 50.4 (11); Health status of Malaysian pilgrims attending Hajj in 2007 was not reported	Non-usage of face masks during Hajj	Use of face mask during Hajj	Low	
Gautret et al. (24)	Cohort	France; 4 weeks	274 (NR); NR	56 (NR); NR	218 (NR); NR	Adult; 58 (23–83)*; French pilgrims attending Hajj in 2009 had varying health status: 23.7% had diabetes mellitus, 5.5% had chronic respiratory disease, 3.3% had chronic cardiac disease, and 2.2% had other chronic conditions	Non-usage of face masks during Hajj	Use of face mask during Hajj	Low	
Al-Jasser et al. (25)	Cross-sectional	Saudi Arabia; 2 weeks	1,507 (61/7%); 94.4%	328 (NR); NR	216 (NR); NR	Adult; 37.9 (12.2); Hajj pilgrims living in Riyadh city who were performing Hajj in 2010, 18.4% of the pilgrims had chronic diseases including diabetes, hypertension, cardiac diseases, bronchial asthma and renal diseases	Never used face mask during Hajj in Mecca	Use of face mask most of the time during Hajj in Mecca	High	
Al-Jasser et al. (25)				328 (NR); NR	635 (NR); NR		Never used face mask during Hajj in Mecca	Use of face mask sometimes or occasionally during Hajj in Mecca		
Balaban et al. (26)	Cross-sectional	USA; 24.1 weeks*	186 (49.5%); at least 74.2%#	54 (NR); NR	89 (NR); NR	Adult and child; 48.9 (16–89)#; US pilgrims attending the 2009 Hajj, who resided in Michigan and Minnesota, of which 31 pilgrims were with chronic conditions (diabetes, hypertension, asthma), of which 13 were <65 years old	Non-usage of face masks during Hajj	Use of face mask during Hajj	High	

(Continued)

TABLE 1 | Continued

References	Study design	Country of study; study duration	Population size (% men); percentage with recent flu vaccine			Age group, mean age (SD) in years; description of population health status	Description of measures implemented		Overall quality	
			Overall	Control	Intervention		Control	Intervention	Reporting	Methods
Kim et al. (27)	Cross-sectional	South Korea; 3 weeks	7,449 (42.3%); 23.1%	2,082 (NR); NR	466 (NR); NR	Child; 12.97 (3.03); School-aged children between 7 and 18 years old, attending schools in Seodaemun-gu, Seoul, some children had the following conditions: asthma ($n = 171$), atopy ($n = 891$), cardiac disease ($n = 20$), renal disease ($n = 12$), liver disease ($n = 11$), diabetes ($n = 6$)	Non-usage of face mask	Continued use of face mask	High	
Kim et al. (27)				2,082 (NR); NR	2,819 (NR); NR		Non-usage of face mask	Irregular use of face mask		
Gautret et al. (28)	Cross-sectional	France; 3 consecutive Hajj seasons	360 (NR); 31.6%∧	167 (NR); NR	193 (NR); NR	Adult; 60.6 (22–85)[#]: French pilgrims attending Hajj from 2012 to 2014 were with the following comorbidities: 55.1% had a chronic disease; 30.2% with hypertension, 27.5% with diabetes, 8.4% with chronic cardiac disease, 7.6% with chronic respiratory disease, 1.3% with immune deficiency, and 0.3% with chronic renal disease	Non-usage of face masks during Hajj	Use of face mask during Hajj	Low	
Hashim et al. (29)	Cross-sectional	Malaysia; 1 week	468 (56.2%); 65.2%	80 (NR); NR	322 (NR); NR	Adult and child; 52.52 (10.15); 60% of the Malaysian pilgrims attending the 2013 Hajj had at least one medical illness: 26.5% had hypertension, 15.4% had diabetes mellitus, 9.0% had allergic rhinitis, 5.6% had bronchial asthma and others (3.6%)	Non-usage of face masks during Hajj	Use of face mask during Hajj	High	
Uchida et al. (30)	Cross-sectional	Japan; 1 week	10,524 (51%); 48.1%	5,050 (NR); NR	5,474 (NR); NR	Children; 9.45 (7–12)[#]; 10.6% of the schoolchildren recruited from elementary schools in Matumoto City had underlying diseases	Non-usage of face masks	Use of face mask at any place or time during the 2014/2015 influenza season (response provided by guardians of the children)	High	

(Continued)

TABLE 1 | Continued

References	Study design	Country of study; study duration	Population size (% men); percentage with recent flu vaccine			Age group, mean age (SD) in years; description of population health status	Description of measures implemented		Overall quality	
			Overall	Control	Intervention		Control	Intervention	Reporting	Methods
Emamian et al. (31)	Nested case–control	Saudi Arabia; During Hajj	95 (57.9%); 75.8%	38 (NR); NR	57 (NR); NR	Adult; NR, but 54.7% of the pilgrims were <60 years and 47.3% were ≥60 years old; 38.95% of recruited Hajj pilgrims had at least systemic disease, defined as asthma, diabetes mellitus, hypertension, chronic obstructive pulmonary disorder and cardiovascular diseases)	Non-usage of face masks during Hajj	Use of face mask during Hajj	Low	
Zhang et al. (32)	Retrospective case–control	China; 2 weeks	41 (48.8%); NR	26 (NR); NR	15 (NR); NR	Adult and child; Not reported but age demographics are as follows: 19.5% were <20 years old, 46.3% were between 20 and 40 years old, and 34.2% were >40 years old; Health status of passengers on the flight from New York to Hong Kong, including a stopover in Vancouver, was not reported	Non-usage of face masks during any leg of the flight	Use of face mask during either leg of the flight: (1) New York to Vancouver, (2) Vancouver to Hong Kong, (3) New York to Hong Kong, and (4) Hong Kong to Fuzhou	Low	

Median (interquartile range); #mean (range).

TABLE 2 | Key findings of wearing surgical face masks on ARI incidence.

References	Outcome measured and ascertainment	Outcome definition	% Population infected (infected/population size)		Reported summary risk estimate: risk ratio (RR), odds ratio (OR), or hazard ratio (HR) (95% CI); *p*-value (if significant)			Key findings
			Control	Intervention	RR	OR	HR	
Aiello et al. (19)	Self-reported ILI through weekly survey on ILI symptoms, and clinical diagnosis of ILI by study nurse during scheduled visits	ILI defined as presence of cough and at least 1 constitutional symptom (fever/feverishness, chills, or body aches)	32.1% (177/552)	26.2% (99/378)			0.9 (0.77–1.05)[a]	**Decreased but not statistically different ILI incidence rate** ($p > 0.025$) in face mask-only group, compared with control group from 4th week of intervention onwards Adjusted rate ratio[a] (95% CI): 4th week: 0.72 (0.53–0.98) 5th week: 0.65 (0.42–0.98) 6th week: 0.58 (0.34–1.00)
Simmerman et al. (20)	Laboratory-confirmed secondary influenza episode	Positive rRT-PCR result on days 3 or 7 or a fourfold rise in influenza HI antibody titers with the virus type and subtype matching the index case	19.2% (58/302)	22.7% (66/291)				No significant difference in odds for secondary influenza infection in mask-wearing group compared with original control group provided with unrelated health education and no relevant non-pharmaceutical intervention Adjusted OR[b] (95% CI) 1.16 (0.74–1.82) No significant difference in individual-level secondary attack rate across all experimental arms, including original control arm (Pearson χ^2 for difference among the three intervention arms, adjusted for within-household correlation of 0.18 = 0.63)
Aiello et al. (21)	Self-reported ILI through weekly survey on ILI symptoms, and clinical diagnosis of ILI by study nurse during scheduled visits	Presence of cough and at least 1 constitutional symptom (fever/feverishness, chills, or body aches)	13.8% (51/302)	11.8% (46/291)			1.1 (0.88–1.38)[c]	No significant reductions in ILI or laboratory-confirmed influenza incidence in the face mask only group compared with the control, through the entire intervention duration, regardless whether summary estimates were adjusted[c] or unadjusted
	Laboratory-confirmed influenza episode (only tested when ILI was self-reported/clinically diagnosed)	Positive RT-PCR result	4.3% (16/302)	3.1% (12/291)			0.92 (0.59–1.42)[c]	
Suess et al. (18)	Self-reported ILI, defined by the presence of fever with cough or sore throat	Presence of fever and cough or sore throat	34.1% (14/41)	17.1% (6/35)	0.61 (0.2–1.87)[d]			No significant difference in secondary attack rate across the groups, regardless whether the case was defined with ILI or laboratory-confirmed influenza definition, and after stratification for influenza season, virus subtype or timing of the first household visit (*p*-values ranged from 0.16 to 0.57) **70% reduction in odds of laboratory-confirmed influenza incidence** in mask-only group when per-protocol analysis was utilized Control OR (95% CI): reference Mask group OR[d] (95% CI) 0.30 (0.1–0.94), p=0.04

(Continued)

TABLE 2 | Continued

References	Outcome measured and ascertainment	Outcome definition	% Population infected (infected/population size)		Reported summary risk estimate: risk ratio (RR), odds ratio (OR), or hazard ratio (HR) (95% CI); *p*-value (if significant)			Key findings
			Control	Intervention	RR	OR	HR	
								Significant reduction in odds of laboratory-confirmed influenza incidence in households who implemented intervention <36 h after symptom onset in index case. Intervention includes mask-only and mask and hand hygiene interventions in households Control OR (95% CI): reference Mask group + mask and hand hygiene group OR[e] (95% CI) 0.16 (0.03–0.92), p=0.04
	Laboratory-confirmed influenza episode	Positive qRT-PCR result, with fever (>38.0), cough, or sore throat	46.3% (19/41)	17.6% (6/34)	0.39 (0.013–1.17)[d]			
Barasheed et al. (22)	ILI determined subjectively (questionnaire responses and symptom diaries) and objectively (results of testing on nasal swabs with point-of-care diagnostic test and nucleic acid tests)	Subjective or proven fever plus one respiratory symptom (dry/productive cough, runny nose, sore throat, shortness of breath), positive results in both point of care test (QuickVue A+B Influenza and Nucleic acid test for influenza and other respiratory viruses)	52.8% (28/53)	30.6% (11/36)*				Significantly lower ILI incidence in mask group than in control group ($p = 0.04$) Significantly lower rate of ILI in subjects who wear facemasks for >8 h compared with those who wear them for ≤8 h ($p = 0.01$) *Proportion with ILI symptoms in following group (%):* >8 h: 1/35 (2.9%) ≤8 h: 8/35 (22.9%)
Deris et al. (23)	Self-reported ILI during stay in Mecca, through a self-administered questionnaire	Triad of cough, sore throat, and fever (WHO definition)	32.4% (34/105)	42.9% (121/282)		1.57 (0.98–2.52)		No significant difference in ILI incidence in mask group than in control group ($p > 0.05$) **Significant difference** in sore throat incidence (OR 1.89; 95% CI 1.20–2.97, $p = 0.006$), and duration of sore throat and fever between mask wearing and control groups *Mean duration (SD) for mask vs. no mask:* Sore throat: 2.0 days (7.0) vs. 0.0 days (5.0), $p = 0.008$ Fever: 2.0 days (4.0) vs. 1.0 days (3.0), $p = 0.039$

(Continued)

TABLE 2 | Continued

References	Outcome measured and ascertainment	Outcome definition	% Population infected (infected/population size)		Reported summary risk estimate: risk ratio (RR), odds ratio (OR), or hazard ratio (HR) (95% CI); *p*-value (if significant)			Key findings
			Control	Intervention	RR	OR	HR	
Gautret et al. (24)	Self-reported ILI during their stay in Saudi Arabia and participation in the Hajj ritual, through a post-travel questionnaire	triad of cough, sore throat and fever (WHO definition)	3.6% (2/56)	9.2% (20/218)	2.57 (0.62–10.66)			No significant effect of preventive measures implemented on occurrence of cough, sore throat, rhinorrhea, voice failure, shortness of breath, and gastrointestinal symptoms during Hajj Preventive measures include vaccination, wearing a face mask, washing of hands, and use of hand disinfectants or disposable handkerchief
Al-Jasser et al. (25)	Self-reported URTI during Hajj in Makkah or within 2 weeks from return to Riyadh, through phone interview	Presence of at least one of the constitutional symptoms (fever, headache, myalgia) and one of the local symptoms (running nose, sneezing, throat pain, cough with or without sputum)	54.9% (180/328)	45.4% (98/216)	RR: 1.21 (1.03–1.42)##,⋆			**Significantly decreased risk** for URTI for those using face mask most of the time during Hajj, compared with those who never used it ($p = 0.014$) or only used it sometimes (0.045) during the Hajj **Significantly lower** URTI incidence in pilgrims who stayed at least 8 days in the Hajj area RR (95% CI) 0.78 (0.65–0.92), $p = 0.006$
Al-Jasser et al. (25)			55.2% (181/328)	53.7% (341/635)	RR: 1.17 (1.00–1.38) ##,⋆			
Balaban et al. (26)	Self-reported respiratory illness[f] during their Hajj stay, through a telephone or in-person interviews within 14 days of pilgrims' return	Presence of one or more of the following localizing signs or symptoms: cough, congestion, sore throat, sneezing, or breathing problems	33.3% (18/54)	41.6% (37/89)		1.42 (0.70–2.88)[f]		No significant difference in odds for ILI incidence between the mask and control groups ($p > 0.05$) **Reduced odds** for ILI incidence when the social distancing, hand hygiene and contact avoidance were practiced *OR[f] (95% CI) for the following protective behaviors:* Social distancing: 0.44 (0.22–0.90), $p = 0.02$ Hand hygiene: 0.36 (0.14–0.94), $p = 0.03$ Contact avoidance: 0.51 (0.24–1.11), $p = 0.06$
Kim et al. (27)	Laboratory-confirmed influenza A(H1N1) infection	Positive RT-PCR, influenza rapid antigen test, or viral cultures results	5.8% (120/2082)	3.0% (14/466)		0.51 (0.3–0.88)*		**Significant difference** in protective effects of facemask use and H1N1 infection ($p = 0.004$) **49% reduction** in odds for H1N1 infection with continuous facemask use, compared with occasional use or non-usage of facemasks
Kim et al. (27)			5.7% (119/2082)	5.8% (164/2819)		1.02 (0.83–1.25)*		
Gautret et al. (28)	Self-reported episodes of cough during Hajj travel, through a post-travel questionnaire	Presence of cough	78.4% (131/167)	81.9% (158/193)	RR: 1.04 (0.94–1.16)			No significant difference in cough prevalence between the mask wearing and control groups ($p = 0.477$) No significant effect of preventive measures practiced in reducing cough prevalence Preventive measures include frequent hand washing, use of hand sanitizer, disposable tissues or face mask, and influenza and/or invasive pneumococcal disease vaccination

(Continued)

TABLE 2 | Continued

References	Outcome measured and ascertainment	Outcome definition	% Population infected (infected/population size)		Reported summary risk estimate: risk ratio (RR), odds ratio (OR), or hazard ratio (HR) (95% CI); *p*-value (if significant)			Key findings
			Control	Intervention	RR	OR	HR	
Hashim et al. (29)	Self-reported respiratory illness[g] while on Hajj in Saudi Arabia through pro-forma distributed before travel	Presence of at least one of the following respiratory symptoms: cough, subjective fever, or sore throat (non-ILI) or a triad of the listed symptoms (ILI)	NR	NR		1.65 (0.79–3.47)		No significant difference in odds for respiratory illness between the mask and control groups ($p > 0.05$) **Significant reduction** in odds for respiratory illness for groups with the following factors: previous experience of hajj or umrah, and those with good hand hygiene *OR[g] (95% CI) for the following factors:* Previous Hajj experience: 0.24 (0.10–0.56) Previous umrah experience: 0.19 (0.07–0.52) Good hand hygiene: 0.35 (0.16–0.79)
Uchida et al. (30)	Clinically diagnosed influenza episode by physician, reported by child's guardian through questionnaire at the end of 2014/2015 influenza season	Seasonal influenza	21.4% (1080/5050)	19.5% (1069/5474)		0.86 (0.78–0.95)*,[8]		Influenza incidence was associated with the following protective measures: mask wearing ($p = 0.003$), hand washing ($p < 0.001$), gargling ($p < 0.001$), and vaccination in this season (0.004) **Significant protective effect** of wearing a mask or vaccination during the influenza season against seasonal influenza incidence OR[h] of vaccination during influenza season (95% CI) 0.87 (0.79–0.95), $p = 0.004$ **Increased odds** of seasonal influenza incidence when gargling or hand washing was practiced OR[h], gargling (95% CI) 1.32 (1.18–1.47) OR[h], hand washing (95% CI) 1.45 (1.27–1.64)
Emamian et al. (31)	Clinical diagnosis of respiratory tract infections by study staff, at point of entry to Mecca and Medina	All types of respiratory tract infections including tonsillitis, pharyngitis, laryngitis, sinusitis, otitis media, bronchitis, pneumonia, and influenza, except common cold	28.9% (11/38)	36.8% (21/57)		0.64 (0.23–1.78)		No significant difference in respiratory tract infections between mask wearing and control group ($p > 0.05$) No significant effect of other demographic variables or protective measures on odds of respiratory tract infection incidence Demographic variables include gender, age, years of education, room contact with other patients, room size, mean duration in holy places daily, body mass index, presence of systemic diseases, and smoking status Protective measures include mask usage, influenza vaccination status, salt water gargling, and use of personal prayer carpet

(Continued)

TABLE 2 | Continued

References	Outcome measured and ascertainment	Outcome definition	% Population infected (infected/population size)		Reported summary risk estimate: risk ratio (RR), odds ratio (OR), or hazard ratio (HR) (95% CI); *p*-value (if significant)			Key findings
			Control	Intervention	RR	OR	HR	
Zhang et al. (32)	Laboratory-confirmed influenza A(H1N1)pdm09 infection, through a post-travel telephone interview	Positive PCR result (RT-PCR and standard PCR) between 21 May to 4 June 2009	34.6% (9/26)	0% (0/15)		0.00 (0–0.71)*		**Decreased odds** of H1N1 infection in the mask group compared with the control group Significantly higher proportion of face mask usage in controls than in cases at all legs of the flight New York–Vancouver leg: $p = 0.037$ Vancouver–Hong Kong leg: $p = 0.018$ New York–Hong Kong leg: $p = 0.018$ Factors not associated to a case-passenger include exposure to any lavatories or specific lavatories, talking with other passengers, moving around the aircraft, and reported hand hygiene during the New York to Hong Kong leg ($p > 0.05$)

*$p < 0.05$.

##*Reference group was wearing mask most of the time.*

[a]*Adjusted for age, sex, race/ethnicity, handwashing practices at baseline, sleep quality, stress, alcohol consumption, and flu vaccination.*

[b]*Adjusted for household-level and individual-level characteristics (unspecified) in multivariable logistic regression analyses.*

[c]*Adjusted for gender, race, ethnicity, smoking status, physical activity, and having ever received a vaccination for influenza, intracluster correlation coefficient: −0.0005.*

[d]*Adjusted for age, sex, timely therapy of the index, vaccination of household contacts, time spent at home.*

[e]*Adjusted for age, sex, timely therapy of the index, time spent at home.*

[f]*Adjusted for (1) demographic and health factors (age, gender, education, whether respondent was US-born, health risk factors, seasonal influenza vaccination in the previous 12 months, influenza A(H1N1) vaccination before Hajj, and taking medication for respiratory illness during or post-Hajj), (2) travel-related factors (length of trip, international travel in the previous 12 months, and whether respondent had made a previous Hajj), and (3) influenza A(H1N1) knowledge and attitudes (if respondent received pre-travel health information, level of influenza A(H1N1) knowledge, perceived severity of influenza A(H1N1), and noticing influenza A(H1N1)-related health messages during the Hajj).*

[g]*Adjusted for previous experience of Hajj or umrah, contact with pilgrims having respiratory illness and good practice of hand hygiene.*

[h]*Adjusted for gender, grade, underlying disease, sibling, regularly go out, vaccination in this season, mask wearing, hand washing, influenza in previous season.*

implemented SM usage with hand hygiene practices in the general community. These studies were excluded from the main analysis, but the relevant data are also extracted and presented in **Supplementary Table 8**.

Quality Assessment

Included studies were individually evaluated for their reporting and methodological quality using methods described in (**Appendix B**). For observational studies, reporting quality was evaluated using the STrengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (34, 35), and methodological quality was evaluated using the National Heart, Lung, and Blood Institute (NHLBI) quality assessment tool for Quality Assessment Tool for Observational Cohort, Cross-sectional and Case–Control Studies (36). For interventional studies, reporting, and methodology qualities were, respectively, assessed using the Consolidated Standards of Reporting Trials (CONSORT) statement (37), and Cochrane's Risk of Bias Tool (RoB 2.0) for cluster-randomized trials (38).

Statistical Analysis

Pooled ORs with their corresponding 95% CIs were estimated with a random-effects model and the generic inverse variance method. The inbuilt RevMan calculator was used to estimate each study's OR and the corresponding 95% CIs when raw event data were available, otherwise the reported ORs were utilized. The estimated OR was subsequently utilized to calculate the log(OR) and standard errors of each individual study with the RevMan calculator. The I^2 statistic and Cochran *Q*-test was used to evaluate statistical heterogeneity, where heterogeneity was characterized as minimal (<25%), low (25–50%), moderate (50–75%), or high (>75%) and was significant if $p < 0.05$. Subgroup analyses analyzing the effects of (1) study design (interventional vs. observational), (2) outcome ascertainment (self-reported or clinically diagnosed ARI episode vs. laboratory-confirmed ARI episode), (3) age, and (4) study setting (hajj setting vs. school setting vs. flight setting) on the protective effect of wearing SM on ARI incidence was also explored. Publication bias for studies included in the meta-analysis was assessed with conventional and contoured funnel plots. All statistical tests were two sided and performed using Review Manager 5.3, except for funnel plots that were generated with STATA 13 (StataCorp, Texas).

All stages of screening, data extraction, and study quality assessments were conducted in duplicate by MW, SG, and PC. Discrepancies were resolved by consensus with JP at the end of each procedure before moving on to the next stage of analysis.

RESULTS

Screening Results and Characteristics of Included Studies

A total of 503 unique studies identified through our literature search were screened after the removal of 120 duplicates, and inclusion of two additional studies identified from external sources. The full texts of 57 potential studies were further assessed for eligibility and a total of 15 studies were selected for final inclusion into the review. The studies included in this systematic review are five cluster-randomized controlled trials (cluster RCT), seven cross-sectional studies, one cohort study, one nested case–control study, and one retrospective cohort study. All five RCTs and a cross-sectional study (29) were excluded from the meta-analysis. The reported summary statistics of three cluster RCTs were appropriately adjusted to account for the cluster design but were not the same measure [two studies reported HRs (19, 21), one study reported OR (18)]. Conventionally, only the same summary statistics across studies can be pooled using the generic inverse variance method when raw event data were unavailable. Hence, these reported summary statistics from these three cluster RCTs could not be pooled via the inverse generic variance method, whereas the remaining two cluster RCTs did not report summary statistics (20, 22). Thus, a total of 10 observational studies were included in the meta-analysis. The flow chart of the screening process and specific reasons for article exclusion are shown in **Figure 1**.

To provide readers with a general idea on the causal effect of SM usage on ARI incidence, the authors also explored combining the different summary measures reported using the generic inverse variance method (**Figure 4**). Summary statistics for all cluster RCTs were pooled after calculating the RRs for studies not reporting any summary statistics, with the RevMan calculator. Nonetheless, the authors did not consider the pooled estimate from cluster RCTs as part of the main meta-analysis results due to possible inaccuracy of the pooled estimate. Inaccuracy is likely present due to different summary measures across studies and the crude summary statistics [which were not adjusted for the clustering and other confounders present in the original study (39)] utilized to generate the pooled summary estimate.

An overview of the study characteristics is presented in **Table 2**. A total of 23,892 participants between 7 and 89 years old involved across 15 studies from 11 countries were included in this review. The health status of participants in all studies was all mixed, except for two studies which did not specify the health status of their participants (19, 21). Design features of seven studies suggested a retrospective cohort study design, although these studies were reported as cross-sectional (23, 25, 26, 29) or observational design (24, 30), or had no reported study design (28). The remaining eight studies had design features that corresponded to their reported study designs.

It is worthy to note that 8 of the 15 studies examined the effectiveness/efficacy of SM in hajj settings (22–26, 28, 29, 31) whereas the remaining investigated the same effect in students living on- or off-campus [4 studies (19, 21, 27, 30)], in households [2 studies (18, 20)], and in a flight setting [1 study (32)]. Of the five interventional studies included in this review, only one study compared hand washing with SM usage; the remaining four studies compared basic education—hand hygiene and/or SM usage and/or infection control–with mask wearing. The 10 observational studies included mainly compared the general lack of SM usage with its general use among participants; only 2 observational studies explored the effects with varying extents of SM usage on ARI prevention (25, 27).

Systematic Review of Surgical Face Mask Wearing on ARI Incidence

Key findings on the effectiveness/efficacy of SM usage on ARI incidence are summarized in **Table 3**. Most studies assessed ARI incidence through self-reported influenza-like illness (ILI) as the sole ($n = 4$) or one of the outcomes together with laboratory-confirmed influenza ($n = 2$). The remaining studies assessed ARI incidence through laboratory-confirmed influenza ($n = 3$), clinically defined influenza ($n = 1$) or study-defined respiratory outcomes encompassing respiratory illness ($n = 2$), (upper) respiratory tract infections ($n = 2$), and cough ($n = 1$). A variety of summary risk estimates were reported when used, with seven studies reporting ORs, four studies reporting RRs, and two studies reporting HRs.

Across the studies, mixed effects of SM use on ARI incidence were observed, ranging from significantly decreased incidence (22, 25, 27, 30, 32) to no significant difference (18–21, 23, 24, 26, 28, 29, 31) compared with non-usage of SMs. Infection rates were generally lower in groups with SM usage, except in six studies (20, 23, 24, 26, 28, 31). Moreover, lower proportions of participants with ARI were consistently observed in groups who wore SMs for a longer duration [>8 vs. ≤8 h (22)] or more persistently [frequent/continued SM usage vs. occasional/irregular SM usage vs. non-SM usage (25, 27)] when studies stratified findings according to varying levels of SM usage. This suggests that varying extents of SM usage is associated with SMs' effectiveness in ARI prevention. Nonetheless, this difference in infection rates between groups were not significantly different ($p > 0.05$) in all but one study [$p = 0.04$ (22)].

It is worthy to note that when Suess et al. (18) analyzed data from compliant participants (i.e., per-protocol analysis), only a 70% reduction in odds of laboratory-confirmed secondary influenza episode was observed among household members with SM usage compared with household members without it (OR 0.30, 95% CI 0.10–0.94; $p = 0.04$) (18). A significant reduction in odds of laboratory-confirmed secondary influenza incidence was also observed in households who implemented interventions (used SM solely or in conjunction with hand hygiene practices) <36 h after symptom onset of the index case, regardless of participant compliance to the interventions (OR 0.16, 95% CI 0.03–0.92; $p = 0.04$).

Meta-Analysis of Surgical Mask Wearing on ARI Incidence

The estimated pooled odds ratio suggests that SM usage is not associated to preventing ARI incidence, and hence ineffective in preventing ARI incidence in non-healthcare settings. This is because the protective effect of SMs did not reach statistical significance (95% CI 0.8–1.15), although it lowered odds of ARI incidence by 4% compared with non-usage (pooled OR 0.96, **Figure 2**). Nonetheless, moderate heterogeneity was detected across the pooled studies ($I^2 = 58\%$, $p = 0.006$; **Figure 2**), indicating certain inconsistency in the findings on efficacy of SMs in ARI prevention.

The protective effect of SMs was more evident among children, demonstrated by a 15% lowered odds of ARI incidence (pooled OR 0.85; **Figure 2**). In contrast, increased odds for ARI incidence were observed among the adult-and-child and the adult-only populations with SM usage. SM usage was estimated to increase odds for ARI incidence in the adult-and-child population by 16% (pooled OR 1.16; **Figure 2**) and by 2% increased odds in the adult-only population (pooled OR 1.02; **Figure 2**). Nonetheless, the associations in all mentioned sub-populations were non-significant (95% CI: children-only: 0.75–1.40, adult-and-children: 0.4–2.89, adult-only: 0.75–1.40; **Figure 2**), indicating the ARI incidence was not associated with increased harm or protection from SM usage. Unexplained heterogeneity between studies were still observed in each subpopulation, particularly in the adult-only subpopulation ($I^2 = 56\%$, $p = 0.04$; **Figure 2**), with no statistically significant subgroup differences detected ($p = 0.58$; **Figure 2**). This suggests that age group of participants does not modify the effect of SM usage on ARI incidence, and hence is unlikely to be a factor behind the differential effects observed across pooled studies. Conventional and contoured funnel plots of the studies pooled in the meta-analysis suggests a slight asymmetry in the areas of mid to high statistical significance on the right side of the funnel plot (**Supplementary Figures 1, 2**). However, publication bias is unlikely to be the underlying cause of the observed plot asymmetry, as much as there is a lack of studies realizing a statistically significant harm associated with SM usage on ARI incidence. Subgroup analysis of the studies according to whether the ARI episode was laboratory-confirmed or not (i.e., self-reported or clinically confirmed) showed differential results on the effectiveness of SM usage on ARI prevention. A non-significant protective effect with SM usage was demonstrated when ARI incidence was laboratory-confirmed (pooled OR 0.82, 95% CI 0.63–1.07; **Figure 3**). When ARI incidence was self-reported or clinically confirmed, a non-significant harmful effect with SM usage was shown (pooled OR 1.10, 95% CI 0.84–1.45; **Figure 3**). Nonetheless, the subgroup difference detected was not statistically significant ($p = 0.14$; **Figure 3**). Unexplained inconsistencies in study findings was also detected within each subgroup, especially in the laboratory-confirmed outcomes subgroup where significant moderate heterogeneity was detected ($I^2 = 68\%$, $p = 0.02$). This indicates that the outcome ascertainment method used in pooled studies does not influence the association between SM usage and ARI incidence, and thus an unlikely cause for differential effects observed across pooled studies.

When studies were stratified by study settings, results suggest that SM use has limited protective effect, and may even be harmful in mass gathering settings such as Hajj (pooled OR 1.10, 95% CI 0.45–1.45; **Figure 4**). However, in enclosed settings such as in schools or flights, a statistically non-significant protective effect against ARI was observed with SM use in studies conducted in schools, the same as that observed amongst children-only studies (**Figure 4**). This was attributable to the exact same studies included in these two subgroups.

Subgroup analysis of the outcome according to study design could not be performed in this review as there were insufficient interventional studies with suitable data to generate a pooled summary estimate.

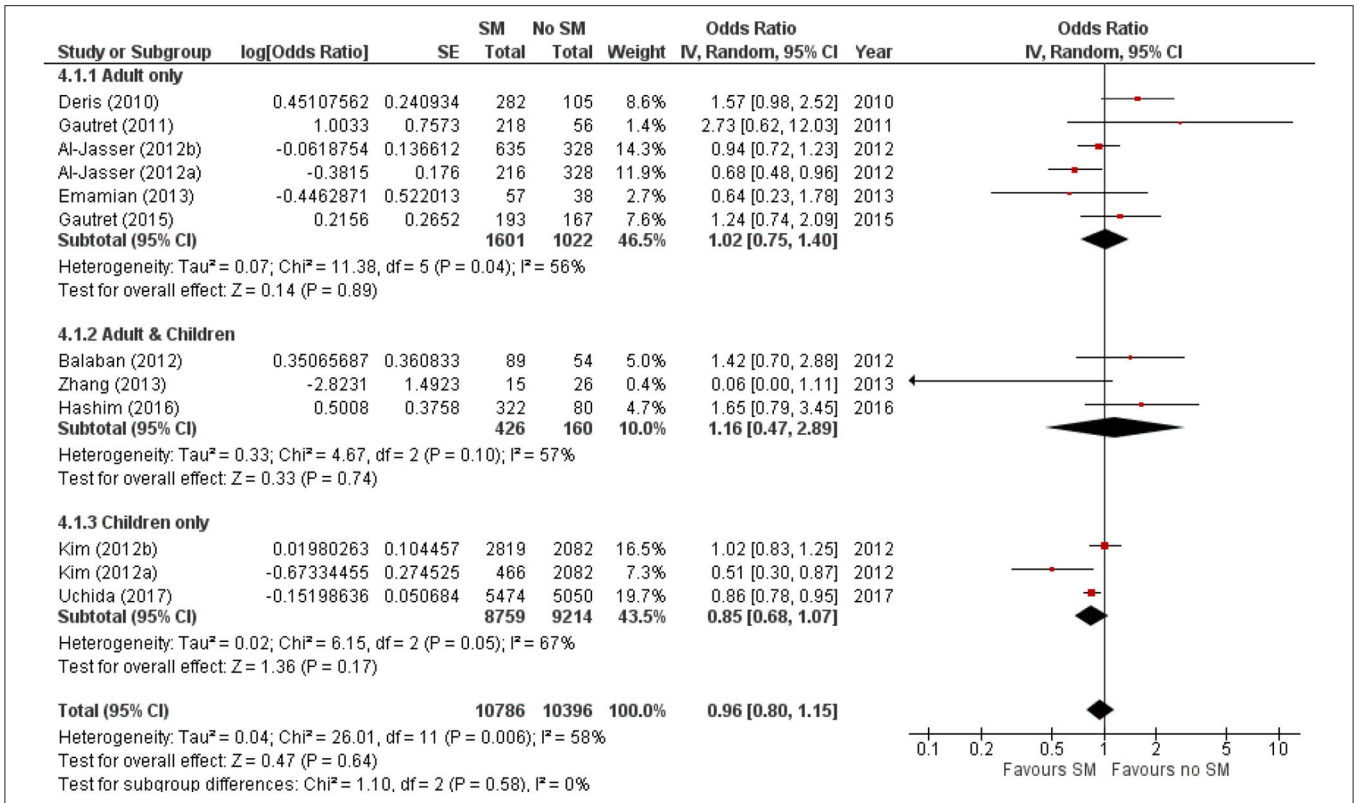

FIGURE 2 | Pooled odds ratio for ARI incidence when surgical masks were worn, compared with not wearing surgical masks, in observational studies (n = 10 studies) estimated with the generic inverse method and a random-effects model. SM, surgical face mask; No SM, no surgical face mask; OR, odds ratio.

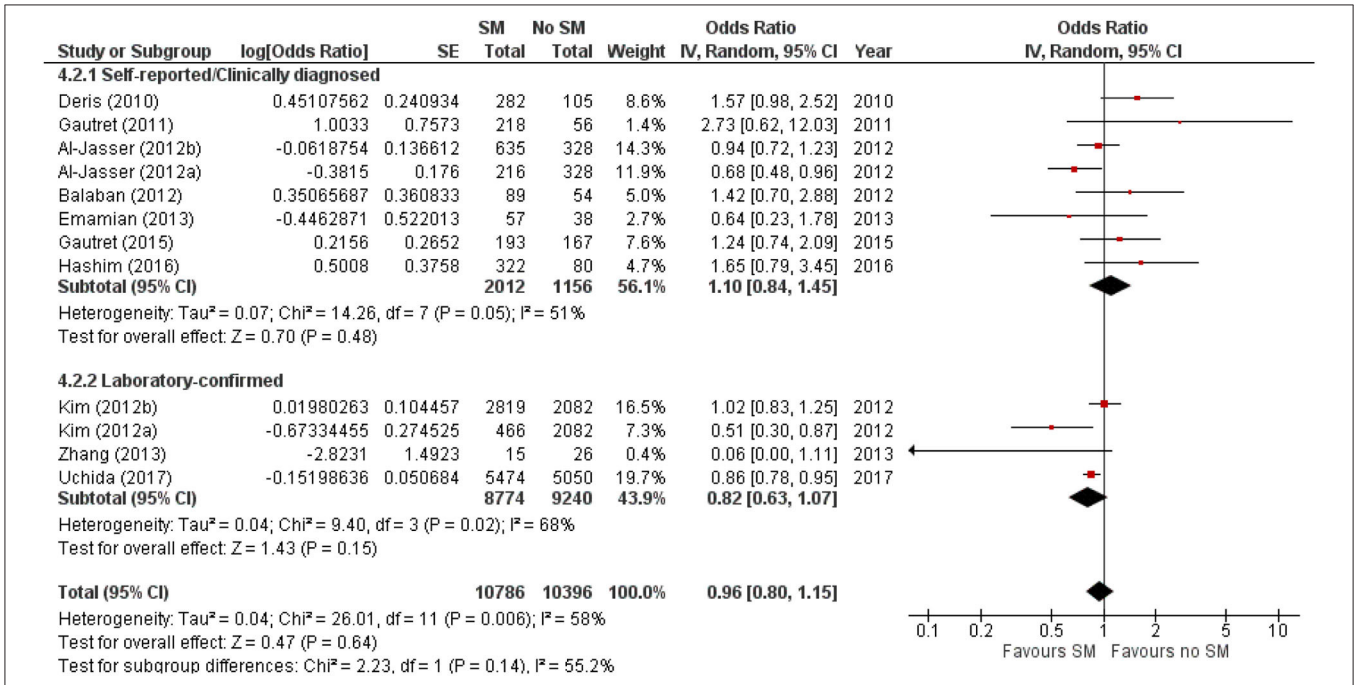

FIGURE 3 | Subgroup analysis of ARI incidence when surgical masks were worn compared with not wearing surgical masks, according to outcome ascertainment methods, estimated with the generic inverse method and a random-effects model. SM, surgical face mask; No SM, no surgical face mask; OR, odds ratio.

TABLE 3 | Summary of reporting and methodological quality of randomized controlled trials.

Reporting quality[a]	Study design	Randomized controlled trials						
	Author, year / Section/domain	Aiello et al. (19)	Simmerman et al. (20)	Aiello et al. (21) (1)	Aiello et al. (21) (2)	Suess et al. (18) (1)	Suess et al. (18) (2)	Barasheed et al. (22)
	Title, abstract, and introduction	Fair	Fair	Fair		Fair		High
	Methods	High	High	High		High		High
	Results	Fair	High	High		High		Low
	Discussion	High	High	High		High		High
	Other information	High	Low	High		High		Low
	Overall	High	High	High		High		Fair
Methodological quality[b] (risk of bias judgement)	Randomization	High	High	High	High	High	High	High
	Timing of identification and recruitment of individual participants in relation to timing of randomization	High	Low	High	High	Low	Low	Low
	Deviations from intended interventions	Some concerns	Low	Low	Low	High	High	Some concerns
	Missing outcome data	Low	Low	Low	Low	Low	Low	Low
	Measurement of outcome data	Some concerns	Low	Low	Some Concern	Low	Some concerns	High
	Selection of the reported result	High	Low	Low	High	Low	Low	High
	Overall	High	High	High	High	High	High	High

[a]Reporting quality assessed with the Consolidated Standards of Reporting Trials (CONSORT) statement. [b]Methodological quality assessed with the Cochrane's Risk of Bias Tool (RoB 2.0) for cluster-randomized trials.

Aiello et al. (21) (1) and Suess et al. (18) (1) assess risk of bias in the following outcome: laboratory-confirmed influenza episode. Aiello et al. (21) (2) assesses risk of bias in the following outcome: self-reported ILI episode. Suess et al. (18) (2) assesses risk of bias in the following outcome: clinically confirmed ILI episode.

However, the authors did estimate the pooled summary statistic on the effects of SM wearing on ARI incidence in cluster RCTs and found a similar non-significant protective effect of SM usage on ARI incidence (**Figure 5**). A 13% reduction in ARI incidence was noted with SM usage, compared with non-SM usage or implementation of hand-hygiene practices, although this reduction is not statistically significant (pooled summary statistic: 0.87, 95% CI 0.74–1.04; **Figure 4**). Nonetheless, the authors would like to highlight that the estimated pooled summary statistic only intends to provide a general idea on the direction of relationship between SM usage and ARI incidence. The pooled summary statistic in **Figure 4** does not intend to, and is unable to quantitatively summaries the effects of SM usage on ARI incidence across the cluster RCTs included in this review. This is largely a result of the inaccuracy arising from reasons mentioned in Section Screening results and Characteristics of included studies.

Reporting and Methodological Quality Assessment

High overall reporting quality was generally observed in the cluster RCTs, whereas only half of the observational studies had high overall reporting quality (25–27, 29, 30). The remaining observational studies had a low overall reporting quality, of which low-quality reporting was found in methods and results section of a study (31), and in either of the section in the remaining four studies (23, 24, 28, 31, 32) (**Tables 3**, **4**).

Methodological quality was poor in general across all studies. All cluster RCTs are at a high risk of overall bias, indicating poor overall methodological quality (**Tables 3**, **4**). The generally high overall risk can be attributed to a high risk of bias from randomization as subversion was likely absent in all cluster RCTs. Bias from timing of identification and recruitment of individual participants in relation to timing of randomization is also likely to be present in two studies with baseline imbalances across groups that suggest the likelihood of recruitment bias (19, 21). Suess et al. (18) was at high risk of bias from reported deviations that arose because of trial context that may have affected the outcome. Three studies also measured multiple outcomes but reported only a single outcome (22), or measured the same outcome at multiple instances but only reported the outcome at a single instance (19, 21), rendering them at high risk of bias from selective reporting. The observational studies were generally at low risk of selection bias, except for three studies which were at moderate risk of selection bias (23, 24, 26). However, unclear or low participation rate was a common issue identified in all cross-sectional and cohort studies included in this review. All but three studies (23, 27, 29) did not specify the number of eligible subjects identified in the course of the study, of which two studies had a participation rate <50% (27, 29). Most studies were also at high risk of misclassification and detection bias, with only three studies at low or moderate risk of bias in these two domains (24, 27, 31). In the misclassification bias domain, a moderate- to high-risk recall bias mainly exists due to the use of retrospectively collected participant-reported exposures to assess exposure across all studies. It was also unclear whether a study utilizing a self-administered questionnaire was able to assess exposures across participants consistently because interpretation

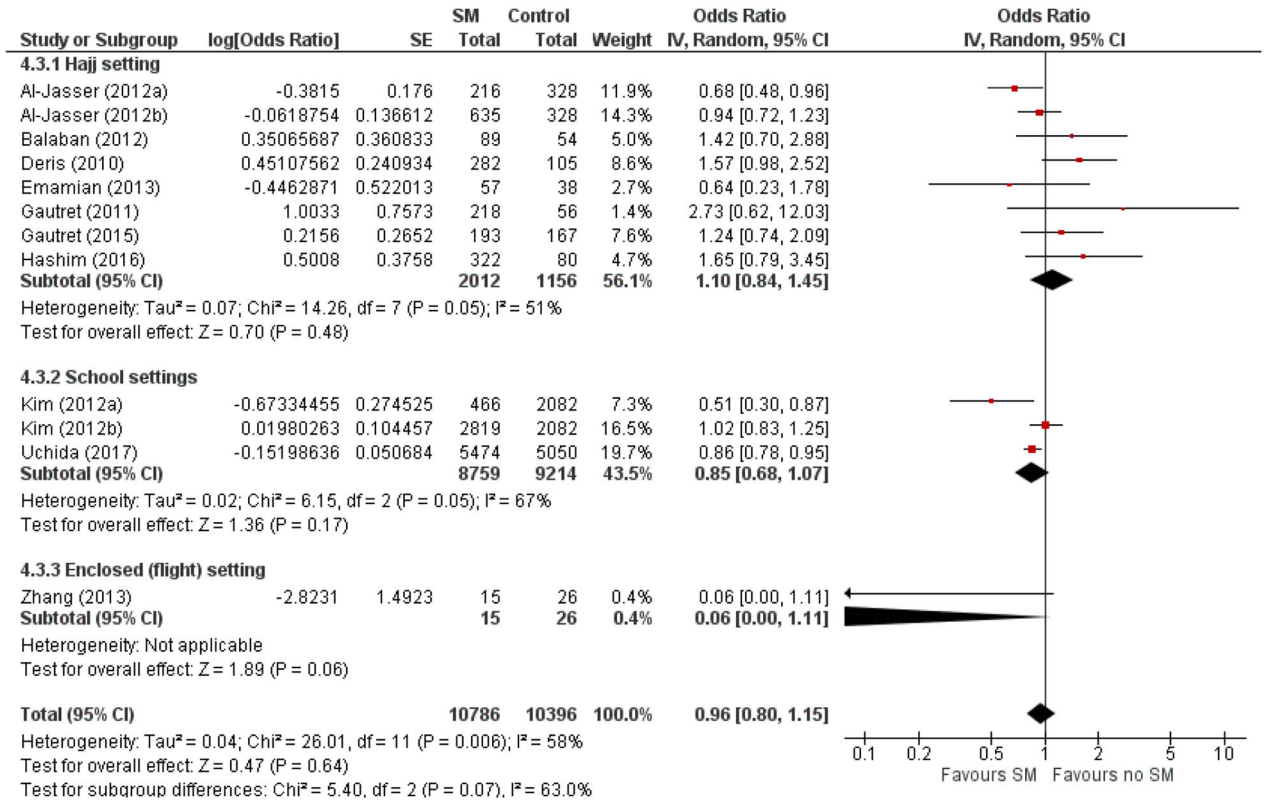

FIGURE 4 | Pooled summary statistic for ARI incidence according to study settings, estimated with the generic inverse variance method and a random-effects model. SM, surgical face mask; No SM/HH, no surgical face mask or hand hygiene practices; OR, odds ratio.

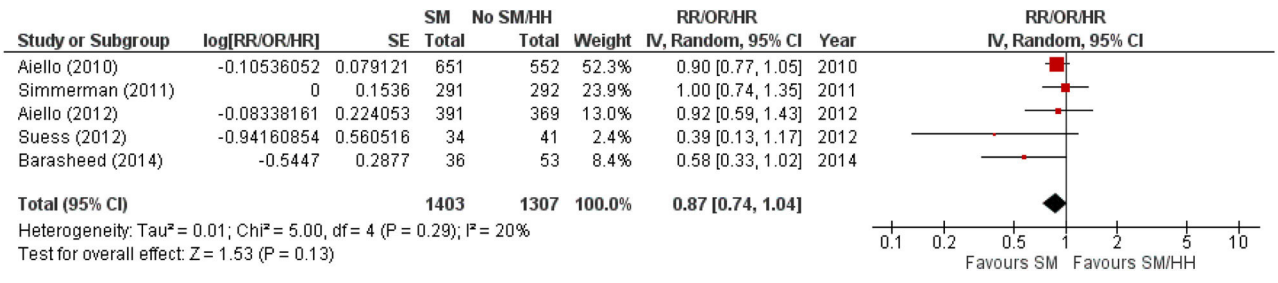

FIGURE 5 | Pooled summary statistic for ARI incidence when surgical masks were worn compared with not wearing surgical masks in cluster randomized controlled trials, estimated with the generic inverse variance method and a random-effects model. SM, surgical face mask; No SM/HH, no surgical face mask or hand-hygiene practices; RR, risk ratio; OR, odds ratio; HR, hazard ratio.

may vary across participants (23). Most of the observational studies were also at high risk of other biases, mainly arising from lack of sample size justification and/or attrition rates >20%.

Detailed results for the quality assessment of included studies can be found in **Supplementary Tables 5–7**.

DISCUSSION

Effectiveness of Surgical Mask Usage on ARI Incidence in Non-healthcare Setting

Our results found that SM usage had a non-significant protective effect in reducing the risk of ARI among asymptomatic individuals in non-healthcare settings (pooled OR 0.96, 95% CI 0.8–1.15; **Figure 2**). The protective effect is also observed within or outside healthcare settings [healthcare setting pooled OR = 0.53; 95% CI 0.16–1.71 (17); community pooled RR = 0.78, 95% CI 0.51–1.20 (40)], regardless of those who were infected or uninfected (15). This contrasts with a review by Jefferson et al. (16), which found face mask to be the best performing intervention compared with other physical non-pharmaceutical interventions studied across different population and settings. Nonetheless, SM was only found to be significantly protective against SARS in the case–control subgroup (pooled OR 0.32, 95% CI 0.26–0.39), and studies related to SM usage was largely based

TABLE 4 | Summary of reporting and methodological quality of observational studies.

	Study design	Cohort and cross-sectional studies								Case–control	
	Author, year	Deris et al. (23)	Gautret et al. (24)	Al-Jasser et al. (25)	Balaban et al. (26)	Kim et al. (27)	Gautret et al. (28)	Hashim et al. (29)	Uchida et al. (30)	Emamian et al. (31)	Zhang et al. (32)
Reporting quality[a]	Section/domain										
	Title, abstract, and introduction	High	Fair	High	High	High	High	High	High	High	Fair
	Methods	Fair	Low	High	High	High	High	High	High	Low	Low
	Results	Low	High	High	High	High	Low	High	High	Low	High
	Discussion	High	Fair	High	High	Fair	Fair	Fair	High	Fair	Fair
	Other information	Fair	Low	High	Low	Low	Low	Fair	Fair	Low	Low
	Overall	Low	Low	High	High	High	Low	High	High	Low	Low
Methodological quality[b]	Selection	Fair	Fair	High	Fair	High	High	High	High	High	High
	Misclassification	Low	Fair	Low	Low	Low	Low	Low	Low	Low	Low
	Detection	Low	Low	Low	Low	Fair	Low	Low	Low	High	Low
	Confounding	Fair	Low	Low	High	Fair	Low	High	High	High	High
	Other (inappropriate sample size, attrition)	Fair	Low	High	Low	Low	Low	Fair	Low	Low	Low

[a]*Reporting quality assessed with the STrengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement.* [b]*Methodological quality assessed with the National Heart, Lung and Blood Institute (NHLBI) Quality Assessment Tool for Observational Cohort, Cross-sectional, and Case–Control Studies.*

in the healthcare setting and among healthcare workers (16). This limits the generalizability of the study given the different compliance in SM wearing between healthcare workers and the general population.

This review observed a non-significant protective effect that was more prominent in the younger age group (pooled OR 0.85, 95% CI 0.75–1.40; **Figure 2**). This contradicts with an experimental study that assessed transmission reduction potential by personal respirators, surgical masks, and homemade masks. The study attributed a significantly less protective effect of all types of mask usage for children, plausibly due to an inferior fit of masks on their smaller faces (41). The observed age-specific difference in effect is also likely because majority of the observational studies among the adult and adult-and-children populations were conducted in Hajj settings. As shown in **Figure 4**, there were differential effects of SM use in mass gatherings such as Hajj, and other enclosed settings in schools or flights. The annual Hajj, which involves as many as 2 million pilgrims nested in highly dense areas for a prolonged period, cannot be generalized to a regular community. Other conditions favoring the spread of infectious diseases include the physical exertion of pilgrims in overcrowded conditions, limited access to resources, humid conditions, and low compliance to mask usage due to religious beliefs (42–44). The combined effects of greater compliance with mask usage and hygiene practices in a more controlled environment could amplify the protective effect of SM usage among children.

SM use was protective against ARI incidence when outcomes were laboratory-confirmed episodes (pooled OR 0.82, 95% CI 0.63–1.07; **Figure 3**), but harmful when outcomes were self-reported or clinically diagnosed. The contrasting observations might be attributed to the subjective nature of self-reported or clinically diagnosed outcome and retrospective collection of self-reported outcomes in most included studies. Such data collection methods are liable to inaccuracies from the participants' judgment of personal condition and recall bias (45). Effects of SM usage in studies utilizing self-reported or clinically diagnosed episodes could also have been diminished by (1) participants overstating the actual experience of illness at the baseline or understating the condition at the end point, and (2) the inability to detect asymptomatic carriers. Conversely, a laboratory-confirmed outcome is more objective and does not require any participant judgment, enabling more accurate evaluation of ARI even among asymptomatic participants.

The effectiveness of face mask in source control hinges on the specific mode of transmission of etiological agent. Studies included in this review measured influenza or ILI, which are collectively caused by a broad range of viruses of varying infectivity and transmission routes (46). Direct and indirect contact are the primary transmission routes of respiratory syncytial virus and adenovirus, which causes ILI, whereas SARS is mainly spread through contact and droplet transmission (3, 47). Influenza is thought to be primarily transmitted through droplet expulsion, although evidence supporting airborne transmission is growing (48). A recent study found significant reduction in influenza virus emitted through

droplets and not aerosols produced by infected individuals after SM usage (13). Another study showed that SM is more effective at reducing influenza viral RNA copies in coarse particles >5 μm (25-fold) than fine aerosols <5 μm (2.8-fold) emitted by an infected wearer (12). SM's effectiveness in preventing influenza decreases with decreasing particle size. As short-range aerosol inhalation is currently the main transmission mode of SARS-CoV-2 in the ongoing COVID-19 pandemic, the usage of SMs may not be highly effective to filter these fine aerosols completely.

The effectiveness of SM usage at reducing environmental risk faced by uninfected individuals remains unclear as existing evidence is limited to mechanistic challenge on masks with largely conflicting results. A study observed lower amount of influenza virus by 1.1- to 55-fold with an average of 6-fold with varying SM design (49). Conversely, SM has also been found to allow penetration of particles as small as 0.04–0.2 μm (influenza virus: 0.08–0.12 μm) (50). Specifically, Bae et al. (51) reported a low effectiveness of filtering SARS-CoV-2 on the basis of the small particle size as SARS-CoV (0.08–0.14 μm). At the time of conducting this review, research found increasing evidence of asymptomatic transmission of SARS-CoV-2 (10, 11). A sweeping change in recommendations to encourage SM usage by the general public was made amidst growing concerns of an increasing asymptomatic infected population, to prevent asymptomatic infected individuals from exposing uninfected individuals to the virus. Despite limited evidence on SM's effectiveness in reducing SARS-CoV-2 transmission due to its plausible airborne transmission mode and small viral particle size, the mechanistic feasibility of masking combined with large-scale uptake by populations might reap effectiveness that have yet to be measured in clinical trials (52).

Effectiveness of Hand Hygiene on ARI Incidence

Three studies included in this review also found no significant protective effect of SM coupled with hand-sanitizer use (SM+HH) on ARI incidence (18, 19, 21) compared with control groups [adjusted HR (95% CI) 0.87 (0.73–1.02) (19), 0.78 (0.57–1.08) (21), adjusted OR (95% CI) 0.62 (0.23–1.65) (18); Appendix D, **Supplementary Table 8**]. However, the results could have been limited by differential protective effect conferred by different types of hand sanitizer used and potential improper application. Gel-based sanitizers were used in two studies (19, 21) and the remaining study likely used liquid-based sanitizer (18). The superiority of liquid-based hand sanitizers to its gel-based counterparts may have resulted in the lack of effectiveness observed (53). More recent evidence also points to increased effectiveness of hand sanitizer in reducing microorganism burden when properly applied in accordance to EN 1500 standards (54).

Nonetheless, WHO recommends that masks are only effective when used in tandem with proper and frequent hand hygiene (55). Findings from a household cluster RCT (56) suggested the risk of influenza transmission is significantly low when healthy family members practice SM usage and frequent hand hygiene within 36 h of symptom onset of an infected family member [adjusted OR 0.16, 95% (CI 0.03–0.92), $p = 0.04$ (18)]. There is also evidence on hand washing with soap and/or hand sanitizer's effectiveness in removing influenza virus (57, 58). These conflicted findings supported inconclusive findings from a review on hand hygiene's protective effect in the community setting (59). Hence, although hand hygiene shows potential in reducing influenza infection and transmission, its effectiveness depends on the types of hand hygiene practiced (e.g., hand sanitizer, washing with soap, and water), usage frequency, proper application, and the setting in which practices are implemented.

Apart from use of SM and hand hygiene, other non-pharmaceutical interventions commonly employed in conjunction will also influence the risk of ARI in the community (60). However, the effectiveness of these measures is beyond the scope of this review and has been extensively evaluated in this other recently published review (61).

Strengths and Limitations

A strength of this review lies in the comprehensive outcomes examined, utilizing an extensive list of pathogens referenced from Jefferson et al. (16) in our search strategy. This compares with the review by Xiao et al. (40) which examined laboratory-confirmed influenza outcomes, or that by Cowling et al. (15) which focused on influenza, flu, and respiratory infections. The period in which evidence for this review was collected also sets it apart from existing reviews. Jefferson et al. (16) explored studies from 1980 to 2010 and Xiao et al. (40) went as far back as 1946. By including studies published in the past decade only, this review presents evidence more representative of the current and rapidly evolving environmental and social–behavioral factors. Next, no limits were imposed on health status and age of populations studied. This diversification increases the representativeness and generalizability of our findings to a community profile. Collectively, the study constitutes an update of SM efficacy investigated in Jefferson's review (16).

This review also complements the review by Xiao et al. (40) in terms of intervention and population evaluated. We focused on assessing SM usage, a more feasible apparatus for the general public as opposed to more intricate facial protective gear included in Xiao et al. (40), that should be reserved for healthcare workers or vulnerable populations. Unlike Xiao et al. (40), this review focused solely on assessing SM's efficacy among the uninfected population. In addition, the included studies were assessed for reporting and methodological qualities, providing additional insight to the reported findings and inadequacy in existing studies to constitute strong evidence on SM efficacy.

However, limitations exist in this review. First, the study settings were largely homogenous. More than half of the studies included were conducted during Hajj, which is unique and distinctly different from the general community setting. This limits the external validity of our results. Second, the authors pooled adjusted summary measures of studies included in the meta-analysis, but residual confounding may still exist in the reported summary measures. Third, the RCTs included cannot be pooled accurately as summary estimates reported

were incompatible for a meta-analysis. Analysis of pooled RCT data, where SM use is better complied with and purposely differentiated between groups, would have constituted more robust evidence on the efficacy of SMs between wearers and non-wearers. Fourth, poor methodological quality was determined across all studies included in this analysis. This review highlights a paucity in well-conducted research examining the efficacy/effectiveness of SMs against ARI incidence in the general community. Non-standardization of methodologies and assessed outcomes inhibited accounting for inconsistencies in compliance to SM usage by the study populations, likely undermining the effectiveness of SMs in preventing ARI. On the contrary, compliance tend to be unusually high during epidemics due to increased risk perception (62). Next, although the funnel plots only showed slight asymmetry (**Supplementary Figures 1**, **2**), there is a likelihood of publication bias as we only searched published literature. Lastly, there is diminished relevance for SM use in resource constrained conditions amidst epidemics. The strict focus on SM in this review was to examine the effectiveness of personal protection equipment that was accessible for the general population. However, there is extensive substitution of SM using reusable cloth masks or face coverings in the current pandemic due to supply constraints. Their efficacy/effectiveness in preventing ARI have not been widely evaluated, and future trials should compare the efficacy/effectiveness of reusable cloth masks to a standard (either SMs or even respirators) to inform policies on cloth mask usage. Nonetheless, such trial findings need to be interpreted with caution as cloth mask production is not regulated, and the quality and construct between products in this category may vary widely. This is unlike SMs which have a consistent quality and construct due to them being a regulated product under 21 CFR 878.4040 (63).

Existing studies are characterized by weak methodologies and a lack of overall significant effect, possibly constrained by small sample sizes. Well-designed, executed, and adequately funded trials are needed to provide robust evidence on SM efficacy in reducing transmission in the general community. Larger studies could be beneficial given their increased sensitivity to small effect sizes, and RCTs have the added benefit of establishing causality over observational studies (although it may be more resource intensive). Well-designed *in vivo* studies on uninfected individuals wearing SM could also be conducted to investigate the proportion of virus of varying sizes that can be blocked by SM via droplet or aerosolized transmission. These studies would form the basis of strong evidence to inform policies and practices on SM usage in the community during times of pandemic.

CONCLUSION

Our review found that SMs were not associated to ARI incidence, indicating that SMs may be ineffective in preventing respiratory illness when worn by an uninfected individual in the general community. However, given the weak methodologies across studies assessed and the possibility of residual confounding, an absence of evidence cannot be simply regarded as an evidence of absence. SM usage cannot be a standalone strategy to protect against infection, but ought to be used together with other physical intervention methods such as hand hygiene and social distancing to combat multiple modes of virus transmission in the community.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JP conceptualized the study. MW, SG, and PC drafted the article, screened the studies, and extracted data. MW analyzed data and prepared the figures and tables. JP validated the final screened studies and analysis, critically edited draft, and interpretation of the findings. All authors have read and agreed to the published version of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.564280/full#supplementary-material

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Centrality of G6PD in COVID-19: The Biochemical Rationale and Clinical Implications

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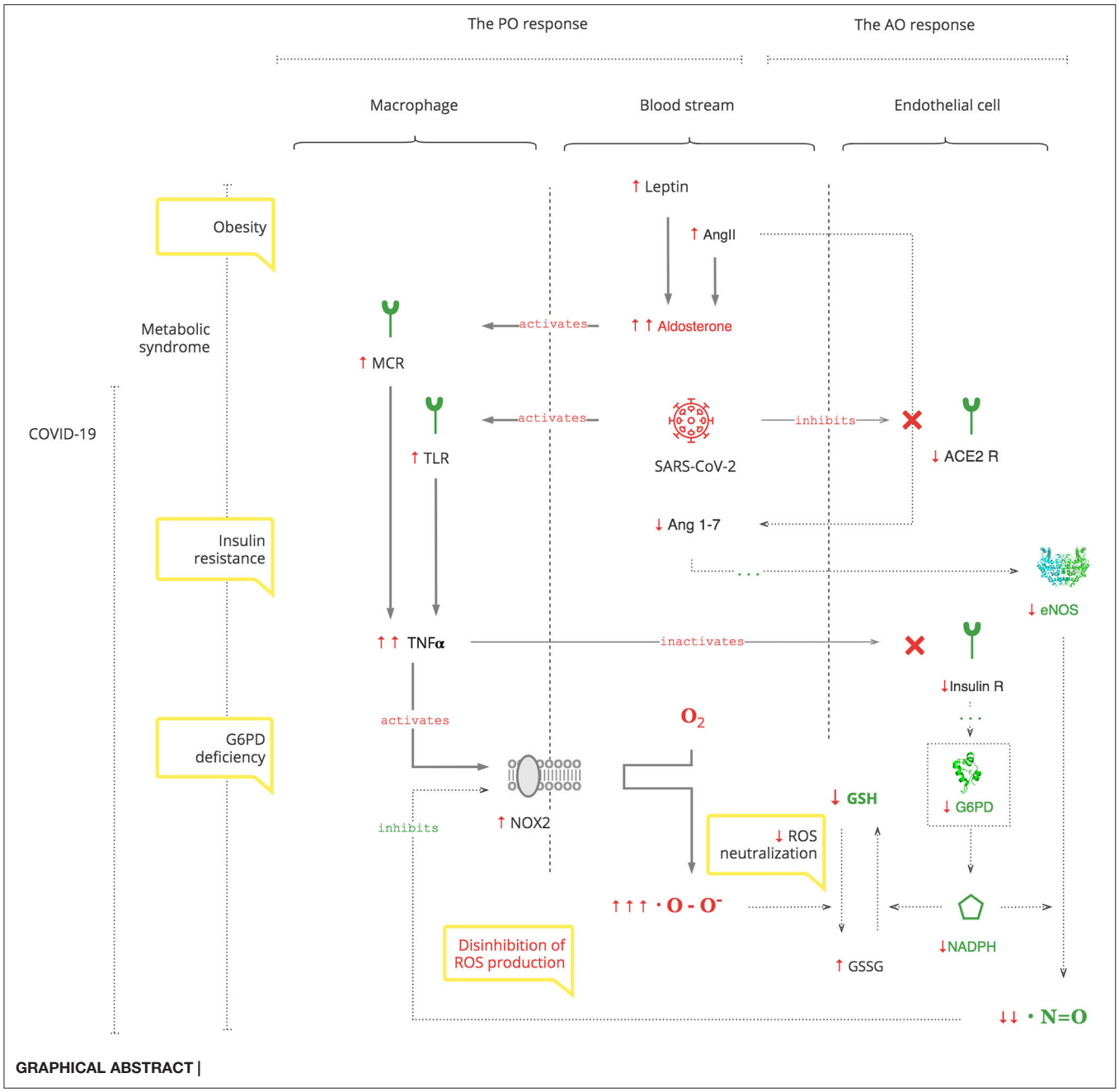
Introduction: COVID-19 is a novel and devastating disease. Its manifestations vary from asymptomatic to lethal. Moreover, mortality rates differ based on underlying health conditions and ethnicity. We investigated the biochemical rationale behind these observations using machine reasoning by the sci.AI system (https://sci.ai/). Facts were extracted and linked from publications available in nlm.nih.gov and Europe PMC to form the dataset which was validated by medical experts.

Results: Based on the analysis of experimental and clinical data, we synthesized detailed biochemical pathways of COVID-19 pathogenesis which were used to explain epidemiological and clinical observations. Clinical manifestations and biomarkers are highlighted to monitor the course of COVID-19 and navigate treatment. As depicted in the **Graphical Abstract**, SARS-CoV-2 triggers a pro-oxidant (PO) response leading to the production of reactive oxygen species (ROS) as a normal innate defense. However, SARS-CoV-2's unique interference with the antioxidant (AO) system, through suppression of nitric oxide (NO) production in the renin- angiotensin-aldosterone system (RAAS), leads to an excessive inflammatory PO response. The excessive PO response becomes critical in cohorts with a compromised AO system such as patients with glucose-6-phosphate dehydrogenase deficiency (G6PDd) where NO and glutathione (GSH) mechanisms are impaired. G6PDd develops in patients with metabolic syndrome. It is mediated by aldosterone (Ald) which also increases specifically in COVID-19.

Conclusion: G6PD is essential for an adequate immune response. Both G6PDd and SARS-CoV-2 compromise the AO system through the same pathways rendering G6PDd the Achilles' heel for COVID-19. Thus, the evolutionary antimalarial advantage of the G6PDd cohort can be a disadvantage against SARS-CoV-2.

Keywords: COVID-19, glucose-6-phosphate dehydrogenase (G6PD), reactive oxygen species, nitric oxide - NO, glutathione, aldosterone (Ald), Metabolic syndrome



GRAPHICAL ABSTRACT |

INTRODUCTION

Based on our previous discussion about the basic mechanisms of coronavirus disease 2019 (COVID-19) pathogenesis (1), in this paper, we will focus on particular parts of it to explain the present clinical and epidemiological observations.

The human host defends itself against infection through its immune response with two cooperative phases.

1. The first phase occurs early, is innate and nonspecific, and has two components.

(a) A pro-inflammatory pro-oxidant (PO) system mediates inflammation. It attacks pathogens with free radicals of the reactive oxygen species (ROS). However, when there is increased production and/or decreased neutralization, a heightened level of ROS occurs, and excessive levels cause collateral damage to normal cells and is referred to as "oxidative stress," "cytokine storm," and "systemic inflammatory response syndrome (SIRS)."

(b) An anti-inflammatory antioxidant (AO) system balances the PO response (2).

2. The second phase occurs with a delay and is adaptive and specific. It is mediated through antibody expression.

These two arms of immune response usually eradicate the pathogen (3). However, in COVID-19, both phases are delayed due to suppression of the host's gene expression by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)'s nsp1 protein (4).

Wu et al. (5) demonstrated, *in vitro*, that glucose-6-phosphate dehydrogenase deficiency (G6PDd) cell lines are vulnerable to coronavirus infection. There are two types of G6PDd: congenital and acquired. Congenital G6PDd is the most prevalent enzyme deficiency in the world, affecting 4.9% of the global population. It evolved against malaria and predominates in specific ethnic cohorts such as the Mediterranean, Asian, and African (6). Interestingly, these cohorts have been particularly affected by the COVID-19 pandemic (7–10). Acquired G6PDd develops in patients with underlying health conditions, especially the metabolic syndrome (11). The metabolic syndrome is prevalent and it spreads acquired G6PDd worldwide.

This paper presents a detailed description of how SARS-CoV-2 affects the innate PO and AO responses and how G6PDd potentiates COVID-19. In addition, we highlight accompanying clinical manifestations and biomarkers that are useful to monitor the clinical course and navigate treatment.

METHODS

We used the sci.AI machine reasoning system (https://sci.ai/) to operate on publicly available datasets from nlm.nih.gov and Europe PMC. The process consisted of two stages: Representation and Reasoning.

Representation algorithms translate unstructured individual papers, documents, and files from heterogeneous sources into embeddings and graphs of entities relations. It goes beyond classic Named Entity Recognition (NER) and arbitrarily recognizes individual and composite biological entities and how they relate to each other. For example, in this sentence: "Obese patients with MetSyn had a significantly lower nitric oxide production rate (0.21 ± 0.13 μmol/h per kg; $P = 0.009$) than healthy normal-weight individuals (0.63 ± 0.30 μmol/h per kg), whereas nitric oxide (NO) production rate was intermediate in obese patients without MetSyn (0.49 ± 0.22 μmol/h per kg; $P = 0.33$)" (12); the machine recognizes the conditions "obesity" and "metabolic syndrome" and recognizes substance "nitric oxide" and links it to CHEBI:16480. Ultimately, "lower nitric oxide production rate" in the context of "metabolic syndrome" is recognized as a biomarker.

The second, Reasoning stage, synthesizes knowledge based on a subset of findings that appear to be relevant to COVID-19. The discovery process was triggered by textual queries "SARS" and "ARDS." Traversing through the interlinked representations computed at the first stage produced multiple subgraphs. We progressively refined the generated knowledge and, in the last step, linked these excerpts to synthesize biochemical pathways to help explain the pathophysiology of COVID-19. We translated complex pathways into clinically relevant applications, conforming to our clinical observations.

Pathways were constructed iteratively; it is not a result of one time inference. Generally speaking, typical machine learning algorithms approximate previous data distributions. In contrast, our reasoning algorithm is based on graph traversing and utilizes biochemical properties in context. It allows to avoid bias caused by frequently mentioned terms, for example, angiotensin-converting enzyme 2 (ACE2). Subgraphs were interactively validated by a domain expert at every iteration. For instance, the term "SARS" mentioned together with "TLR" and "ACE2" led to the creation of two axes as described in our previous work (1):

– TLR/TNFα/NADPH oxidase (NOX2)/ROS, which is positively regulated, and

– ACE2/NOS3/NO, which is negatively regulated by SARS.

Both axes turn out to be composed mainly of canonical pathways: renin–angiotensin system, glutathione (GSH) metabolism, pentose phosphate pathway, aldosterone (Ald) synthesis and secretion, and NO production. When we placed all these pathways on the same canvas, reduced nicotinamide adenine dinucleotide phosphate (NADPH) appeared to be the cofactor of both axes and, in turn, is produced by glucose-6-phosphate dehydrogenase (G6PD). This biochemical rationale, together with the worldwide prevalence of congenital and acquired G6PDd, is consistent with COVID-19 outcomes at individual and epidemiological levels.

A limitation of our research is that it focuses on the centrality of G6PD. Yet, we acknowledge that there is certainly other biochemistry relevant to COVID-19 that remains open for investigation.

RESULTS AND DISCUSSION

Based on machine reasoning of data from 30M papers, we demonstrate the results.

Severe Acute Respiratory Syndrome Coronavirus 2 Affects the Innate Immune Response

The PO System

The PO system is triggered by SARS-CoV-2, as for any pathogen, through toll-like receptors (TLRs) on macrophages, the first-line cell of innate defense (13). As depicted in **Figure 1**, this results in tumor necrosis factor-alpha (TNFα)-induced inflammation, which has two clinically relevant molecular effects: inactivation of insulin receptor signaling on endothelial cells (see **Graphical Abstract**) and activation of NOX2 on macrophages (14, 15). This response is acute and transient. Activated NOX2 produces ROS, particularly superoxide anion (O2*-) from oxygen (O2). Then a hydroxyl radical (OH*) is produced through the Fenton reaction (16). It destroys microorganisms (17). At the same time, it stresses the host's cells, especially platelets, lymphocytes, erythrocytes, and muscle cells (18–20). Muscle cell damage manifests with rhabdomyolysis (21). Damage of erythrocyte membranes results

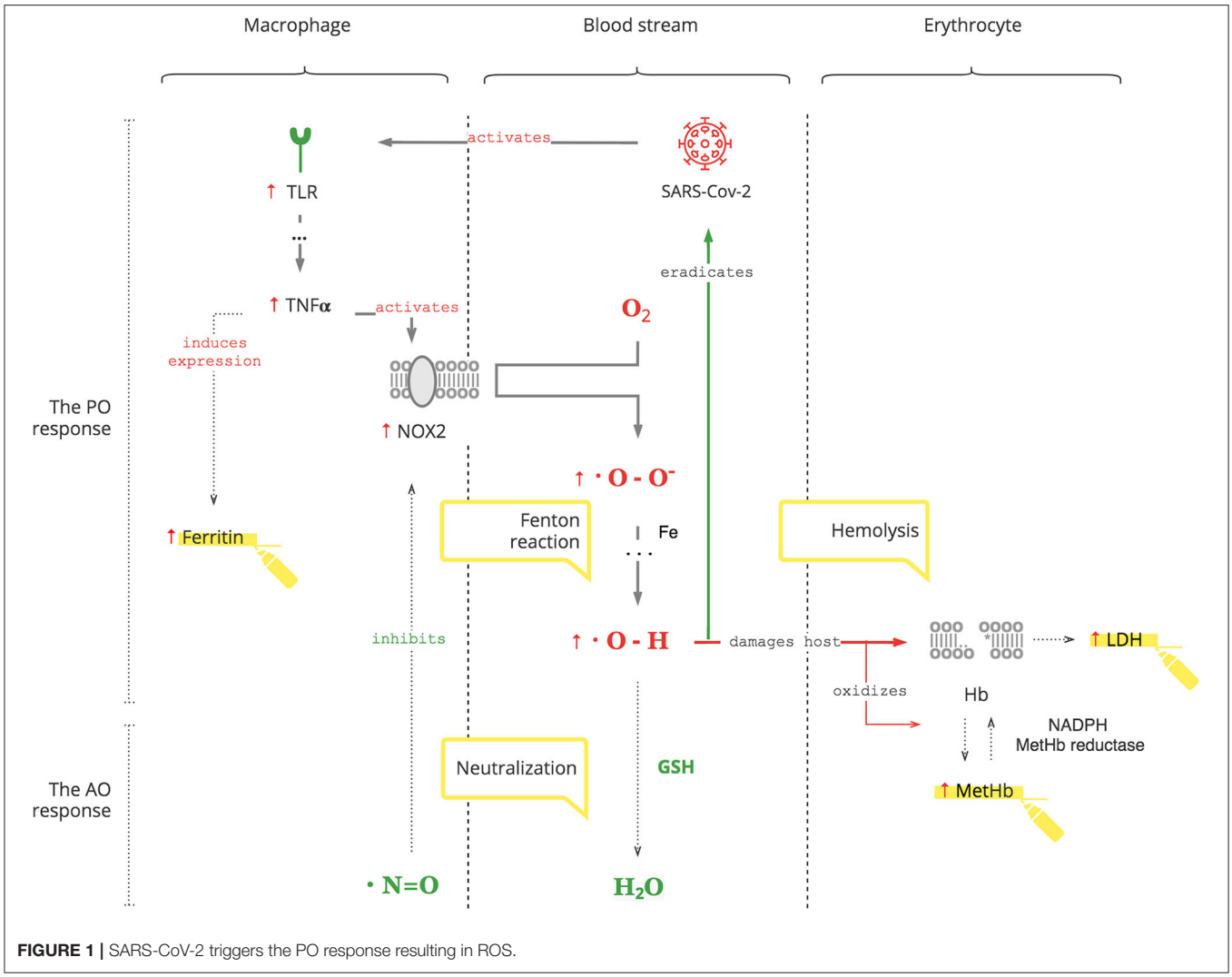


FIGURE 1 | SARS-CoV-2 triggers the PO response resulting in ROS.

in latent hemolysis leaking lactate dehydrogenase (LDH), and hemoglobin (Hb) is oxidized to methemoglobin (MetHb) (22–24).

Clinical Pearls

- Hyperglycemia occurs during COVID-19. It is transient and reversible if there is no antecedent insulin resistance. Otherwise, underlying insulin resistance is aggravated by the stress of infection (25).
- Ferritin production is induced by TNFα and can be used to monitor the degree of the PO response (26).
- Thrombocytopenia and lymphopenia reflect the degree of oxidative stress and can be followed as biomarkers (27, 28).
- Since statins cause rhabdomyolysis as a complication, avoid these drugs in COVID-19 patients (29, 30).
- Erythrocytes are decreased due to latent hemolysis, which can be monitored by LDH levels (31).
- Increased MetHb makes SpO_2 calculation inaccurate. It causes a low SpO_2 by pulse oximetry in patients with a normal PaO_2 (32, 33). This can be misleading and can result in an unnecessary administration of O_2, the substrate of ROS.

Thus, SARS-CoV-2 interacts with the innate PO system, and adequate ROS levels are a first-line antimicrobial defense.

The AO System

The AO system balances the PO response through two central mechanisms: suppression of ROS production by NO and ROS neutralization by GSH (34, 35).

As depicted in **Figure 2**, SARS-CoV-2 binds to the ACE2 receptor in order to enter cells and, in turn, destroys this receptor. The ACE2 receptor is involved in the protective ACE2/endothelial nitric oxide synthase (eNOS)/NO pathway of the renin–angiotensin–aldosterone system (RAAS). It leads to the suppression of eNOS, the most prevalent isoform of

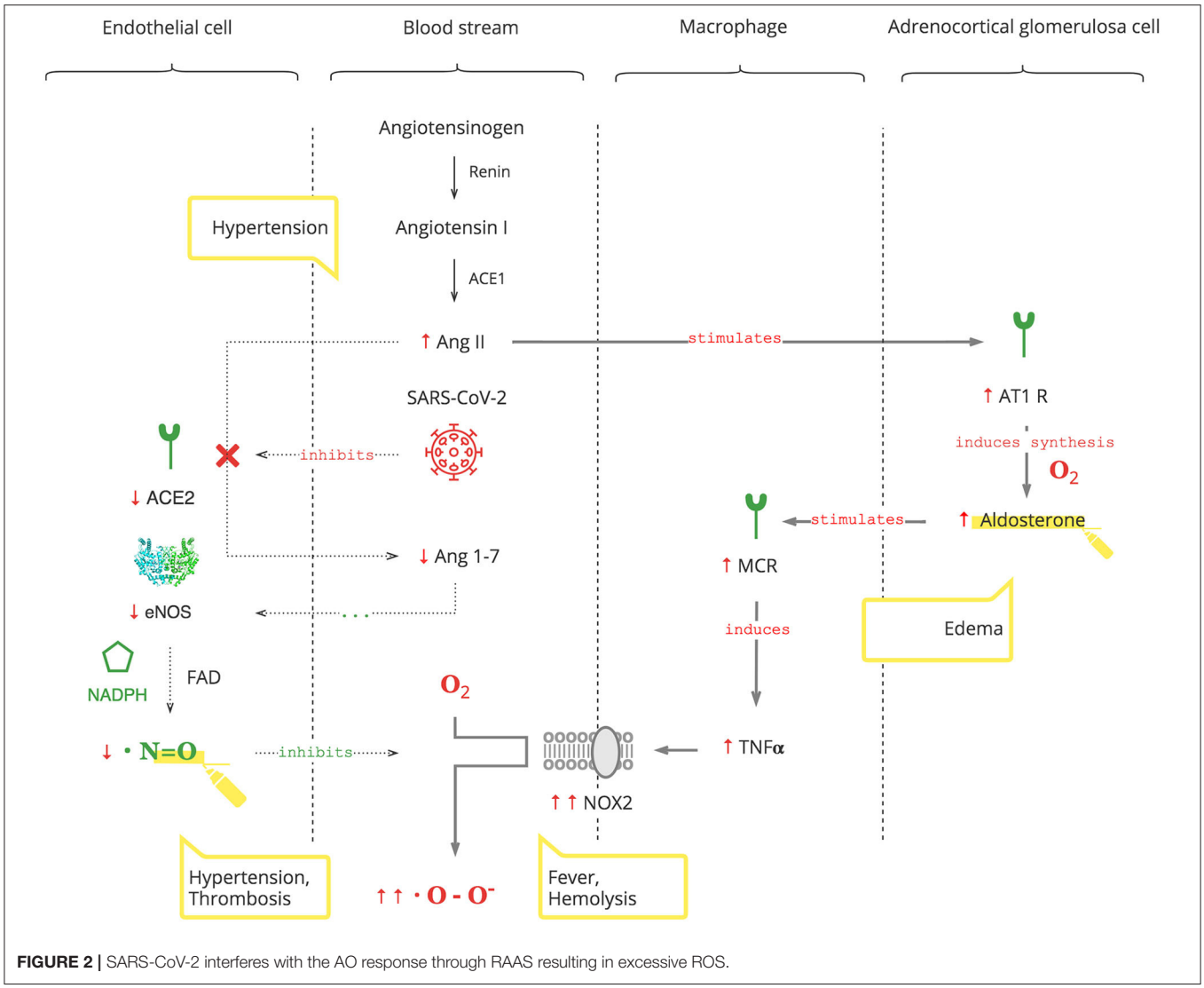


FIGURE 2 | SARS-CoV-2 interferes with the AO response through RAAS resulting in excessive ROS.

NOS, and consequently decreased NO levels (36). In addition to its antioxidant property, NO is also essential for vasodilation, prevention of platelet aggregation, and inhibition of the replication of SARS-CoV (37, 38).

Suppression of ACE2 activity also leads to an inability to convert angiotensin II (Ang II) to angiotensin 1-7 (Ang 1-7). Ang II is a potent vasoconstrictor and also stimulates Ald (39). This results in a transient increase in Ald that induces TNFα through mineralocorticoid receptor (MCR) on macrophages (40). NOX2 hyper-activation by TNFα, which is induced by the virus and Ald, and its disinhibition by virus-induced NO inhibition perpetuate ROS production, making it excessive.

It is also noteworthy that adrenocortical glomerulosa cells are extremely sensitive to dissolved O_2 blood levels (41). And fever shifts the O_2-Hb dissociation curve to the right, lowering the affinity of Hb to O_2, further contributing to ROS production (42).

Clinical Pearls

- An increased Ald level is a specific biomarker of the SARS-CoV-2 infection. This is acute, transient, and Ang II-dependent.
- The virus-induced decrease in NO and increase in Ald (NO/RAAS dysbalance) render the immune response to COVID-19 excessive. This manifests with fever and hematological complications, especially progressive hemolysis, and thrombus formation (43).
- Vasoconstriction, mediated by NO/RAAS dysbalance, is a main pathophysiological component of COVID-19-associated acute respiratory distress syndrome (ARDS) and manifests as acute vascular distress syndrome (AVDS) (44).
- Pulmonary edema is potentiated by elevated levels of Ald and aggravates ARDS (45).
- Excessive O_2 therapy can be deleterious.

Thus, SARS-CoV-2 interferes with the AO system, rendering the PO response excessive. Moreover, SARS-CoV-2-induced

increases of Ald aggravate the condition, especially in patients with underlying health conditions.

The Role of Underlying Health Conditions

Individuals probably contract COVID-19 at similar rates. However, once infected, some persons do worse than others. The inoculum of infection may be an important variable (46) but will not be further discussed here. We will focus on the role of underlying health conditions. And we will relate these to the PO and AO immune responses that we discussed above.

As noted above, COVID-19 induces an excessive PO response. This needs to be balanced by AO mechanisms: NO and GSH. As depicted in **Graphical Abstract**, these two mechanisms are dependent on the cofactor NADPH (47, 48). It is produced mainly by G6PD in a rate-limiting manner in the pentose phosphate pathway (PPP) of glucose metabolism. In addition to NO and GSH, there are several other systems that require NADPH and compete for it: macrophage NADPH oxidase (NOX2) for antimicrobial defense, NADPH methemoglobin reductase for Hb recovery, and thyroid NADPH oxidase for triiodothyronine (T3) production (49). The inability of G6PD to supply enough NADPH for the excessive immune response, along with these other demands, aggravates G6PDd. Thus, G6PD is essential for both components of innate immune response and, particularly, for the AO system to balance the PO system (50).

NO and GSH are also dependent on flavin adenine dinucleotide (FAD). FAD production is catalyzed by T3, which requires NADPH for its synthesis by thyroid NADPH oxidase (51). Thus, G6PDd ultimately decreases T3, NO, and GSH, thereby compromising the body's defensive mechanisms.

Acquired G6PDd

While congenital G6PDd is well known, its acquired deficiency is less appreciated. It accompanies insulin resistance (52) and hypertension (53, 54), grouped together as the metabolic syndrome. In addition, advancing age also lowers it (55). We demonstrate the biochemical rationale of these findings and why these cohorts do worse with COVID-19.

As depicted in **Figure 3**, adipocytes secrete leptin. Obesity-induced hyper-leptinemia is chronic and progressive and directly stimulates Ald (56). Moreover, leptin suppresses atrial natriuretic peptide (ANP), which helps to "escape" Ald activity (57). Increased Ald, as discussed previously, results in increased TNFα (37). In addition to NOX2 activation, chronic TNFα stimulation also causes insulin resistance (14, 58).

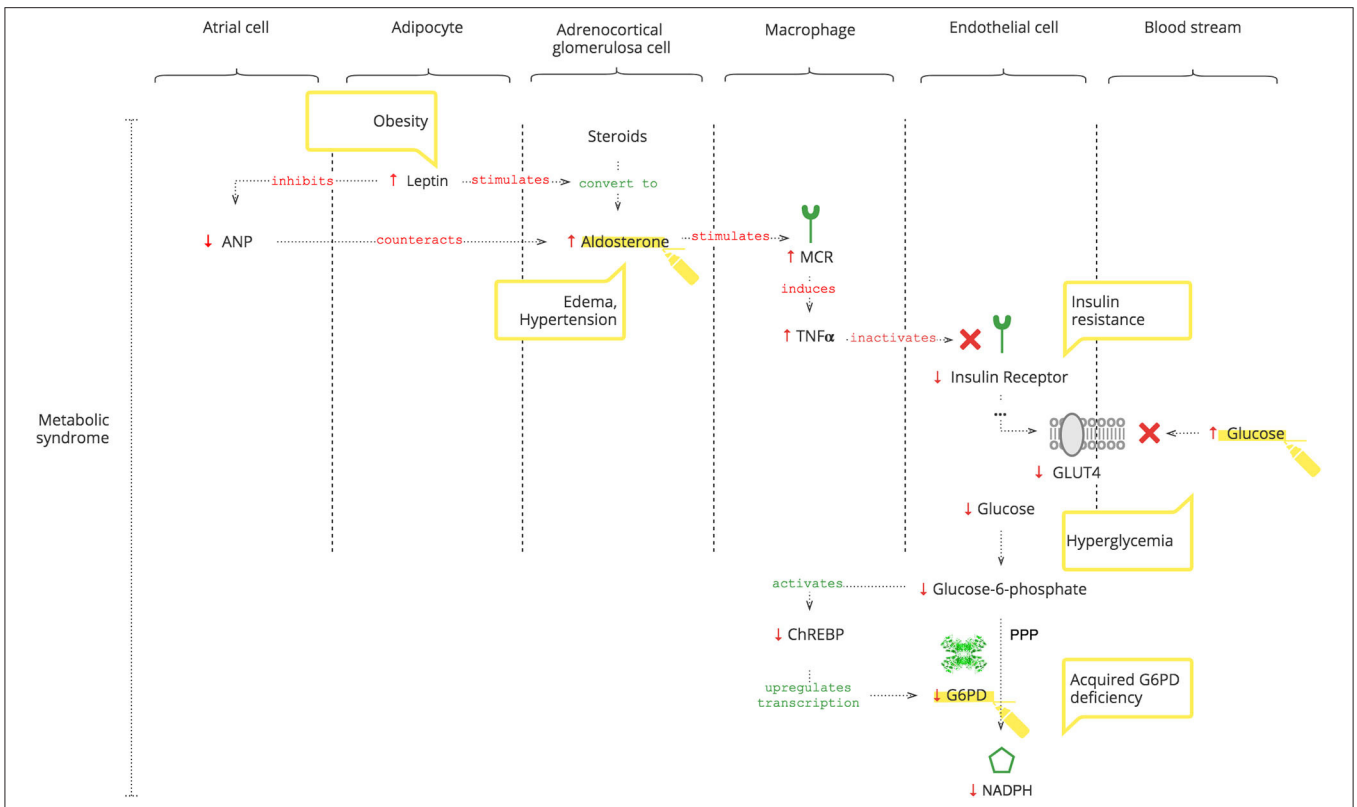


FIGURE 3 | How the metabolic syndrome leads to acquired G6PDd.

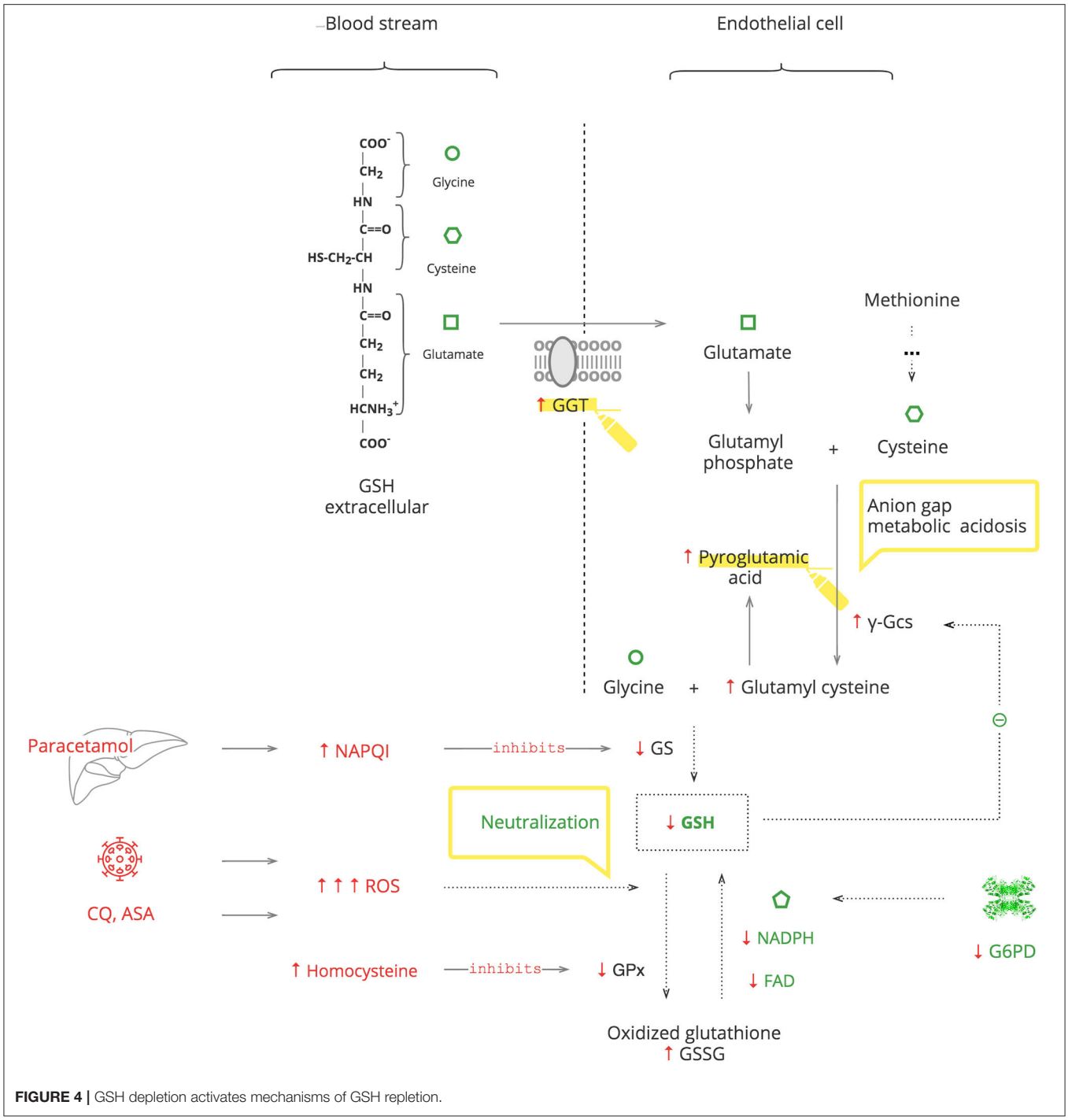


FIGURE 4 | GSH depletion activates mechanisms of GSH repletion.

Under normal conditions, insulin receptor signaling is required for glucose entrance into cells. Glucose is phosphorylated to glucose-6- phosphate, which activates the carbohydrate response element-binding protein (ChREBP) (59). ChREBP regulates the expression of rate-limiting enzymes in glucose metabolism, in particular G6PD (60). Thus, decreased intracellular glucose results in decreased G6PD gene expression and, consequently, lower NADPH (61).

Moreover, Liao et al. (62) showed that there is no significant difference in the expression of TNFα between G6PDd and normal patients.

Clinical Pearls

- Even when SARS-CoV-2 can no longer be detected and antibodies have formed, clinical manifestations of inflammation may ensue due to Ald-triggered TNFα.

- Leptin-induced increase of Ald is chronic, progressive, and AngII-independent, and it is not controlled by the RAAS, so angiotensin II receptor blockers (ARBs) and ACE inhibitors can be ineffective as antihypertensives (63).
- Decreased ANP levels in patients with metabolic syndrome render them vulnerable to COVID-19-induced acute increased Ald (64, 65).
- In COVID-19 patients with metabolic syndrome, hypertension, edema, and hyperglycemia accentuate.
- Chronic hyperglycemia can cause insulin resistance and can be a biomarker of developing G6PDd (66, 67).
- Laboratory values of G6PD levels and resulting NADPH activity can differ for several reasons: highly variable glucose level-dependent G6PD gene expression; the unique rate-limiting catalyzation of NADPH production; and the overload of immune mechanisms competing for NADPH, especially in patients with developing G6PDd.
- T3 levels reflect the NADPH activity but also can be involved in thyroid gland disorders.
- Metabolic syndrome-related chronic G6PDd can be aggravated by COVID-19-induced insulin resistance.
- As a consequence, patients with metabolic syndrome have a decreased level of NO and exogenous NO treatment can be considered (12, 68).
- Optimal control of underlying chronic diseases helps defend against COVID-19.

Thus, metabolic syndrome causes G6PDd. And G6PDd, by reducing NO, dysbalances the immune response to COVID-19. In addition, GSH plays a critical role as discussed below.

The Role of GSH System

GSH is an essential endogenous antioxidant. As depicted in **Figure 4**, it is composed of three amino acids: glycine, cysteine, and glutamate. The sulfhydryl (-SH) moiety of cysteine is responsible for the neutralization of toxic substances, both endogenous such as ROS and exogenous such as xenobiotics. During this reaction, GSH is oxidized to its inactive form, GSSG. The recycling requires NADPH and FAD (69).

GSH depletion can be caused by G6PDd, which leads to an inability to recycle it (70, 71). It can also be caused by excessive levels of toxic substances that overload the capacity for its neutralization. Furthermore, the GSH system can be compromised by exogenous substances, e.g., the paracetamol metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which inactivates glutathione synthetase (GS) of GSH production, and by endogenous substances, e.g., homocysteine, which inactivates glutathione peroxidase (GPx) of GSH function (72, 73). The body responds with γ-glutamyl transferase (GGT) upregulation to replete intracellular amino acids from extracellular GSH and also by *de novo* production of cysteine from methionine (74, 75). These amino acids then enter the γ-glutamyl cycle. When there is abundant GSH, it suppresses its own production by blocking γ-glutamyl cysteine synthase (γ-Gcs). Otherwise, GSH depletion results in increased γ-Gcs, leading to accumulation of pyroglutamic acid (76).

Clinical Pearls

- Exogenous stresses such as infection, medications, e.g., chloroquine (CQ), aspirin (ASA), and medical procedures, are accompanied by increased ROS production, which exacerbates GSH deficiency (77–80).
- In COVID-19, paracetamol is used as an antipyretic to avoid NSAIDs, and it accentuates GSH deficiency (69).
- Exacerbation of G6PDd manifests with fever and hematologic complications, especially hemolytic anemia. If a patient's Hb decreases after 2–3 days on certain treatments, e.g., CQ or O_2 therapy, and the LDH level has increased, G6PDd should be considered.
- In critically ill patients, severe G6PDd manifests with transient hypothyroidism also known as the "low T3 syndrome" or the "euthyroid sick syndrome" (81).
- Patients with metabolic syndrome have increased levels of homocysteine. Consider folic acid and/or cyanocobalamin deficiency in these patients to prevent aggravation of GSH depletion (82, 83).
- Severe GSH deficiency clinically manifests with unexplained anion gap metabolic acidosis. This should be considered as pyroglutamic acidosis until proven otherwise. This acidemia, by itself, is not clinically important, but it is a sign of serious metabolic stress (73).
- An increased level of GGT can be used as a biomarker of GSH depletion.

CONCLUSION

G6PD activity is essential for the adequate functioning of both the PO and AO components of the innate immune response to counteract COVID-19-induced immune dysregulation. Therefore, in COVID-19 patients, inadequate G6PD activity should be considered and can be monitored with biomarkers. Recognizing these interactions is critical to avoid inappropriate treatment. "*Primum non nocere*."

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors contributed to the work equally. All authors read and approved the final manuscript.

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Epidemiological Characteristics and Clinical Outcomes of Coronavirus Disease Patients in Northwest China: High-Volume Research From Low Population Density Regions

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Background: Few studies have reported the transmission characteristics of coronavirus disease (COVID-19) in low-density populations. This study has therefore analyzed the epidemiological characteristics and clinical outcomes of COVID-19 patients in Northwestern China, an area with low population density.

Methods: From January 21 to March 11, 2020, data from patients diagnosed with novel coronavirus pneumonia (NCP) in areas of Northwestern China with lower population densities were retrospectively analyzed. Certain variables were categorized as numbers and percentages, with the ratio between resident patients (no history of going out during the epidemic) and imported patients representing the contagiousness of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) responsible for COVID-19. Hospitalization time was also calculated.

Results: A total of 617 COVID-19 patients were reported in Northwestern China, and the morbidity and mortality rates of 0.000005 and 0.011, respectively. Further analysis showed that the morbidity was inversely proportional to population density and distance from Wuhan City. This study enrolled 473 confirmed cases; among these patients, there were 248 residents and 225 imported cases with a ratio of 1:1. The youngest and oldest patients were 1 and 94 years of age, respectively, with a median age of 42 years. Fifteen (3.2%) patients were children or infants. Two patients were pregnant, and one patient gave birth to a healthy baby with negative results during her disease course. About 17.3% of patients (82 cases) were healthy carriers without any symptoms during their disease course. One male patient (0.2%) had recurrence of a positive test result 4 days after discharge. The median hospitalization time was 16.0 days, ranging from 2.0 to 43.0 days. Further analysis showed that age ($P = 0.03$) and severity status ($P < 0.001$) were significantly correlated with hospitalization time.

Conclusions: The morbidity and mortality rates of COVID-19 patients in the regions with a low population density were lower than those of the national average in China. All populations were susceptible to infection by SARS-CoV-2. Asymptomatic patients with positive results should be taken seriously, and the hospitalization time of patients is associated with their age and severity status.

Keywords: COVID-19, low density population, public health intervention, epidemiological characteristics, outcomes

INTRODUCTION

Coronavirus disease (COVID-19) (1), also called novel coronavirus pneumonia (NCP) by Chinese officials, emerged in Wuhan City in late December 2019 and remains an ongoing outbreak that has spread globally (2). After critical management of COVID-19 by restricting transmission and treating patients, the number of infected people from China has declined but has sharply increased outside China, especially in Middle Asia, Europe, and the United States (3). On March 3, 2020, the World Health Organization (WHO) reported that more than 132,000 cases had been diagnosed from 123 countries and regions, with more COVID-19 cases reported in Europe every day than were reported in China at the height of its pandemic (4). In addition, recent studies have shown that the infection rate of COVID-19 has been higher than that of Severe Acute Respiratory Syndrome (SARS) (5). To identify the infection source, transmission route, and susceptible population, researchers have focused on investigating the epidemiology of COVID-19 in densely populated areas of China (6, 7); however, only few have reported on regions with lower population densities. Northwestern China is adjacent to Middle Asia and far from the outbreak area; moreover, it has a low population density, inconvenient transport system, and insufficient medical facilities. The potential differences in this epidemic disease in other countries and regions are not clear. We consequently performed this study on the clinical features of COVID-19 cases in these regions compared to epidemiology and clinical prognosis of NCP patients from Northwestern China to contribute to NCP prevention and control and to help other countries similar to the geography and demographic distribution of Northwestern China.

MATERIALS AND METHODS

Study Design

From January 21 to March 11, 2020 (January 24 was the Chinese New Year), all patients diagnosed with NCP from seven provinces or autonomous regions in Northwestern China with a low population density were retrospectively analyzed. The diagnostic criteria were based on the seventh edition of the National New Coronavirus Pneumonia Diagnosis and Treatment Program (8) developed by the National Health Commission of the Peoples' Republic of China. The patient inclusion criteria were the following: (1) positive severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) result by reverse transcription-polymerase chain reaction (RT-PCR); (2) receiving treatment at a designated hospital; (3) definite outcome (discharge or death); and (4) adequate clinical information and available follow-up data. This study was approved by the ethics committee of Tangdu Hospital. Written informed consent was waived due to the nature of open-access data. The last follow-up was on March 11, 2020.

Setting and Population Density

Northwestern China is adjacent to Middle Asia, far from the outbreak epicenter. This region has a low population density, inconvenient transport system, and insufficient medical facilities. Northwest China consists of four autonomous regions (Tibet Autonomous Region, Xinjiang Uygur Autonomous Region, Ningxia Hui Autonomous Region, and Inner Mongolia Autonomous Region) and three provinces (Shaanxi Province, Qinghai Province, and Gansu Province) (**Table 1**). The seven provinces or autonomous regions of Northwestern China cover ∼57.5% of China's total territory, with an area of 5,490,400 km^2; however, its total population from the 2018 census data is only 131,590,000 (9). Hubei Province, located in Central-Eastern China, the COVID-19 outbreak area, has a population density of 318.3 people/km^2. The population density of Northwestern China is, however, relatively low (23.8 people/km^2)—lower even than the national average (145.4 people/km^2). The distances between the capital of the province or the autonomous region and Wuhan City are also shown in **Table 1**.

Data Collection

We obtained data on the exposure history, age, sex, clinical signs and symptoms, diagnosis time, treatment, and outcome from the news reports and press releases reported by the Health Commission of Tibet Autonomous Region, Xinjiang Uygur Autonomous Region, Ningxia Hui Autonomous Region, Inner Mongolia Autonomous Region, Shaanxi Province, Qinghai Province, and Gansu Province. The degree of severity, diagnostic criteria, and discharge criteria refer to the seventh edition of the National New Coronavirus Pneumonia Diagnosis and Treatment Program. All data were collected by Doctors Zhu, Zhang, and Jia, and major disagreements between these three doctors was checked by a fourth reviewer (Doctor Xu). Data are verified with the National Health Commission and the Chinese Center for Disease Control and Prevention.

Follow-Up

The follow-up of patients after discharge was equally important; all discharged patients should continue to be isolated for medical observation for 14 days. During the isolation period,

TABLE 1 | The population and geography features of seven provinces or autonomous regions in Northwestern China.

Region	Distance (km)[a]	Population density	Confirmed cases[b]	Infection density[c]	Morbodity[d]
Shaanxi province	731	187.8	245	0.001	0.000006
Qinghai province	1,597	8.4	18	0.00002	0.000003
Tibet autonomous region	3,568	2.8	1	0.0000008	0.0000003
Xinjiang Uygur autonomous region	3,267	15.0	76	0.00005	0.000003
Ningxia Hui autonomous region	1,468	103.6	75	0.001	0.00001
Gansu province	1,383	58.1	127	0.0003	0.000005
Inner Mongolia autonomous region	1,436	21.5	75	0.00006	0.000003
Hubei province	–	318.3	67,781	0.4	0.001

[a]Distance: between the capital of province or autonomous region and Wuhan city; [b]Confirmed cases: cumulative confirmed cases as at March 11th, 2020; [c]Infection density: Number of confirmed cases/km^2; [d]Morbodity: The proportion of confirmed cases in the total population.

the body temperature, physical signs, and other conditions should be monitored daily in observation. All patients reviewed the pathogenic test of SARS-CoV-2 at last quarantine day, and nucleic acid testing will be performed at any time if any symptoms appear after discharge.

Statistical Analysis

Hospitalization time was defined as the time from the final diagnosis to discharge or death. According to severity status, all patients were divided into group for general, severe, and critical (8). Categorical variables are summarized as numbers and percentages. *T*-tests were performed to compare the differences in hospitalization time among groups; when the cases were not normally distributed, Mann–Whitney U or Kruskal–Wallis H-tests were used. A bilateral $P < 0.05$ was considered statistically significant. All analyses were performed using IBM SPSS Statistics for Windows, version 22.0.

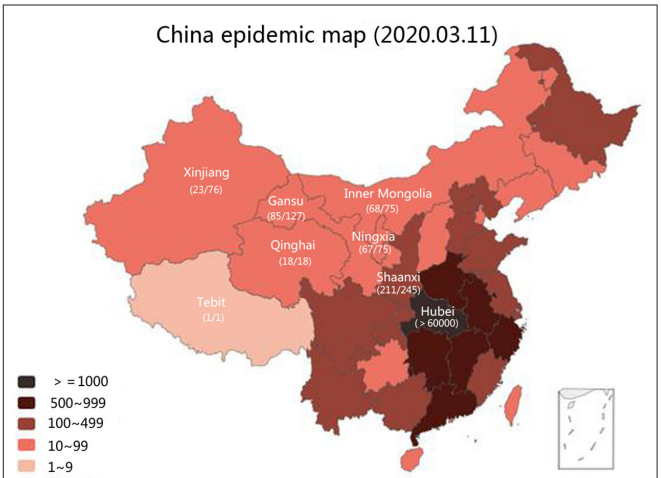


FIGURE 1 | Northwestern China and the epidemic map of China.

RESULTS

Epidemiological Characteristics

As of March 11, 2020, 80,793 patients had been diagnosed with COVID-19 in China; of these, 67,781 were in Hubei Province, while 617 were in seven northwestern provinces or autonomous regions, accounting for 0.8%. The morbidity and mortality rates were 0.000005 and 0.011, respectively, in the low population-density region. The epidemic map of China (**Figure 1**) showed that the cumulative confirmed cases in the seven northwestern provinces or autonomous regions were far less than those in Hubei and other provinces. **Figure 2A** showed that the number of infected patients increased with population density. The Tibet Autonomous Region, with the lowest population density of China, reported only one imported case, without indigenous secondary cases, with an infection density of only 0.0000008 people/km^2. There were 245 cases in Shaanxi Province, which is the most densely populated of these seven provinces, with an infection density of only 0.001 people/km^2. The number of cumulative confirmed cases was associated with the distance between the capitals of the province or autonomous region and Wuhan City (**Figure 2B**, **Table 1**).

According to the inclusion criteria, a total of 473 patients were finally enrolled in our study for analysis (**Table 2**). Of the 473 confirmed cases, one was from the Tibet Autonomous Region, 23 from the Xinjiang Uygur Autonomous Region, 67 from the Ningxia Hui Autonomous Region, 68 from Inner Mongolia Autonomous Region, 211 from Shaanxi Province, 18 from Qinghai Province, and 85 from Gansu Province (**Figure 1**). The first confirmed and discharged patients were reported on January 21 and January 31, 2020, respectively. The peak period for confirmed cases was from January 25 to February 8, 2020 (**Figure 3**). A total of 194 patients (41.0%) had a history of living or traveling in Hubei Province, 33 patients had an absence of contact history, and two patients had traveled from Iran.

Public Health Interventions and Their Effects

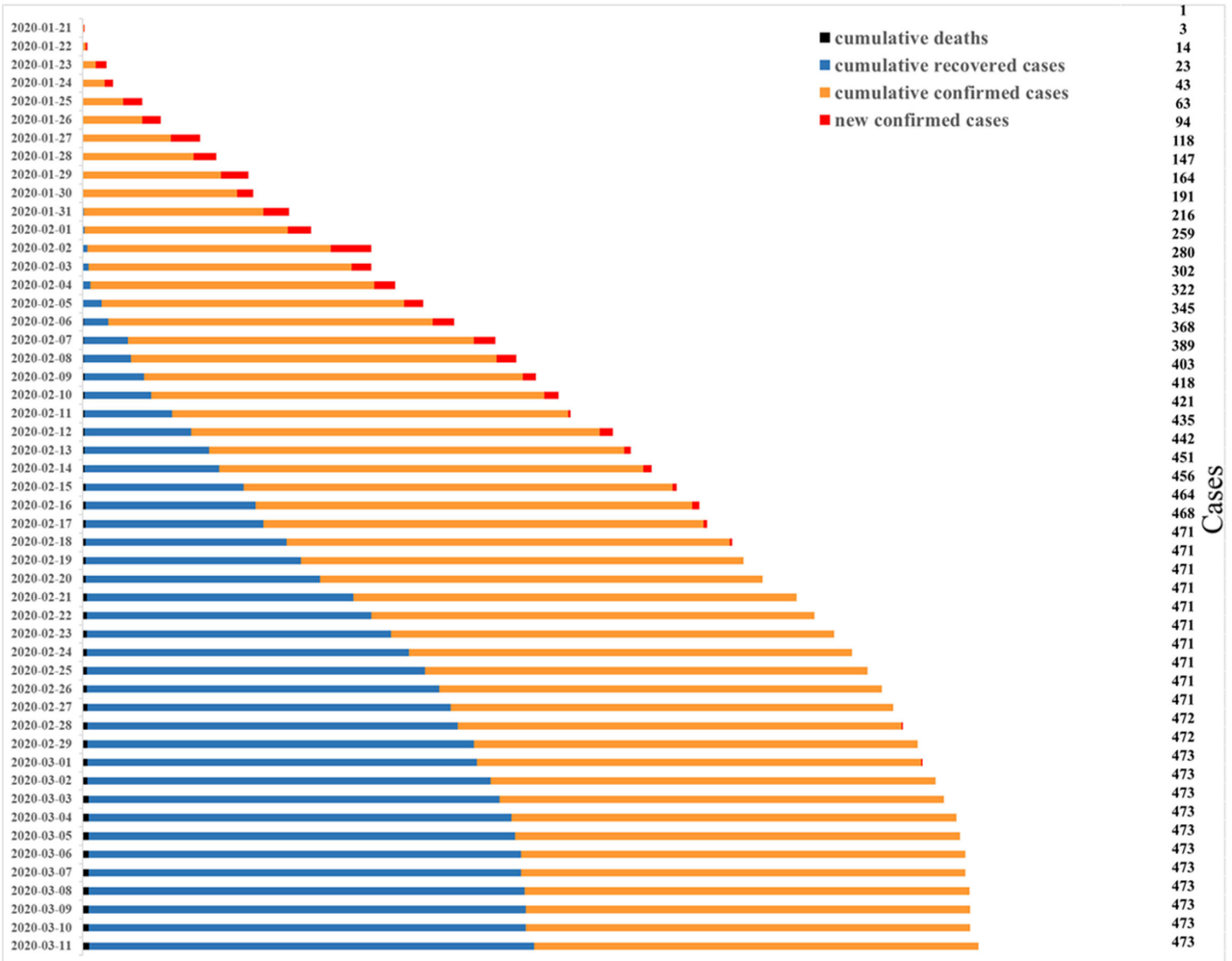
From the beginning of the COVID-19 outbreak in Wuhan, the confirmed cases in Northwestern China have gradually been on the increase. On January 21, 2020, the first imported

FIGURE 2 | Correlation between geographical factors and COVID-19. **(A)** Distribution of cumulative confirmed cases and population density in Northwestern China. **(B)** Distribution of cumulative confirmed cases and distances between the provincial capital and Wuhan City and Northwestern China.

patient emerged, with confirmed local cases increasing with the growth of imported cases within 6 days despite the shutdown of Wuhan on January 23, 2020. Four days after the Wuhan shutdown, the number of new imported patients peaked at 22 cases. Approximately 76.9% of imported cases (173/225) were diagnosed within 10 days of the closure of Wuhan City. To better prevent and control COVID-19 spread, strict countermeasures designed and approved by local authorities were implemented on January 30, 2020, which included raising the public health response level to Class A, setting up health checkpoints in public areas, and locking down the regional border. The daily numbers of imported cases decreased, and more new local cases were confirmed 3 days after the implementation of these control measures. On February 2, 2020, the number of new local patients surpassed imported patients and simultaneously peaked. The next day, the numbers of imported and local patients had significantly decreased and on February 18, 2020 no newly confirmed patients were reported. On February 26, 2020, however, the first imported case from overseas was reported in Northwestern China. The details of the public health events, government anti-virus measures, and related dynamic results are separately displayed in **Figure 4**.

Clinical Characteristics

The final analysis included 473 patients (246 men and 227 women). The youngest and oldest patients were 1 and 94 years of age, respectively, with a median age of 42



Date	2020-01-21	2020-01-22	2020-01-23	2020-01-24	2020-01-25	2020-01-26	2020-01-27	2020-01-28	2020-01-29	2020-01-30	2020-01-31	2020-02-01	2020-02-02	2020-02-03	2020-02-04	2020-02-05	2020-02-06	2020-02-07	2020-02-08	2020-02-09	2020-02-10	2020-02-11	2020-02-12	2020-02-13	2020-02-14	2020-02-15	2020-02-16	2020-02-17	2020-02-18	2020-02-19	2020-02-20	2020-02-21	2020-02-22	2020-02-23	2020-02-24	2020-02-25	2020-02-26	2020-02-27	2020-02-28	2020-02-29	2020-03-01	2020-03-02	2020-03-03	2020-03-04	2020-03-05	2020-03-06	2020-03-07	2020-03-08	2020-03-09	2020-03-10	2020-03-11
cumulative confirmed cases	1	3	14	23	43	63	94	118	147	164	191	216	259	280	302	322	345	368	389	403	418	421	435	442	451	456	464	468	471	471	471	471	471	471	471	471	471	471	472	472	473	473	473	473	473	473	473	473	473	473	473
cumulative deaths	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	2	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	4	5	5	5	5	6	6	6	6	6	6	6	6	6	6	7
cumulative recovered cases	0	0	0	0	0	0	0	0	0	0	1	2	5	6	8	20	26	47	50	63	71	93	113	132	143	168	181	189	214	229	249	284	303	324	343	360	375	386	394	411	414	429	437	450	454	460	460	464	465	465	473
new confirmed cases	1	2	11	9	20	20	31	24	29	17	27	25	43	21	22	20	23	23	21	14	15	3	14	7	9	5	8	4	3	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0

FIGURE 3 | Time distribution of cumulative cases in Northwestern China through March 11th, 2020.

years. Fifteen patients (3.2%) were <12 years of age. Two patients were pregnant. One of them was full-term. She was infected with SARS-CoV-2 during the delivery period and eventually gave birth to a baby girl. Among all patients, 82 (17.3%) were asymptomatic in their disease course. Fever was the most common symptom (53.9%), ∼2.7% of patients presented with diarrhea, and 26.0% of patients with other symptoms. According to severity status, 429 patients (90.7%) were general, 26 patients (5.5%) were severe, and 18 patients (3.8%) were critical. Detailed patient information is listed in **Table 2**.

Clinical Outcomes

The current discharge criteria are based on the seventh edition of the COVID-19 diagnosis and treatment guidelines (8). These include the disappearance of clinical symptoms and two consecutive negative nucleic acid tests with an interval time of over 24 h. Of the 473 patients, 466 met the discharge criteria. One male patient (0.2%) had a recurrence of positive test 4 days after discharge.

From March 11, 2020, seven patients died, corresponding to a mortality rate of 1.1% (7/617), which is lower than that of the national average (3.9%) (10); the other patients were all

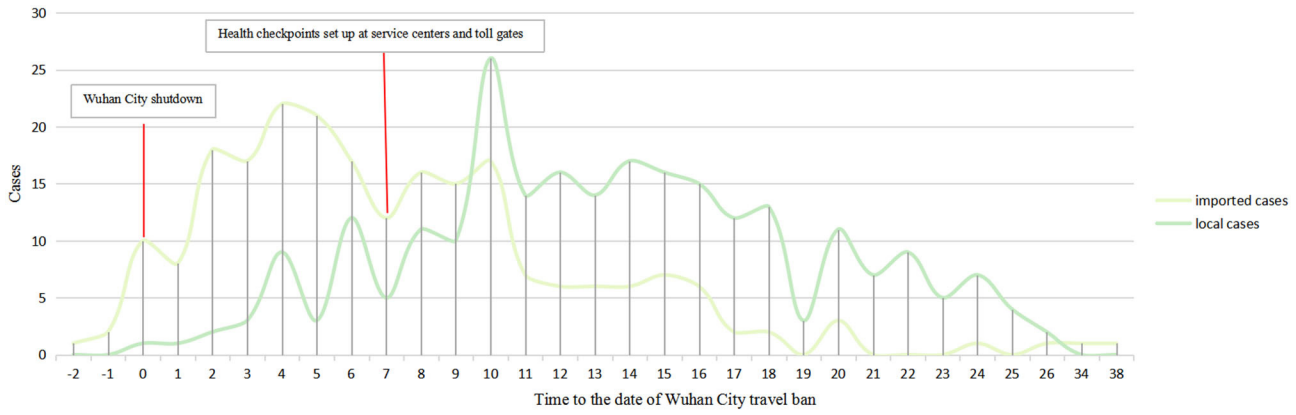


FIGURE 4 | Newly increased COVID-19 cases (local cases vs. imported cases) per day in northwestern China.

TABLE 2 | Clinical characteristics of COVID-19 patients.

Characteristics	N(473)	%
Age, median (range)	42.0 year	1.0–94.0
Gender		
Male	246	52.0%
Female	227	48.0%
Religion		
Shaanxi province	211	44.6%
Qinghai province	18	3.8%
Tibet autonomous region	1	0.2%
Xinjiang Uygur autonomous region	23	4.9%
Ningxia Hui autonomous region	67	14.2%
Gansu province	85	18.0%
Inner Mongolia autonomous region	68	14.4%
Age		
<42	223	47.1%
≥42	250	52.9%
Patient type		
Local cases	248	52.4%
Imported cases	225	47.6%
Exposure history		
Living or traveling in Hubei	194	41.0%
Contact with confirmed patients	244	51.6%
Without contact history	35	7.4%
Symptom		
Fever	255	53.9%
Diarrhea	13	2.7%
Other symptom	123	26.0%
Without symptom	82	17.3%
Severity status		
General	429	90.7%
Severe	26	5.5%
Critical	18	3.8%

discharged (**Figure 3**). The median hospitalization time was 16.0 days, ranging from 2.0 to 43.0 days. Further analysis showed that age ($P = 0.03$) and severity status ($P < 0.001$) were significantly correlated with hospitalization time (**Table 3**). While patients presenting with diarrhea had shorter hospitalization times than those of patients presenting the other symptoms, the difference was not of statistical significance (16.0 vs. 17.0 vs. 14.0 vs. 14.0 days, $P = 0.091$).

Of the seven patients who died, four were male and three were female (**Table 4**). Only one patient was younger (aged 48 years); the other patients were aged over 70 years (70, 73, 76, 77, 82, and 89 years). According to disease severity two and five of these patients were categorized as severe and critical, respectively. Only two patients died without comorbidity; three patients were diagnosed with hypertension, and two patients were diagnosed with chronic obstructive pulmonary disease (COPD).

DISCUSSION

Three months ago, the COVID-19 outbreak started in Wuhan City (1), the capital of Hubei Province, which is located in the central region of China and is known as "Chinese Chicago"—a developed transportation hub for advanced railway, water carriage, and aviation systems. The population density of Wuhan is 1,249 people/km^2. The main transmission routes of SARS-CoV-2 include droplets and aerosols (11). Compared to other infectious diseases, such as SARS and Middle East Respiratory Syndrome (MERS), COVID-19 thus has higher infectivity (5). A previous study proved that high population density catalyzes the spread of COVID-19 (12). To our knowledge, the present was the first study to assess the impact of government public health interventions on the spread of COVID-19 in low population density areas. A total of 617 patients were identified in these regions, accounting for 0.8% of the total number of Chinese cases. The prevalence of COVID-19 in Northwest China was 0.000005, which is lower than the national average (0.00006).

The lower morbidity in Northwest China might be attributed to its low population density and geographical distance from the epicenter. A previous study proved that high population densities catalyze the spread of COVID-19 (12), a finding consistent with our results. In our study, compared to high-density areas, low-density areas had lower morbidity. In

TABLE 3 | Clinical outcomes of COVID-19 patients.

Variable	*N*	Hospitalization time (days)	Percentiles	*P*
In total	473	43.0	–	
Gender				0.323
Male	246	17.0	(12.0, 21.0)	
Female	227	16.0	(11.0, 20.0)	
Age				0.030
<42	223	16.0	(11.0 ,20.0)	
≥42	250	17.0	(12.0, 21.0)	
Patient type				0.196
Local cases	248	16.0	(11.0, 20.0)	
Imported cases	225	17.0	(12.0, 21.0)	
Exposure history				0.303
Living or traveling in Hubei	194	17.0	(12.0, 21.0)	
Contact with confirmed patients	244	16.0	(11.0, 21.0)	
Without contact history	35	16.0	(11.0, 20.0)	
Symptom				0.091
Fever	255	17.0	(13.0, 21.0)	
Diarrhea	13	14.0	(10.75, 21.5)	
Other symptom	123	16.0	(12.0, 20.0)	
Without symptom	82	14.0	(10.75, 20.0)	
Severity status				<0.001
General	429	16.0	(11.5, 20.0)	
Severe	26	20.5	(14.75, 26.0)	
Critical	18	23.5	(16.25, 32.25)	

TABLE 4 | Clinical characteristics of seven patients died from COVID-19.

No	Age	Gender	Exposure history	Symptom	Severity status	Comorbidity
1	89.0	Male	Contact with confirmed patients	Cough	Critical	Hypertension, cerebral infarction, gout
2	82.0	Female	Contact with confirmed patients	Cough	Severe	Hypertension, Alzheimer disease
3	77.0	Male	Living or traveling in Hubei	Without symptom	Critical	Hypertension, COPD
4	73.0	female	Contact with confirmed patients	Fever	severe	Hypertension
5	76.0	Female	Without contact history	Without symptom	Critical	Hypertension, Atrial fibrillation, Chronic bronchitis
6	70.0	Male	Without contact history	Without symptom	Critical	Without
7	48.0	Male	Without contact history	Fever, cough and diarrhea	Critical	Without

addition to population density, the regions and autonomous regions in this study are far from the urban center of China. Compared to other regions closer to Hubei, the lower incidence in the seven northwestern provinces farther from Wuhan was related to the geographical distance. Our findings confirmed that Xinjiang and Tibet Autonomous Region, far from Wuhan, had a much lower incidence than in Shaanxi Province, which is relatively close to Wuhan. The traffic conditions and proportion of floating population in Wuhan also implied that distance is a factor affecting the epidemic (13).

Moreover, public health interventions, advocated by the government, also played an important role in preventing and controlling the spread of COVID-19. Tian et al. reported that the Wuhan City shutdown delayed the spread of COVID-19 to other cities for 2.91 days (95% confidence interval [CI]: 2.54–3.29 days) (14). A simulation analysis by Chinese scholars proved that both quarantine and traffic blockage were significant methods to control the outbreak (15). The economic level, the ability of government management and the activities of medical treatment have great impact on the incidence rate and mortality rate of different regions (16, 17). In our study, public health interventions designed and approved by local authorities included setting up health checkpoints in public areas and locking down the regional border. Four days after the implementation of control measures, the number of imported and local cases decreased significantly, and no newly confirmed cases occurred after 19 days, Surprisingly, in the first 3 days after restricting activities, the number of new cases increased, which may be related to the familial spread of COVID-19.

Related research confirmed family clustering sin the spread of COVID-19 (18).

In addition, quarantine and medical observation of potentially infected populations are effective methods of epidemic control (19), especially in screening asymptomatic patients. It remains controversial whether asymptomatic patients are contagious. A study from Henan Province of China showed that an asymptomatic carrier transmitted COVID-19 virus to her five family members (20); therefore, the management of asymptomatic patients is critical for preventing outbreaks. We reported that ∼17.3% of patients were asymptomatic carriers, all of whom were detected during the period of quarantine or medical observation.

Early research has suggested that infants and young children belong to a disease-exempt population. However, the results of later studies (21, 22) refuted these initial findings. In our study, the youngest confirmed patient was only 1 year of age and 15 infants or children accounted for 3.2% of the total cases. It is worth noting that the confirmed specimen was a stool sample from a 1-year-old child, which indirectly proved the possibility of fecal–oral transmission of COVID-19, especially in children (23, 24).

In China, the current discharge criteria are based on the seventh edition of the COVID-19 diagnosis and treatment guidelines (8). These consist of the disappearance of clinical symptoms two consecutive negative nucleic acid test results at least 24 h apart. There are, however, sporadic case reports of recurrence worldwide (25), and we also observed one patient (0.2%) with recurrence of positive test 4 days after discharge.

The mortality rate of patients diagnosed with COVID-19 in this study was 1.1%, which was significantly lower than those reported in Wuhan City, Europe, and Central Asia (3). A possible reason for the low mortality rate is that, although the medical resources of Northwestern China are relatively insufficient compared to those of the eastern regions of China and European countries, every single patient can receive sufficient medical support, and most of the cases were imported young patients who left Wuhan City for work and had returned home (13).

The limitations of this study cannot be ignored. First, the laboratory results of patients could not be further analyzed due to the data came from news reported by the CDC in the provinces and autonomous regions; Second, although we have compared the incidence and mortality of COVID-19 in low-density areas with the national average, the data in other high-density areas outside the epidemic area were deficient; Finally, further analysis was limited because of the small sample size of this study.

In summary, the results of this study revealed that population density and distance from the epicenter are important factors affecting the prevalence of COVID-19. Public health interventions are useful for COVID-19 prevention and control. Additionally, the management of asymptomatic patients and children is more efficient when attempting to restrict the outbreak. Finally, the hospitalization time of patients was associated with their age and severity status.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The study was approved by the review board of Tangdu hospital of Air Force Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

TJ, JZha, and SX participated in study design and study conception. JZhu, QZ, WuW, JC, YX, WeW, XW, MW, HW, and ZZ performed data analysis. JZhu, QZ, CJ, and SX recruited patients. JZhu, QZ, CJ, WuW, and JC drafted the manuscript. All authors provided critical review of the manuscript and approved the final draft for publication.

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Epidemiological Characteristics and Clinical Features of Patients Infected With the COVID-19 Virus in Nanchang, Jiangxi, China

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Objectives: The 2019 novel coronavirus disease (COVID-19) pandemic is the biggest public health crises in the 21st century. While most patients infected with the COVID-19 virus have no to moderate symptoms, there is currently limited clinical information about these patients.

Methods: In this study, we retrospectively investigated 41 patients infected with the COVID-19 virus in Nanchang, Jiangxi province, China, from February 4 to March 2, 2020. Nanchang is about 260 km southeast of Wuhan, the initial epicenter of the COVID-19 pandemic. We retrieved information on patient demographics, physical examination results, epidemiology, clinical manifestations, underlying conditions, laboratory analyses, radiological images, and treatment outcomes.

Results: Most patients (70.7%) had a history of close contact with patients with confirmed COVID-19, and 16 patients (39.0%) showed a high degree of family clustering. All 41 patients had no to moderate symptoms. The median age was 39.9 years and common symptoms of illness were fever (69.2%), cough (65.4%), and fatigue (19.2%). The dominant patient group was middle-aged women, with hypertension (14.6%) and chronic liver disease (12.2%) as the most frequent underlying conditions. All patients recovered, with the mean time of viral nucleic acid clearance at 10.6 days. Chest CT scans presented ground-glass opacities in 53.7% of patients while 26.8% had normal CT images. Laboratory results showed that lymphocyte counts, lymphocyte percentages, ESR, CRP, IgG, Fib, and cytokines were correlated to patients' conditions. Approximately 60–90% of patients had abnormally high levels of cytokines IL-4, IL-6, IL-10, and/or TNF-α.

Conclusions: Our results showed variable clinical and laboratory presentations among this group of patients infected with the COVID-19 virus. Though all 41 patients recovered, our results suggest that cytokine levels and other biochemical indicators should be monitored for patients infected with the COVID-19 virus showing no to moderate symptoms to ensure quick access for critical medical attention, if needed.

Keywords: COVID-19, cytokines, asymptomatic, epidemiogy, close contact, biochemical & physiological biomarkers

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INTRODUCTION

The 2019 coronavirus disease (COVID-19) was first identified in December 2019 in Wuhan, China (1). It's caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), commonly known as the COVID-19 virus (2, 3). Since then, the virus has spread globally, resulting in an ongoing pandemic. As of June 4, 2020, over 6 million cases have been reported across 216 countries and territories, resulting in more than 382,000 deaths (4). Within China where the pandemic originated, there have been 84,614 confirmed cases of infection by the COVID-19 virus (data on June 4, 2020) (5). Among the reported cases in China, about 20% of the patients had severe or critical illness, with the remaining 80% showing moderate to no symptom (5). A similar pattern is found globally where over 80% of infected patients have shown moderate to no symptom (4). Among the total of 84,614 cases in China, 932 were reported from Jiangxi (6), a province immediately to the southeast of Hubei province where the pandemic originated. Among the 932 cases in Jiangxi, 931 have since recovered and one patient died (6). There has been no active COVID-19 indigenous case in Jiangxi province since February 27, 2020, except for two imported cases from abroad on March 21, 2020 and March 28, 2020, respectively.

Based on sequence analyses, the Coronaviridae Study Group (CSG) of the International Committee on Taxonomy of Viruses revealed that the virus causing COVID-19 was similar to that causing the severe acute respiratory syndrome (SARS) epidemic in 2003 (7, 8). However, unlike patients infected with SARS-CoV, those infected with the COVID-19 virus typically have a longer incubation period, and with the majority of infected patients showing apparently no to moderate symptoms (9, 10). A large number of studies have shown that these asymptomatic to moderate cases have contributed significantly to the transmission and spread of the virus in communities throughout the world (11, 12). Indeed, the large proportion of asymptomatic carriers with no to moderate symptoms represents among the most difficult challenges to the prevention and control of the pandemic. Thus, understanding the epidemiological and clinical features of these patients could help future prevention and control of COVID-19.

Several studies have investigated the epidemiology and clinical features of COVID-19 patients in China. Those studies have come from either Wuhan, Hubei province, the original epicenter of the outbreak or large metropolitan areas such as Beijing and Shenzhen where travel between Wuhan and these cities were very frequent in the initial phase of the outbreak (December 2019 to January 23, 2020) before the lockdown of Wuhan. In contrast, cases from other provinces, including Jiangxi, located immediate to the southeast of Hubei, have not been reported. Compared to Wuhan (a mega-transport hub in central China), Jiangxi province is relatively isolated, and not a typical travel destination for tourists, especially in winter months. Thus, even though it was close to Wuhan, the number of cases in Jiangxi province was relatively small, with a cumulative 932 cases in a population of 45.2 million, a prevalence of COVID-19 at ∼0.002% and a case-fatality rate of 0.11% (1/932). At present, the epidemiological and clinical feature of COVID-19 in Jiangxi is not known. The objective of this study is to retrospectively analyze the epidemiological and clinical characteristics of patients infected with the COVID-19 virus in a designated hospital for treating COVID-19 patients in the capital of Jiangxi province, Nanchang.

MATERIALS AND METHODS

Study Population

We retrospectively reviewed the medical records of inpatients between February 4, 2020 and March 2, 2020 at the Ninth Hospital of Nanchang, the designated hospital for treating patients infected with the COVID-19 virus. For all patients, the diagnostic criteria followed the guidelines for diagnosis and treatment plan of COVID-19 issued by the National Health Commission (trial version 7) (13). Specifically, the tests were based on real-time, reverse-transcription polymerase chain reaction using the primers and probes targeting the *ORF1ab* and *N* genes of the COVID-19 virus, as recommended by the Chinese Center for Disease Control and Prevention (14). There must be a positive laboratory test showing the respiratory specimens containing nucleic acid of the COVID-19 virus in order for the patient to be included in this study.

Ethics Statement

This work, which involved retrospectively reviewing medical records, was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University, and the Ninth Hospital of Nanchang in Jiangxi province, China (License Number: 202004, Date: 13 March 2020). This was a retrospective study without intervention. However, when additional clinical specimens were needed for analyses, informed consent was obtained from all involved subjects.

Data Collection

From the electronic medical record for each eligible patient, we extracted information on demographic characteristics (age, sex, and others), physical examination (highest temperature), epidemiology (Wuhan exposure history, history of contact with confirmed cases, and family and social gathering), and underlying disease conditions (high blood pressure, diabetes mellitus, chronic liver disease, and others). Clinical symptoms such as fever, cough, dyspnea, headache, fatigue, muscle ache, diarrhea, and others were obtained. Laboratory analytical results included white blood cell count (WBC), Lymphocyte percentage (Lym%), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), procalcitonin (PCT), heart function, liver and kidney function, coagulation test, cytokines test, immunoglobulin, cycle threshold (Ct) value in RT-qPCR, etc. Information on chest computerized tomography (CT), drug treatment, and prognosis of disease progression were also retrieved and evaluated. The virus clearance criteria were defined as two continuous negatives of nucleic acid tests according the guidelines for diagnosis and treatment plan of COVID-19 issued by the National Health Commission (trial version 7) (13). All of the patients' laboratory and radiological information were performed at admission, thus, some patients may have missing data.

Case Classifications

All the retrieved cases for patients infected with the COVID-19 virus were classified into the following clinical categories: (i) Asymptomatic: no apparent symptom; (ii) Mild: mild fever without pneumonia manifestation through image results; (iii) Moderate: fever and other respiratory symptoms with pneumonia manifestation through image results; (iv) Severe: fever, respiratory distress with pneumonia manifestation through image results, hypoxia or abnormal results of blood gas analysis; (v) Critical: respiratory failure requiring mechanical ventilation, shock, or other organ failure requiring intensive care unit monitoring and treatment; and (vi) death.

Statistical Analysis

For the statistical analysis, the associations between qualitative/categorical variables were analyzed using the Chi-square test or Fisher's exact test. Continuous variables were expressed as mean ± SD or median (interquartile range; IQR) and analyzed using Student's *t*-test, Mann–Whitney *U*-test or the Kruskal-Wallis *H*-test as appropriate. All tests were two-tailed, with $P < 0.05$ considered as statistically significant. All analyses were performed using SPSS version 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Demographic Characteristics

Between February 4, 2020 and March 2, 2020, a total of 41 hospitalized patients were identified as infected by the COVID-19 virus at the Ninth Hospital of Nanchang. Of the 41 patients, 15 (36.6%), 7 (17.1%), and 19 (46.3%) were categorized into asymptomatic, mild, and moderate groups, respectively (**Table 1**). There was no severe or critical COVID-19 case in this hospital. All 41 individuals were identified as infected by the COVID-19 virus during their medical or home isolation. The median age of all patients was 39.9 years (range 15–83 years), with a female/male ratio of 1.56. Among the 41 patients, 5 (12.2%) were younger than 20 years old; 12 (29.3%) were between the ages of 21 and 40 years old; 19 (46.3%) were in patients aged from 41 to 60 years old; and 5 (12.2%) were aged 61 years and older (**Table 1**).

TABLE 1 | Demographics, underlying diseases, and signs and symptoms on admission of 41 patients with COVID-19: 15 asymptomatic, 7 mild, and 19 moderate cases.

Characteristics demographics	All cases ($n = 41$)	Asymptomatic cases ($n = 15$)	Mild cases ($n = 7$)	Moderate cases ($n = 19$)
Sex ratio (F/M)	1.56	1.5	0.4	2.8
Age in years, mean (range)	39.9 (15–83)	42.7 (18–83)	30.6 (15–48)	41.2 (20–66)
Age groups (years)				
≤20	5 (12.2%)	2 (13.3%)	2 (28.6%)	1 (5.3%)
21–40	12 (29.3%)	4 (26.7%)	3 (42.8%)	5 (26.3%)
41–60	19 (46.3%)	6 (40.0%)	2 (28.6%)	11 (57.9%)
>60	5 (12.2%)	3 (20.0%)	0	2 (10.5%)
Underlying diseases	13 (31.7%)			
Hypertension	6 (14.6%)	2 (13.3%)	1 (14.3%)	3 (15.8%)
Diabetes mellitus	2 (4.9%)	1 (6.7%)	0	1 (5.3%)
Chronic liver disease	5 (12.2%)	2 (13.3%)	0	3 (15.8%)
Signs and symptoms on admission				
Fever	69.2% (18/26)	0	5 (71.4%)	13 (68.4%)
Highest temperature, °C				
37.3–38.0	44.4% (8/18)	0	80.0% (4/5)	30.8% (4/13)
38.1–39.0	44.4% (8/18)	0	20.0% (1/5)	53.8% (7/13)
>39.0	11.1% (2/18)	0	0	15.4% (2/13)
Cough	65.4% (17/26)	0	4 (57.1%)	13 (68.4%)
Fatigue	19.2% (5/26)	0	1 (14.3%)	4 (21.1%)
Dyspnea	7.7% (2/26)	0	0	2 (10.5%)
Muscle ache	7.7% (2/26)	0	0	2 (10.5%)
Headache	3.8% (1/26)	0	0	1 (5.3%)
Diarrhea	3.8% (1/26)	0	0	1 (5.3%)

The most common patient group was middle-aged women, with hypertension (14.6%) and chronic liver disease (12.2%) as the predominant underlying condition. Fever (69.2%), cough (65.4%), and fatigue (19.2%) are the common symptoms.
Data are presented as the median (range), or proportion (%), as appropriate.

TABLE 2 | Epidemiological history, chest CTs, and outcomes of 41 patients with COVID-19.

Characteristics demographics	All cases ($n = 41$)	Asymptomatic cases ($n = 15$)	Mild cases ($n = 7$)	Moderate cases ($n = 19$)
Epidemiological history				
Wuhan exposure history	9 (22.0%)	2 (13.3%)	2 (28.6%)	5 (26.3%)
Contact with confirmed cases history	29 (70.7%)	13 (86.7%)	3 (42.8%)	13 (68.4%)
No clear epidemiological history	3 (7.3%)	0	2 (28.6%)	1 (5.3%)
Family/social gathering	16 (39.0%)	6 (40.0%)	2 (28.6%)	8 (42.1%)
Duration				
Incubation period	9.1 ± 5.1	-	8.4 ± 2.7	8.7 ± 5.6
Duration from onset of symptoms to admission	4.2 ± 3.0	0	5.9 ± 4.3	3.6 ± 2.3
Duration of viral nucleic acid clearance	10.6 ± 4.2	9.7 ± 3.5	10.7 ± 4.6	11.2 ± 4.7
Duration of lesion absorption evidenced on CT images	13.7 ± 6.1	9.3 ± 4.9	-	16.4 ± 5.1
Chest CT scan				
Ground-glass opacity	22 (53.7%)	8 (53.3%)	0	14 (73.7%)
Focal lesion	5 (12.2%)	2 (13.3%)	0	3 (15.8%)
Bilateral lung patch shadow	3 (7.3%)	1 (6.7%)	0	2 (10.5%)
No abnormal lesion	11 (26.8%)	4 (26.7%)	7 (100.0%)	0
Outcomes after treatment				
Discharge	41 (100.0%)	15 (100.0%)	7 (100.0%)	19 (100.0%)
Secondary bacterial pneumonia	2 (4.9%)	0	1 (14.3%)	1 (5.3%)
Abnormal liver function	3 (7.3%)	1 (6.7%)	0	2 (10.5%)

Most patients (70.7%) had a history of close contact exposure with patients with confirmed COVID-19, and 16 patients (39.0%) were related to family/social clustering. Chest CT scans presented ground-glass opacity (53.7%), focal lesion (12.2%), bilateral lung patch shadow (7.3%), and normal CT image (26.8%). The mean time of viral nucleic acid clearance was 10.6 days.

Data are presented as the percentage (%), or mean ± SD, as appropriate.

Among the 41 patients infected with the COVID-19 virus, 9 (22.0%) had a history of recent travel to or living in Wuhan, and 29 patients (70.7%) had close contact with individuals with confirmed COVID-19 viral infection. Known family/social gathering accounted for 39.0% (16/41) of all cases. Of these 16 family/social clustering cases, 7 were due to family members returning from Wuhan, 6 had dinners together with people returning from Wuhan, and 3 unknowingly socialized with others who were later identified as having COVID-19. However, 3 of the 41 cases (7.3%) had no clear epidemiological history (**Table 2**). At least 13 of the 41 cases (31.7%) had underlying disease conditions, including 6 cases of hypertension, 2 cases of diabetes mellitus, and 5 cases of chronic liver disease. The other 28 (68.3%) patients had no known underlying condition (**Table 1**).

Clinical Characteristics

On admission to the Ninth Hospital of Nanchang, the most frequent symptom was fever (18/26, 69.2%) in the mild and moderate disease categories. For patients with fever, an equal proportion (at 44.4% each) suffered a low fever (37.3–38.0°C) and moderate fever (38.1 to 39.0°C). Two patients had high fever (>39°C). Cough (65.4%) and fatigue (19.2%) were also common symptoms in these patients. However, headache, dyspnea, muscle soreness, and diarrhea were relatively rare (**Table 1**). Seven patients were asymptomatic from exposure to admission, however, during the course of hospitalization, 2 asymptomatic ones developed mild fever and cough. Chest CT examination served as an important basis for the diagnosis of COVID-19 (**Figure 1**). Among the 41 patients, 22 showed ground-glass opacities, 5 cases showed focal lesions (patch or strip-shaped shadow), 3 cases showed bilateral lung patch shadow, and the remaining 11 showed no CT scan image abnormality. Interestingly, all seven mild cases showed normal CT scan images while 11 of the 15 asymptomatic cases showed abnormal CT scan images (**Table 2**).

Treatment and Clinical Outcome

Among the 41 cases, the average incubation period was 9.1 days, with the longest being 20 days. The mean time from onset of symptoms to hospital admission was 4.2 days (**Table 2**). Antiviral therapy was given to 33 cases (80.5%) as the initial therapy which included lopinavir/ritonavir with ribavirin or arbidol. All 33 patients also took the traditional Chinese medicine Lotus Qingwen capsules as supplement to the antiviral therapy. The initial therapy followed the guidelines for diagnosis and treatment plan of COVID-19 issued by the National Health Commission (trial version 7) (13). In some cases, the patients also received antibiotic therapy, immunoglobin therapy, and/or bronchial nebulization of interferon-α2β. All 41 cases recovered and were discharged after two consecutive negative nucleic acid tests. They were then isolated at home for 14 additional days. None of the cases developed severe pneumonia, requiring systemic corticosteroids treatment, mechanical ventilation, or

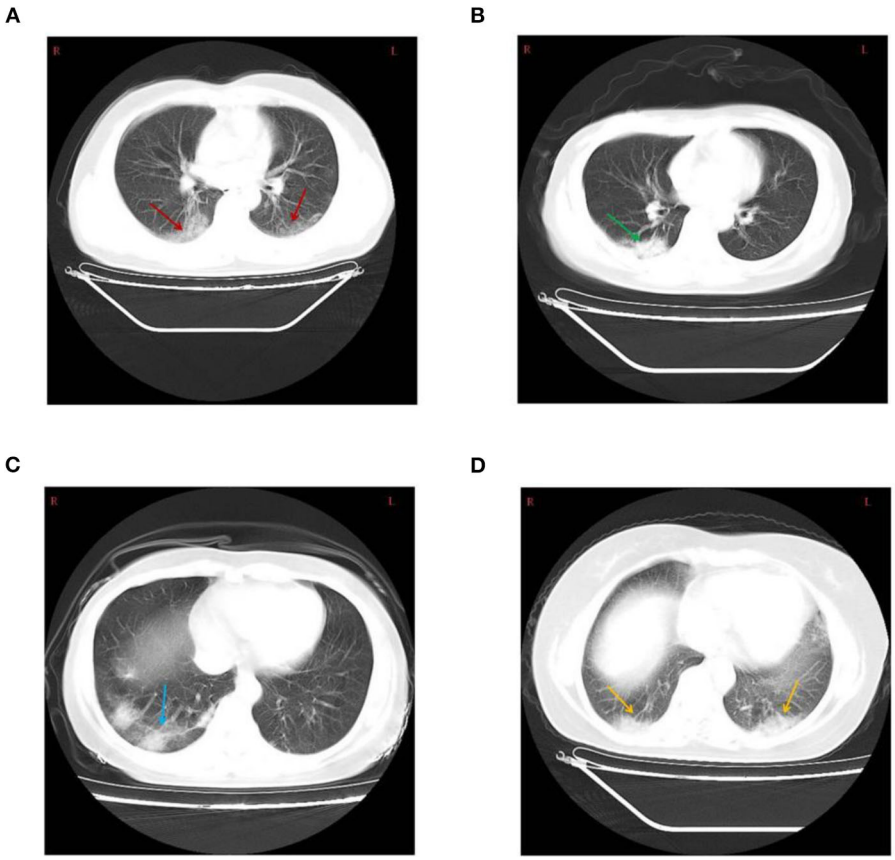

FIGURE 1 | (A) Chest computed tomography showing bilateral diffuse ground-glass opacity (red arrow) in a 43-year-old male patient. **(B)** Patchy shadow of the right lung (green arrow) in a 44-year-old female patient. **(C)** Consolidation and Strip-shaped shadows of the right lung (blue arrow) in a 43-year-old female patient. **(D)** Bilateral lung patchy shadow (yellow arrow) in a 42-year-old female patient.

admission to ICU. In addition, there was no recurrence of clinical manifestations or positive nucleic acid tests in their follow-up visits at 2nd and 4th week after discharge. The duration of viral nucleic acid clearance, defined as the interval from the 1st day of positive nucleic acid tests to the two continuous negative tests, ranged from 4 to 20 days (mean: 10.6 days). The mean duration of lesion absorption as evidenced on CT scan was 13.7 ± 6.1 days. Secondary bacterial pneumonia was empirically diagnosed in two patients, one had a mild COVID-19 symptom and the other had a moderate symptom. However, neither patient had persistent pathology from bacterial infection and both patients recovered from the bacterial pneumonia after antibiotic treatment. Among the 41 cases, three (1 asymptomatic case and 2 moderate cases) had secondary abnormal liver function after antiviral therapy (**Table 2**).

Laboratory Test Results

The laboratory results of the three groups of patients (asymptomatic, mild, and moderate cases) were summarized in **Table 3**, **Supplementary Tables 1, 2**. The results for blood routine markers, conventional inflammatory markers, cytokines, heart function, liver and kidney functions, immunity, and coagulation indicators are all presented in these tables as the median (IQR) or mean ± SD, as appropriate. The averages of the laboratory test results of 41 patients that showed above normal ranges included CRP, ESR, interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and immunoglobulin G (IgG) (**Supplementary Table 1**). Statistical comparisons between asymptomatic cases and mild/moderate cases revealed that patients with relatively severe condition showed significantly higher levels of ESR, IL- 6, TNF-α, and fibrinogen (Fib) ($p < 0.05$) than the asymptomatic cases. However, no statistically significant difference was observed between the two groups of patients in their values of Lym%, CRP, IL-4, IL-10, IgG, or Ct-reading (**Table 3**).

On admission, the majority of patients (37 of 41, 90.2%) had normal values of WBC counts. More than 50% of patients had lymphocyte counts ($<0.8 \times 10^9$ cells/L) and percentages (<20%) below the normal range in all three groups of patients (**Supplementary Table 2**). Between 60 and 90% of the patients had abnormally high cytokine levels in IL-4, IL-6, IL-10, and TNF-α (**Supplementary Table 2**). There were 21 (21 of 41, 51.2%) and 19 (19 of 41, 46.3%) patients with CRP and ESR above the normal range, respectively.

TABLE 3 | Statistical analyses of abnormal biochemical indicators in asymptomatic cases and mild/ moderate cases.

Laboratory test results	Asymptomatic cases ($n = 15$)	Mild/moderate cases ($n = 26$)	*P*-value
Blood routine test			
Lym%, (20.0–40.0)%	20.6 ± 9.8	22.5 ± 9.6	0.59
<20%	6 (40.0%)	15 (57.7%)	0.34
Conventional inflammatory markers			
CRP, (0.0–10.0) mg/L	8.9 (3.9–13.8)	11.9 (5.0–15.9)	0.50
>10 mg/L	6 (40.0%)	15 (57.7%)	0.34
ESR, (0.0–20.0) mm/H	18.0 (13.5–23.5)	26.5 (20.0–56.2)	0.03
>20 mm/H	4 (26.7%)	15 (57.7%)	0.10
Cytokines			
IL-4, (0.0–2.80) pg/mL	5.9 ± 3.4	8.3 ± 4.5	0.09
>2.80 pg/mL	11 (73.3%)	24 (92.3%)	0.17
IL-6, (0.0–5.30) pg/mL	8.6 ± 7.3	18.6 ± 18.1	0.04
>5.30 pg/mL	9 (60.0%)	22 (84.6%)	0.13
IL-10, (0.0–4.91) pg/mL	8.9 ± 2.4	9.8 ± 7.5	0.07
>4.91 pg/mL	6 (40.0%)	19 (73.1%)	0.05
TNF-α, (0.0–2.31) pg/mL	3.2 ± 3.9	6.8 ± 6.5	0.02
>2.31 pg/mL	6 (40.0%)	19 (73.1%)	0.05
Immune protein and complement			
IgG, (7.0–16.0) g/L	15.5 ± 8.1	17.0 ± 10.1	0.36
>16.0 g/L	3 (20.0%)	8 (30.8%)	0.72
Coagulation test			
Fib, (2.0–4.0) g/L	2.7 ± 0.6	3.7 ± 1.1	0.03
>4.0 g/L	1 (6.7%)	11 (42.3%)	0.03
RT-PCR			
Ct-values	28.6 ± 5.1	28.5 ± 4.4	0.98

The patients with relatively severe condition showed significantly high levels of ESR, IL-6, TNF-α, and (Fib) ($p < 0.05$).

Data are presented as the percentages (%), or mean ± SD, as appropriate. Lym%, Lymphocyte percentage; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IL-4, interleukin-4; IL-6, interleukin-6; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α; IgG, immunoglobulin G; Fib, fibrinogen; Ct-values, cycle threshold values.

25–30% of the patients had abnormally high IgG (>16.0 g/L) and Fib (>4.0 g/L) levels (**Supplementary Table 2**). However, abnormal levels of PCT, lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase-MB (CKMB), myohemoglobin (MYO), cardiac troponin T (cTnT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein (TP), total bilirubin (TBIL), blood urea nitrogen (BUN), serum creatinine (Cr), immunoglobulin A (IgA), immunoglobulin M (IgM), complement 3 (C3), complement 4 (C4), prothrombin time (PT), Activation of partial prothrombin time (APTT), thrombin time (TT), and D-dimer (DD) were uncommon among the 41 patients (**Supplementary Tables 1, 2**).

DISCUSSION

In this study, we analyzed the demographic characteristics, physical examination results, underlying disease conditions, laboratory test results, and clinical characteristics of 41 patients infected with the COVID-19 virus who were hospitalized at the Ninth Hospital of Nanchang in Jiangxi Province, China. The 41 patients belonged to asymptomatic to mild and moderate cases, with no severe or critically ill cases. All 41 patients completely recovered. Our analyses identified that most patients had a history of close contact exposure with patients with confirmed COVID-19, including a high degree of family/social clustering. The most common patient group was middle-aged women, with hypertension and chronic liver disease as the predominant underlying disease conditions. The most common symptoms were fever and cough. Chest CT scans showed ground-glass opacities as the main type, followed by normal images, focal lesion and bilateral lung patch shadow. The laboratory results showed that in most COVID-19 cases, lymphocyte counts, lymphocyte percentages, ESR, CRP, IgG, Fib, and cytokines all deviated from the normal ranges. Below, we discuss the clinical significance of our results and their potential implications for the management of COVID-19.

In our study, 9 patients had a history of toured or lived in Wuhan in the early stage of the pandemic. These 9 patients were the primary cases in Nanchang, and most of these patients had mild to moderate symptoms. Twenty-nine of the 41 patients had close contact (e.g., living, dining, or socializing together) with confirmed primary COVID-19 patients in Nanchang. These 29 patients were the secondary cases and the inferred basic reproductive number (Ro) based on our observed cases is 3.22, consistent with published reports on COVID-19 (1, 9). Interestingly, the clinical manifestations of the secondary patients were mostly asymptomatic to mild, consistent with results from earlier studies (15, 16), which might have been due to their low-dose exposure to the virus and/or reduced virulence of the virus. For example, Chen et al. reported that the local cases in Chongqing were generally asymptomatic (16). Among our 41 cases, we were unable to identify the epidemiological history for 3 cases. The subsequent no new case in Jiangxi (including Nanchang) since February 27th (except the two imported cases) indicated that limiting the flow of people, prohibiting large gatherings, as well as encouraging people to stay at home and self-isolation were effective public health policies to reduce the spread of COVID-19 in Nanchang (17).

Our investigation identified more women (18) than men (16) infected with the COVID-19 virus in this hospital. This is different from several previous studies that showed more males than females with COVID-19 (1, 19, 20). The dominant patient group in our sample was in middle-aged people (30–50 years old), similar to the cohorts reported in other studies (16, 21–23). Interestingly, cases of the younger ages were more likely to be asymptomatic, or with mild symptoms, and to have a normal CT image. However, a few older individuals were also asymptomatic, including an 83-year-old in our patient sample. This result suggested that expanding the scope of nucleic acid testing to young people might be crucial to identify potential carriers to prevent the spread the COVID-19 and to reduce the risk of second wave of this pandemic (11, 16). Among the 41 patients, 13 (31.7%) had underlying disease conditions, including 6 with hypertension, 5 with chronic liver disease, and 2 with

diabetes mellitus. Compared with several published studies, the proportion of our cases with underlying diseases was slightly higher (15, 21). However, the relatively small sample size of our study could have contributed to the slight difference.

The most common symptoms of patients with COVID-19 were fever (69.2%), however, high fever was rare. Cough was also common (65.4%), with most of the cough being dry cough without sputum. The other symptoms included fatigue, dyspnea, muscle ache, headache, and diarrhea, each at <20%. Our results are consistent with those reported in previous studies (24, 25). The average incubation period was 9.1 days and the longest was 20 days, higher than the 3–7 days announced by the National Health Commission. The asymptomatic carriers typically had no visible clinical manifestations on admission, and they often did not receive as much attention as symptomatic ones (22). However, it took a similar length of time for the asymptomatic patient group to clear viral nucleic acid as it was for the mild and moderate symptomatic groups ($P = 0.61$). Similarly, no correlation was found between Ct-value and disease severity, patient age, etc. Consequently, within the community, the asymptomatic cases could be a significant source of infections for other people. In the case of governments rushing to re-open the economy, public health should pay special attention to this group of people through active contact tracing and comprehensive health monitoring (11).

The most common chest CT scan of COVID-19 patients were ground-glass opacity (53.7%) (**Figure 1**), which is also the most common image of viral pneumonia (18). Interestingly, normal CT images were found in 26.8% of patients, with no evidence of pneumonia. Furthermore, we found that focal lesions were more likely to occur alone in the right lung, and most cases had variable lesions (**Figure 1**). Previous reports have shown that these lesions may be related to the severity and stage of COVID-19 disease progression (26). Overall, our results showed that care should be taken to use a combined set of criteria to diagnose the severity of COVID-19, separating it from other viral infections.

The mean time from illness onset to visit hospital was 4.2 days, consistent with Tian et al.'s study (27). The mean times of viral nucleic acid clearance and lesion absorptions as evidenced based on CT scan images were 10.6 and 13.7 days respectively. While we found no correlation between the time to viral nucleic acid clearance and time to lesion absorption, previous studies have shown that the lengths of time for both were positively associated with the severity of COVID-19 disease (21). Patients carrying the COVID-19 virus longer and with longer lesion absorption time typically have more severe clinical symptoms. In our cases, while all patients improved and were discharged from the hospital, and no cases of reinfection were found during the 30-day follow-up, a small number of patients developed secondary bacterial pneumonia and abnormal liver function during hospitalization, which required extended hospital stays. Thus, care must be taken to look for co-infections or secondary infections in COVID-19 patients. Indeed, there has been a suggestion that antibiotics should be used in COVID-19 patients to prevent secondary infections (19).

An increasing number of biomarkers have been identified as related to the severity of COVID-19 disease. For example, one study indicated that CRP and LDH might be good predictors of COVID-19 severity (23). Several researchers have also proposed the use of neutrophil-to-lymphocyte ratio as an independent risk factor for predicting the severity of COVID-19 (28). In addition, a recent study showed that abnormal coagulation results, especially markedly elevated D-dimer and FDP were common in deaths with severe COVID-19 (29). Consequently, guidelines for managing COVID-19 patients are continuously updated. The most recent COVID-19 diagnosis and treatment program (7th edition) published by the National Health Commission of China suggested using indicators such as low lymphocyte counts, high levels of CRP, ESR, CK, AST, and LDH, and high levels of cytokines in severe patients (13). In our study, the mean of most laboratory examinations at the time of admission for most participants with no to moderate symptoms were normal. However, a large proportion of patients had lymphocyte counts, lymphocyte percentages, and ESR, CRP, IgG, and Fib levels beyond the normal range. Notably, the levels of several cytokines such as IL-6, IL-10, and TNF-α increased dramatically after COVID-19 viral infection, with the degree of increases positively correlated with the severity of the disease. However, with patients improving, the cytokine levels gradually returned to normal. According to Ricardo et al. (30), the COVID-19 cytokine storms were likely the results of the interplay between inflammation and coagulation. Guidelines for the diagnosis and treatment of SARS-CoV-2 infected pneumonia were first published on January 30th, 2020, and it recommended that cytokine monitoring be applied to improve the curative rate and reduce mortality (31). Indeed, several studies showed that dynamic monitoring of cytokine levels, and several inflammatory biochemical indicators were important to predict the changes of patients' conditions and to carry out effective interventions as early as possible (19).

This research presented the clinical characteristics of patients infected with the COVID-19 virus in Nanchang, Jiangxi Province. Though we identified several important features in our data, our study has certain limitations. Specifically, our patient population was only part of the infected population in Nanchang and we were only able to collect data from patients who were either directly admitted to the Ninth Hospital of Nanchang or transferred from other hospitals to the Ninth Hospital of Nanchang. In addition, even though we analyzed all the known COVID-19 patients visiting our hospital, our patient population might not be representative of the entire COVID-19 patient population in Nanchang where severe/critical cases may be present in other hospitals in Nanchang. In order to obtain more accurate results, it would be better to conduct large-scale multicenter studies to extend the collection of more patient data in the province. Furthermore, the study data were retrospectively analyzed and mainly collected from patients at admission, with about 2% data missing. Additional information such as antibody testing for all patients should provide valuable information about the potential susceptibility of people with prior exposure to the COVID-19 virus.

CONCLUSIONS

The COVID-19 pandemic is the biggest public health crises in the world. Various international and national public health organizations have been working hard to prevent and control

the spread of the virus and effectively manage patients with COVID-19 viral infections. Having epidemiological and clinical information from early cases in China could help us better prepare strategies in other parts of the world, including the potential second wave. In this study, we conducted a comprehensive epidemiological and clinical analysis of 41 patients infected the COVID-19 virus in a specialized hospital designated for treating COVID-19 in Nanchang, Jiangxi province in China. Our analyses revealed that all 41 cases were asymptomatic, mild or moderate, and all have recovered. Most patients had a history of close contact exposure with patients with confirmed COVID-19 and showed a high degree of family clustering. Among these 41 patients, the most common group was middle-aged women. The most common symptoms were fever and dry cough with the majority of patients showing ground-glass opacity in their chest CT scan images. Laboratory analyses showed that most patients had abnormal lymphocyte counts, lymphocyte percentages, and levels of ESR, CRP, IgG, and Fib. Furthermore, the levels of several cytokines were significantly higher in these COVID-19 patients than the normal ranges for healthy individuals, and with the degree of cytokine elevation positively correlated with the severity of the disease. Interestingly, we found little differences in most biochemical test results among the asymptomatic, mild, and moderate group of cases. Our results suggest the close monitoring of cytokine levels and other inflammatory and biochemical indicators should be conducted for all infected patients to help determine their medical needs. Further follow-up studies are needed to determine whether the infected patients are immune to future infections by the COVID-19 virus.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The study was approved by the ethics committee of the Second Affiliated Hospital of Nanchang University, the Ninth Hospital of Nanchang in Jiangxi province, China, and written informed consent was obtained from the patients or their next of kin (License Number: 202004, Date: 13 March 2020). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

J-MH and Y-HC participated in the study design and conceptualization. J-MH, L-CZ, H-BS, and Z-HH participated in the acquisition of data. L-HH, Q-SZ, Y-PH, and X-YF participated in analysis and interpretation of data. J-MH and L-HH participated in drafting of the manuscript and participated in the statistical analysis. J-MH, L-HH, Y-HC, and J-PX participated in critical revision of the manuscript for important intellectual content. Y-HC and J-PX participated in administrative, technical, material support, and participated in study supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.571069/full#supplementary-material

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Longitudinal Changes on Clinical Features in 28 Children With COVID-19 in Shenzhen, China

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Objective: To investigate the clinical characteristics of children with coronavirus disease 2019 (COVID-19) and identify the occurrence of viral shedding of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) during follow-up.

Methods: We retrospectively retrieved data from pediatric patients with COVID-19 from the Shenzhen Third People's Hospital in China. The dynamics of SARS-CoV-2 and antibodies against SARS-CoV-2 were analyzed during hospitalization and after discharge.

Results: From January 23 to March 15, 2020, a total of 28 pediatric patients were diagnosed with COVID-19 and were followed for at least 1 month. The median age was 7 years (IQR 3.5–10) and none of the children progressed to severe COVID-19 during hospitalization. Ten patients tested positive for SARS-CoV-2 1 month after discharge while four patients tested positive during the 2nd month after discharge. Only three of 12 children showed detectable immunoglobulin-M (IgM) on day 5, 18, and 21 after illness onset, respectively.

Conclusions: COVID-19 disease was relatively mild among children while a number did test positive after discharge from the hospital. Public health initiatives should thus adapt control measures targeted toward children.

Keywords: coronavirus disease 2019 (COVID-19), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), pediatric, antibody, clinical features

INTRODUCTION

Coronavirus disease 2019 (COVID-19), a newly emerged respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a global pandemic. According to the "Coronavirus Disease 2019 (COVID-19) Situation Report" from the World Health Organization (WHO), nearly 20 million confirmed cases were reported globally as of August 10, 2020 (1). The current evidence confirms that people of all ages are susceptible to COVID-19, with the latest available information indicating that from January 16, 2020, to February 8, 2020, ∼2, 135 pediatric infections occurred in China (2). With the growing COVID-19 pandemic, more children are at risk of infection and subsequent negative outcomes. Current studies however, have primarily focused on the epidemiological and clinical characteristics and outcomes of infected adults (3, 4). A general understanding of how COVID-19 presents in adolescents and the factors that may lead to more advanced disease in children needs to be understood.

In addition, the presence of antibodies in individuals with COVID-19 lacks conclusive evidence of a protective effect. A previous study determined that 93.1, 82.7, and 64.7% of adults infected with SARS-CoV-2 achieved sero-conversion of total antibody (Ab), immunoglobulin-G (IgG), immunoglobulin-A (IgA), and immunoglobulin-M (IgM) (5). Although the presence of these antibodies to SARS-CoV-2 as being protective or pathogenic has yet to be determined, the production of antibodies does provide support for the routine application of serological testing for the diagnosis and management of COVID-19 patients. To the best of our knowledge, first of all, no other investigations have thus far reported on the dynamic changes of antibodies among pediatric patients. Second, only one study from China found three pediatric cases that tested SARS-CoV-2 positive in the stool samples of patients within 10 days after discharge, even though these patients tested negative for nucleic acid when using throat swab specimens. This implies that children who have recovered might still be possible carriers for the virus (6). Finally, no clinical data have been reported on children who have been followed up for more than 2 months. Thus, it is necessary to investigate the occurrence of antibodies and reoccurrence of SARS-CoV-2 among children who have recovered from COVID-19. Further robust analysis of pediatric patients from multi-centers is still needed in order to better understand how the disease presents in children, what associated outcomes may be linked to COVID-19, and in general, to help support the development of guidelines for the successful treatment and care of children infected with COVID-19 through cooperative researches. Hence, we aimed to investigate the clinical characteristics of children with COVID-19 and identify the occurrence of viral shedding of SARS-CoV-2 during follow-up.

METHODS

Study Design and Participants

We conducted a retrospective review of medical records from 28 pediatric patients with confirmed COVID-19 pneumonia admitted to the Shenzhen Third People's Hospital in China from January 23, 2020 to March 15, 2020. All children were diagnosed according to the WHO interim guidance (7). Records were excluded if the patient was older than 15 years old or without baseline clinical characteristics. This study was reviewed and approved by the Medical Ethical Committee of the Shenzhen Third People's Hospital (approval number 2020-201). Written informed consent was obtained from the guardians of the children enrolled in this study.

Abbreviations: Ab, total antibody; Ab-ELISA, double-antigen sandwich enzyme-linked immunosorbent assay; COVID-19, coronavirus disease 2019; CMIA, Chemiluminescence Microparticle Immuno Assay; CT, computed tomography; CRP, Increasing serum c-reactive protein; IgM, sero-converted immunoglobulin-M; IgM-ELISA, IgM μ-chain capture method; IgG, immunoglobulin-G; IgA, immunoglobulin-A; IQR, interquartile range; IL-6, Increasing serum interleukin-6; RT-PCR, real-time reverse transcription polymerase chain reaction; RBD, receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WHO, World Health Organization.

Confirmation of COVID-19 Infection

The presence of SARS-CoV-2 was detected by real-time reverse transcription polymerase chain reaction (RT-PCR) (8). Two pairs of primers targeting the open reading frame 1ab (ORF1ab) and the nucleocapsid protein (N) were amplified and examined. Each sample was run in triplicate with positive and negative controls set as suggested. Samples identified as positive for SARS-CoV-2 by the local laboratory were confirmed by the key laboratory of the Shenzhen Centers for Disease Control (CDC). These diagnostic criteria were based on the recommendations by the Chinese Center for Disease Control and Prevention (China CDC). All patients were classified as mild, ordinary, or severe cases based on results from chest radiography, clinical examinations, and symptoms (9).

Data Collection and Follow-Up

Two physicians reviewed epidemiological, clinical, laboratory, and radiological characteristics and treatment outcomes using standard data collection forms from electronic medical records. Baseline was defined as the time of first hospital admission due to COVID-19 and all pediatric patients underwent routine SARS-CoV-2 nucleic acid testing every 3 days during hospitalization. Patients were discharged from the hospital after clearance of SARS-CoV-2. Viral clearance was defined by the presence of two consecutive negative results in qRT-PCR detection for SARS-CoV-2 RNA at an interval of 24 h, and the day of the first one of these two tests was considered as the clearance day. Within the 1st month after discharge from the hospital, all patients received at least four additional tests for the presence of SARS-Cov-2 RNA at day 3–7, 13–14, 20–22, and 28–30 after discharge from the hospital. After discharged from the hospital for patients who achieved clearance of SARS-CoV-2, they would be re-admitted to the hospital if they were tested positive for SARS-CoV-2 RNA during follow-up. Patients were categorized as the "early virus clearance group" if they had four consecutive negative results of qPCR detection for SARS-CoV-2 RNA within 1 month after they achieved two consecutive negative results of qPCR detection for SARS-CoV-2 RNA with an interval of 24 h. While patients who had any positive result of qPCR detection for SARS-CoV-2 RNA within the 1st month after two consecutive negative results of qPCR detection for SARS-CoV-2 RNA with an interval of 24 h were categorized as "delayed virus clearance group."

Antibody Testing

The SARS-CoV-2 specific Ab, IgG, IgA, and IgM in plasma was tested using a Chemiluminescence Microparticle Immuno Assay (CMIA). Briefly, antigens containing the receptor-binding domain (RBD) were used as the immobilized and horseradish peroxidase (HRP)-conjugated antigen to detect total antibodies by double-antigen sandwich enzyme-linked immunosorbent assay (Ab-ELISA). IgM was tested by the IgM μ-chain capture method (IgM-ELISA), using the same RBD antigen as the Ab-ELISA. IgA and IgG were tested by indirect ELISA using RBD antigen. The testing kits were provided by Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. Fluorescence intensity was used to measure antibody concentration. The relative fluorescence of sample to control (COI) was used to estimate the

result. When COI was more than one, the result was judged to be positive.

Statistical Analysis

Categorical variables were described as proportions and continuous variables were described using median and interquartile range (IQR) values. The Mann-Whitney test was used to compare the median for continuous variables. The Fisher exact test was used when the data were limited. All significance tests performed were two-sided. *P*-values <0.05 were deemed statistically significant. We also analyzed the longitudinal changes in clinical characteristics of 10 pediatric patients at admission to the hospital, at discharge from the hospital, and at re-admission to hospital due to reoccurrence of SARS-CoV-2. All analyses were carried out using SAS software version 9.4 (SAS Institute).

RESULTS

Demographic, Epidemiological, and Clinical Characteristics

From January 23 to March 15, 2020, a total of 28 hospitalized, pediatric patients were diagnosed with COVID-19 and were followed-up for at least 1 month. As seen in **Table 1**, the median age was 7 years (IQR 3.5–10), and 18 (64.3%) were female. Twenty-seven (96.4%) patients had a close contact with confirmed COVID-19 patients. The median interval between illness onset and admission to the hospital was 1 day (IQR 0–1). Of the participants, 18 (64.3%) presented symptoms at admission to the hospital. The most common symptoms at onset of illness were fever (39.3%), cough (35.7%), and nasal discharge (10.7%). Six (21.4%) patients remained asymptomatic until discharge, while four patients had pulmonary computed tomography (CT) abnormalities, mostly appearing as frosted glass and nodules. Twenty-five patients received interferon-alpha treatment, 19 received antiviral therapy with Lopina-velitonavir, and 14 received treatment with probiotics. None of the children progressed to severe COVID-19. Of the 28 patients with COVID-19, more were older than 7 years ($n = 10$, $p < 0.001$) and the majority had close contact with a confirmed COVID-19 patient ($n = 15$, $p = 0.035$).

SARS-CoV-2 Test Results Among 10 Pediatric Patients Whom Tested Positive During Follow-Up

A total of 10 patients tested SARS-CoV-2 positive at least one time, and 9 (90%) had more than two SARS-Cov-2 positive tests within 30 days after being discharged from the hospital, as seen in **Figure 1**. Eight patients completed 60 days of follow-up after being discharged from the hospital. During 31–60 days after discharge, four patients tested negative, while the other four patients tested positive on day 51, day 54, day 56, and day 76 after illness onset, respectively (**Figure 1**).

Clinical Characteristics of the 10 Patients That Tested Positive During Follow-Up

The most common symptoms at admission to the hospital were fever (40%), cough (30%), and nasal discharge (20%). None of patients had fever and nasal discharge at discharge from the hospital, while three patients had cough during the whole course of their diseases.

At the first time admission to hospital, various proportions (10–60%) of patients had abnormal laboratory tests (**Table 2**). Three patients maintained lymphocytosis throughout the course of their hospitalization, and three patients had increasing alkaline phosphatase at re-admission to hospital compared to the first time of admission to hospital. Beside lymphocyte and alkaline phosphatase, other laboratory testing remained normal.

At the time of admission, a total of eight cases had ground glass and nodular changes on their CT scans. At the time of discharge and at the re-admission to hospital, the lesions were not completely absorbed, with a few nodules and inflammatory lesions remaining.

All 10 children received interferon nebulization therapy during their hospitalization, and seven of them received antiviral therapy. However, antiviral therapy was not continued after discharge. After re-admission to the hospital, 60% of patients received traditional Chinese medicine and 70% of patients received probiotics.

Anti-SARS-CoV-2 IgG and IgM Antibodies in Pediatric Patients With COVID-19

Of 28 pediatric patients, 12 patients tested antibodies against SARS-CoV-2 with a total of 57 plasma samples collected throughout the course of their diseases. The cumulative sero-conversion rate for Ab, IgM, IgA, and IgG was 100.0% (12/12), 25.0% (3/12), 100.0% (12/12), and 100.0% (12/12) during the follow-up period. Within 7 days of illness onset, the sero-conversion rate for Ab, IgA, and IgG was 66.7% (4/6), 66.7% (4/6), and 66.7% (4/6), while all these corresponding rates increased to 100% (9/9) on day 8–14 after onset, and maintained positive until day 30. In addition, we found only three children sero-converted IgM at day 5, day 18, and day 21 after disease onset, respectively (**Figure 2**).

DISCUSSION

There has been a substantial gap in understanding the course and pathogenic features of SARS-CoV-2 infection, especially for pediatric patients. In this study, we analyzed the clinical characteristics and antibody responses to SARS-CoV-2 infection to understand its impact on pediatric patients. This study observed that COVID-19 was relatively mild in pediatric patients, mainly representing non-occurrence of severity of disease and transient abnormal laboratory testing. Moreover, this study revealed that the reoccurrence of SARS-CoV-2 was common in pediatric patients, even during the 2nd month of follow-up after being discharged from the hospital. In addition, this study found a high sero-conversion rate of antibodies against SARS-CoV-2.

TABLE 1 | Clinical features of 28 pediatric patients with COVID-19 at the Third People's Hospital of Shenzhen.

	Total (*n*,%)	<7 years (*n*,%)	≥7 years (*n*,%)	*P*-value
Total number of case	28	13	15	
Age, median (IQR), years	7 (3.5, 10)	2 (2, 5)	10 (7,12)	0.000
Sex				
Male	10 (35.7)	5 (38.5)	5 (33.3)	1.000*
Female	18 (64.3)	8 (61.5)	10 (66.7)	
Close contact with confirmed COVID-19 patients	27 (96.4)	12 (92.3)	15 (100.0)	0.206*
Number of Days between illness onset and admission to hospital	1 (0, 1)	1 (0, 1)	1 (0, 1)	0.486
Symptoms				
Fever	11 (39.3)	6 (46.2)	5 (33.3)	0.488
Cough	10 (35.7)	6 (46.2)	4 (26.7)	0.433*
Nasal discharge	3 (10.7)	2 (15.4)	1 (6.7)	0.583*
Severity of illness				
Ordinary	24 (85.7)	13 (100%)	11 (83.3)	0.102*
Mild	4 (14.3)	0 (0.0)	4 (26.7)	
Asymptomatic[#]	6 (21.4)	3 (23.1)	3 (20.0)	1.000*
Radiological features of CT in Asymptomatic patients				
Bilateral pneumonia	3 (50.0)	1 (33.3)	2 (66.7)	1.000*
Unilateral pneumonia	1 (16.7)	1 (33.3)	0 (0.0)	0.464*
Frosted glass	2 (33.3)	1 (33.3)	1 (33.3)	1.000*
Nodules	1 (16.7)	0 (0.0)	1 (33.3)	1.000*
Normal	2 (33.3)	1 (33.3)	1 (33.3)	1.000*
Treatment				
Lopina-velitonavir	19 (67.9)	8 (61.5)	11 (73.3)	1.000*
Interferon atmotherapy	25 (89.3)	11 (84.6)	14 (93.3)	1.000*
Probiotics	14 (50.0)	6 (46.2)	8 (53.3)	1.000*

**P-values were obtained by Fisher exact test. IQR, interquartile range; CT, computed tomography.*
[#]Asymptomatic was defined as no clinical discomfort in the course of the disease.

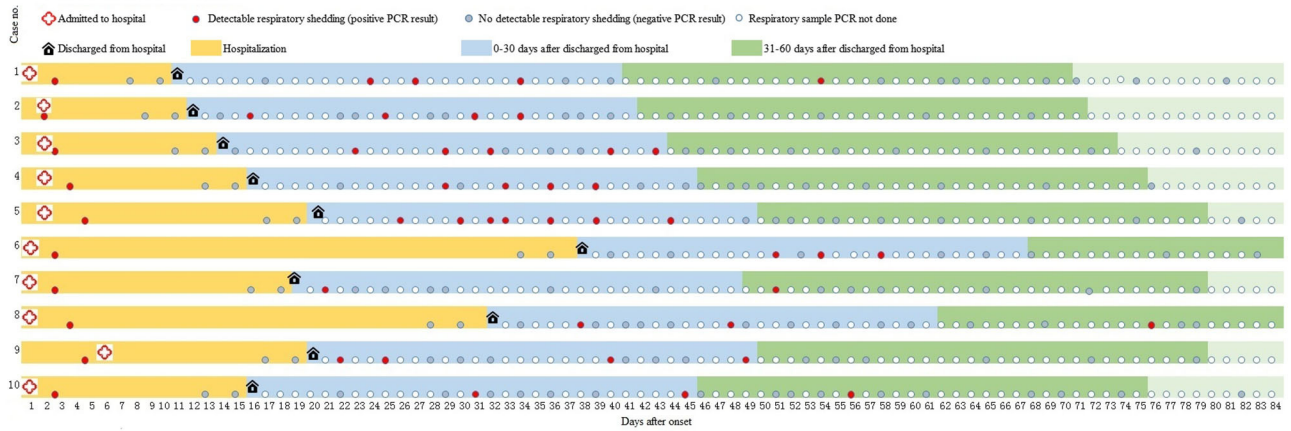

FIGURE 1 | A summary of illness onset, hospitalization, and test results of 10 participants who tested positive for SARS-CoV-2 during follow-up. PCR, polymerase chain reaction.

Investigation of SARS-CoV-2 shedding will be the key for determining the risk of transmission and formulating the criteria for being released from quarantine. In this study, the criteria for being discharged from the hospital were the presence of two consecutive negative results using qPCR detection for SARS-CoV-2 RNA within 24 h. However, 10 patients had reoccurrence of SARS-CoV-2 RNA during the 1st month of follow-up, and four patients tested positive for SARS-CoV-2 at the 2nd month

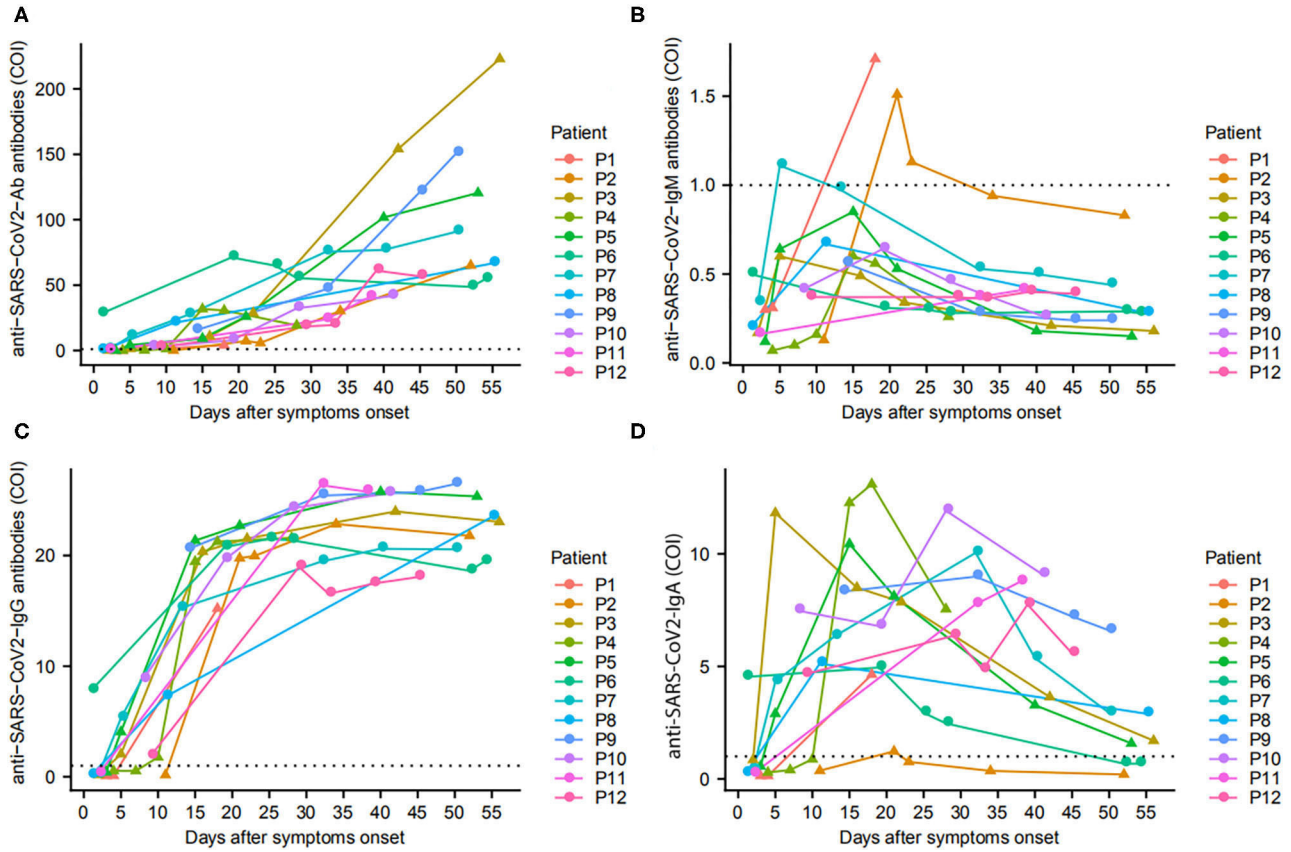

FIGURE 2 | Antibodies in pediatric COVID-19 patients since onset of disease. The changes of total Ab **(A)**, IgM **(B)**, IgG **(C)**, and IgA **(D)** in 12 patients are presented. ▲ represents patients with early virus clearance, while ● represents delayed virus clearance. …means cutoff values for antibody tests.

of after being discharged from the hospital. Various reoccurrence rates of SARS-CoV-2 have also been reported in pediatric and adult patients in other studies (6, 10, 11). However, the study showed that the positive rate of SARS-CoV-2 after discharge in adult group was significantly lower than that in children in this study (12). Although it has not been confirmed whether patients with reoccurrence of SARS-CoV-2 are contagious, the high frequency of reoccurrence of SARS-CoV-2 implies that the virus may be transmitting, even among patients being released from quarantine. However, based on the findings in this study which showed fewer individuals testing positive for the disease 2 months after discharge, viral clearance could be completed within longer period than the suggested quarantine time. Further studies are needed to explore the interaction of host and SARS-CoV-2.

We also found pediatric patients had relatively milder clinical symptoms and laboratory abnormalities at admission to the hospital, which was consistent with several other studies (13, 14). Particularly, none of pediatric patients progressed to severe COVID-19, which was much lower than that in adult patients (15). The large proportion of children with asymptomatic or mild symptoms may lead to larger public health uncertainty, as it could be difficult to identify pediatric patients, resulting in an increased risk of intra-family transmission and general on-going transmission in China. Thus, though severe disease manifestation is not as expected in this specific patient population, public health messaging should still be adamant about the proper prevention and management strategies in children. Age appropriate resources and support for parents should be considered when updating national care and treatment guidelines.

Compared to PCR, serological testing is advantageous with faster turn-around time, high-throughput, and less workload. Based on the accumulated understanding of host antibody responses during infection, the presence of serum IgM and IgG antibody against SARS-CoV-2 has been added as one confirmation criteria of SARS-CoV-2 infection (16). Long et al. (17) reported that within 19 days after symptom onset, all 285 patients who were tested were positive for IgG against SARS-CoV-2. This study found that at 8–14 days after illness onset, 100% of pediatric patients tested positive for Ab, IgA, and IgG, suggesting that pediatric patients may achieve sero-conversion earlier than adult patients. A previous study showed a strong positive correlation between clinical severity and antibody titer 2 weeks after illness onset (5), but did not assess this causal relation as all patients

TABLE 2 | Clinical characteristics of the 10 that tested positive for SARS-CoV-2 during follow up.

	Admission to the hospital	Discharge from the hospital	Re-admission to the hospital
Symptoms			
Fever	4 (40.0)	0 (0.0)	0 (0.0)
Cough	3 (30.0)	3 (30.0)	3 (30.0)
Nasal discharge	2 (20.0)	0(0.0)	0 (0.0)
Asymptomatic	2 (0.0)	7 (70)	7 (70.0)
Laboratory examination*			
Abnormal white blood cell count	2 (20.0)	0 (0.0)	0 (0.0)
Neutropenia	2 (20.0)	0 (0.0)	0 (0.0)
Lymphocytosis	3 (30.0)	3 (30.0)	3 (30.0)
Increasing aspartate aminotransferase	1 (10.0)	0 (0.0)	0 (0.0)
Increasing alkaline phosphatase	6 (60.0)	/	9 (90.0)
Increasing lactate dehydrogenase	1 (10.0)	0 (0.0)	0 (0.0)
Procalcitonin	3 (30.0)	0 (0.0)	0 (0.0)
Increasing serum interleukin-6 (IL-6) level	1 (10.0)	0 (0.0)	0 (0.0)
Increasing serum c-reactive protein (CRP) level	2 (20.0)	0 (0.0)	0 (0.0)
Chest radiographic imaging			
Normal	2 (20.0)	2 (20.0)	2 (20.0)
Bilateral pneumonia	4 (40.0)	4 (40.0)	4 (40.0)
Unilateral pneumonia	4 (40.0)	4 (40.0)	4 (40.0)
Frosted glass	2 (20.0)	3 (30.0)	0 (0.0)
Nodules	4 (40.0)	5 (50.0)	3 (30.0)
Treatment			
Antiviral therapy	7 (70.0)	0 (0.0)	0 (0.0)
Interferon atmotherapy	10 (100.0)	0 (0.0)	0 (0.0)
Traditional Chinese medicine	2 (20.0)	0 (0.0)	6 (60.0)
Probiotics	7 (70.0)	0 (0.0)	7 (70.0)

**Normal range of laboratory examinations: white blood cell count: 5–12 × 10^9 /L. neutrophil count is 1.8–6.3 × 10^9 /L. lymphocyte count: 1.1–3.2 × 10^9 /L. aspartate aminotransferase: 21–72 U/L. alkaline phosphatase: 38–126 U/L. lactate dehydrogenase: 313–618 U/L. Procalcitonin: <0.01 ng/ml. IL-6: <7 pg/ml. CRP: <8 mg/L.*

presented with mild disease. Whatsoever, this study provides important evidences that total antibody and IgG antibody could be used to understand the epidemiology of SARS-CoV-2 infection, to assist in diagnosing and managing COVID-19 pediatric patients.

In addition, we found that the positive rate of IgM antibody in children was only 25% during follow-up, which was significantly lower than the result of adults in our previous study (5). The reason of the disparity between children and adults remains unclear. The positive rate of IgM antibody might varies in different virus. The low positive rate of IgM antibody also exists in children infected with influenza A, but it can reach 41–51% after influenza B infection (18). Therefore, it is necessary to collect large samples and regular data with dense detection time-points to explore the positive results of IgM antibody.

There were some limitations in this study. First, the sample size was small and the study was a case series. Though these findings may be valuable early data and add to the existing literature, the results may not be generalizable to the entire infected population. Second, serum samples were collected from 12 individuals to assess the dynamics of antibodies against SARS-CoV-2. The results may be biased to sample selection. Third, the influence of reoccurring SARS-CoV-2 virus on disease progression and transmission has not yet been confirmed. Further longitudinal studies assessing these factors in pediatric patients could help improve diagnostic and treatment criteria in this unique population.

CONCLUSIONS

Among the 28 pediatric patients diagnosed with SARS-CoV-2 infection, the clinical symptoms of the disease were relatively mild, however the reoccurrence of SARS-CoV-2 was common even after two consecutive negative tests. While additional cohorts are needed to understand this phenomena, this study highlights the need to reconsider and improve existing prevention and control measures, particularly in children.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

ZZ and LL conceptualized and designed the study, and reviewed and revised the manuscript. XL and JL designed the data collection instruments and drafted the initial manuscript. ZH, MH, and QC collected data, carried out the initial analyses, and reviewed and revised the manuscript. TX, LW, and HW completed blood sample collection and serum antibody detection. QH coordinated and supervised data collection, and critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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Increasing Age, the Existence of Comorbidities, and Corticosteroid Treatment in Combination With Antiviral Therapy Prolongs the Recovery of SARS-COV-2-Infected Patients, Measured as the Conversion From Positive to Negative rtPCR: A 239 Patients' Retrospective Study

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Background: Severe acute respiratory syndrome (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-COV-2), has become a global pandemic in the past months. An overall defined treatment has not yet been established. Therefore, it is important to summarize and report treatment experiences and identify patient groups that have a significantly higher risk of an adverse clinical outcome.

Methods: Two hundred thirty-nine COVID-19 patients were recruited from January 25 to February 15, 2020. Demographic, clinical, laboratory, treatment management, and outcome data obtained from patients' medical records were evaluated.

Results: Patients who recovered from PCR positive to negative within 2 weeks had significantly lower erythrocyte sedimentation rate (ESR) and higher C-reactive protein (CRP) levels than those recovered post 2 weeks. During antiviral treatment, COVID-19 patients with older age, comorbidities, and corticosteroid treatment required a significantly longer time to turn from PCR positive to negative COVID-19 result.

Conclusion: PCR tests are of great importance to evaluate the recovery of COVID-19-positive patients, and ESR could be an indirect indicator to monitor SARS-COV-2 activity. Furthermore, our data suggest that older age, the existence of comorbidities, and corticosteroid treatment of COVID-19 patients during antiviral treatment could prolong the duration of conversion from SARS-COV-2 positive to negative.

Keywords: COVID19, SARS-CoV-2, RTPCR, age, comorbidity, corticosteroid

INTRODUCTION

In December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) was isolated, and the resultant disease was termed as a novel coronavirus severe acute respiratory syndrome (COVID-19) (1). In the meantime, COVID-19 has become a global pandemic, causing enormous damage to human health and the economy worldwide. One hundred eighty-eight countries/regions are affected, and more than 30 million people have been confirmed positive for COVID-19 (WHO COVID-19 Weekly Epidemiological Update, 5 Oct, 2020). Currently, many therapeutic agents for COVID-19 have not proven to be effective or are still in clinical trials as confirmed cases continue to increase worldwide (2). It is promising that China, severely affected by SARS-COV-2, has shown a static or declining trend since the pandemic began. By September 2020, a large number of COVID-19-positive patients had recovered in China and other countries. However, recent data indicated that a second COVID-19 wave is ongoing in Europe (3) and most likely in other parts of the world (4). It is therefore essential to summarize and report the treatment experiences for salvaged patients for each affected area and to carry out further studies.

Due to the similarity of the clinical features between SARS-CoV-2 infection and other viral diseases (5, 6), merely confirming COVID-19 patients based on clinical symptoms does not seem to be sufficient. It is believed that the majority of patients experiences asymptomatic or mild courses of the infection (7). Under these circumstances, real-time polymerase chain reaction (rtPCR) screening tests have become the gold standard for both confirming COVID-19-positive patients and evaluating the clinical outcome as well as discharging patients (8, 9). It is now generally accepted that SARS-CoV-2-positive patients can be discharged from the hospital if two consecutive rtPCR results are negative at intervals of 24 h (10). The time between positive and negative results of rtPCR determines the hospital stay and the associated costs for society. Given the considerable shortage of medical and financial resources worldwide (11), it will become increasingly important to identify patient groups that have a significant risk of extended hospital stays and to choose the right treatment strategy.

The Hunan province bordered directly on the Hubei province and was one of the first provinces in China where SARS-COV-2 infections occurred. To date, 1,014 out of 1,019 confirmed SARS-COV-2 patients have recovered in Hunan province (12). In this study, we report the clinical experience in epidemiology, clinical features, and treatment management of 239 recovered COVID-19 patients from Changsha First Hospital. One focus of this work reports on the clinical course between SARS-COV-2-positive and SARS-COV-2-negative rtPCR results.

METHODS

Patients

In this retrospective, single-center study, we recruited patients from January 25 to February 15, 2020, at The First Hospital of Changsha in Changsha, Hunan province, China, which is a designated hospital for COVID-19. Inclusion criteria of a nucleic acid PCR test for patients in this study were the following: (1) epidemiological conditions—(i) history of travel to or residence in Wuhan district or potential contact with people from Wuhan within 14 days, (ii) contact with confirmed Covid-19 patients within 14 days, (iii) contact with people from Wuhan district or pandemic area within 14 days, (iv) contact with people who had a fever or other respiratory symptoms within 14 days, and (v) clustered cases (two or more confirmed cases in small areas like families, schools, offices or workplace within 14 days); (2) clinical condition—(i) patients with fever or respiratory symptoms, (ii) radiological changes in the lung, and (iii) white blood cells decreased or stayed normal, while lymphocyte counts decreased. In addition, patients with at least one epidemiological condition and one clinical condition, as well as patients with no epidemiological conditions but with two clinical conditions, were tested by rtPCR. Inclusion criteria for patient's evaluation of the present study were as follows: (i) patients who were diagnosed with COVID-19 according to a positive rtPCR result at the time of admission; (ii) symptoms recovered after hospitalization; (iii) rtPCR was negative twice at intervals of 24 h before discharge; and (iv) received antiviral drugs treatments. The study was approved by The First Hospital of Changsha Hospital Ethics Committee, and written informed consent was obtained from all patients before enrolment.

Procedure

rtPCR was routinely done for patients on days 1, 3, 7, and 10 after admission and tested every 3 days if patients stayed more than 10 days after admission. The clinical classification of patients was based on the Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia in China (Trial Version 7) (13). Patients with mild symptoms and no sign of pneumonia on imaging were considered as mild cases, patients with fever and respiratory symptoms and radiological findings of pneumonia were considered as moderate cases, while patients meeting any of the following criteria were considered as severe cases: (1) respiratory distress ($\geqq$30 breaths/min); (2) oxygen saturation $\leq$93% at rest; (3) arterial partial pressure of oxygen (PaO2)/fraction of inspired oxygen (FiO2) $\leqq$300 mmHg (l mmHg = 0.133 kPa); and (4) patients with chest imaging that showed obvious lesion progression within 24–48 h (>50%). Demographic, clinical, laboratory parameters, treatment management, and outcome data were obtained from patients' medical records.

Treatment Management

All patients received supportive and antiviral treatments. Supportive treatments were staying in bed, protein–calorie supplementation, maintenance of hydroelectrolyte balance, and oxygen inhalation were routinely given to all COVID-19-positive patients. For severe patients, oxygen therapy, mechanical ventilation, rescue therapy, circulation support, replacement therapy, or immunotherapy was used. Antiviral drugs were applied according to the following schema: Lopinavir/Ritonavir (200/50 mg per pill, twice a day) and Arbidol (200 mg, three times a day) were used for no longer than 10 days; α-interferon (five million *U* or equivalent dose each time for adults, twice

a day) and Novaferon (20 μg, twice a day) were applied by aerosol inhalation; three or more drugs combination should be avoided (13). All patients received at least one antiviral drug during hospitalization, and the drug could be changed if rtPCR results were positive for more than 1–2 weeks. Patients with type 1 respiratory failure, increasing lung lesion, and severe symptoms (shock, organ dysfunction) received additionally methylprednisolon, 40 mg, twice a day.

Outcomes

The number of COVID-19 patients in our study was summarized by age, gender, and symptoms (**Table 1**). The time from the rtPCR positive to the negative result was defined as the time between the admission date with SARS-COV-2 positive and the first time a negative rtPCR result during hospitalization. The time of an rtPCR result from positive to negative was compared among groups divided by demographic data (age, gender), medical history (with or without chronic diseases), symptoms (with or without typical symptoms), blood inflammation markers [erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)], and treatment management (antiviral drugs and corticosteroids).

Statistical Analysis

Results are represented in the tables and figures (mean ± SEM). The Mann–Whitney *U* test was used to compare two single groups. The difference among multiple groups was compared by the Kruskal–Wallis H test, followed by Dunn's multiple comparison test. Statistical analysis was performed using the GraphPad Prism Software (Version 8, El Camino Real, CA, USA). $p < 0.05$ was considered as the minimum level of statistical significance.

TABLE 1 | Demographics of 239 patients admitted to The First Hospital (January 25–February 25) with COVID-19.

GENDER [NUMBER OF PATIENTS (PERCENTAGE)]	
Female	121 (50.6%)
Male	118 (49.4%)
Age [years (range)]	
Mean	44.15 (1–84)
Mean in female	46.94 (1–84)
Mean in male	44.08 (8–82)
Age [NUMBER OF PATIENTS (PERCENTAGE)]	
≤18	11 (4.6%)
18–29	28 (11.7%)
30–39	46 (19.2%)
40–49	54 (22.6%)
50–59	41 (17.2%)
60–69	38 (15.9%)
≥70	21 (8.8%)
CLASSIFICATION [NUMBER OF PATIENTS (PERCENTAGE)]	
Non-severe patients	201 (84.1%)
Severe patients	38 (15.9%)

RESULTS

Demographics

All patients (239 in total) with SARS-COV-2 infection confirmed by rtPCR were included in this study. The average age is 44.15, and middle-aged patients accounted for the main proportion. There were no gender differences in the selected study group (**Table 1**).

The Duration for rtPCR Result From Positive to Negative

All patients were tested by rtPCR and confirmed SARS-COV-2 positive at the time of hospital admission, and reviewed on days 1, 3, and 7 and every 3 days afterwards when necessary. Most patients (72.8%) in our study cohort recovered from a positive COVID-19 rtPCR result to a negative one within 1–4 weeks after hospital admission (**Table 2**).

Patients' Symptoms Did Not Affect the Duration of rtPCR Result From Positive to Negative

We summarized patients' distribution of each symptom at onset respectively, and the overview of multiple symptoms. Fever (67.3%), cough (58.2%), fatigue (33.9%), and shortness of breath (12.6%) were the most common symptoms, and most patients (60.3%) experienced more than one symptom at the onset. Rhinorrhea seems not related to COVID-19 (**Table 3**). We also compared the time for rtPCR result from positive to negative between patients with and without four of the most common symptoms (fever, cough, fatigue, and shortness of breath). As depicted in **Figure 1**, there was a significant difference in the clinical symptoms and the time of change for an rtPCR result from positive to negative (**Figure 1**).

Patients With Lung Lesion Progression Needed More Time to Recover From a Positive rtPCR Result to a Negative One During Hospitalization

Chest computed tomography (CT) scans were routinely performed on patients in the study to determine lung lesions at the time of hospital admission and reviewed every 3–5 days afterwards. At the time of hospital admission, 24 patients (10.0%) had no lung lesions, 96 patients (40.2%) had single lung lesion, and 119 patients (49.8%) had multiple lung lesions. No significant difference was found among lung lesions of patients at admission (**Figure 2A**). Although all patients' lung lesions

TABLE 2 | The duration from nucleic acid PCR positive to negative.

Mean time (days)	**16**
≤1 week	35 (14.6%)
1–2 weeks	97 (40.6%)
2–4 weeks	77 (32.2%)
>4 weeks	30 (12.6%)

improved significantly at discharge in our study, 98 patients (41.0%) experienced progression of their lung lesions during hospitalization. It is worth noting that patients who experienced lung lesion progression took significantly more time to convert rtPCR results (**Figure 2B**).

TABLE 3 | Clinical symptoms of patients at admission.

TYPICAL SYMPTOMS [NUMBER OF PATIENTS (PERCENTAGE)]	
Fever	161 (67.3%)
Cough	139 (58.2%)
Fatigue	81 (33.9%)
Shortness of breath	30 (12.6%)
Pharyngalgia	27 (11.3%)
Myalgia	23 (9.6%)
Diarrhea	20 (8.4%)
Headache	18 (7.5%)
Dizziness	10 (4.2%)
Nausea and vomiting	8 (3.3%)
Rhinorrhea	5 (2.1%)
Without symptoms	17 (7.1%)
With only 1 symptom	65 (17.2%)
With 2 symptoms	70 (23.9%)
With more than 2 symptoms	87 (36.4%)

Blood Inflammation Makers Could Be Useful Indicators for the Duration of Change for rtPCR From Positive to Negative

ESR and CRP are classical test parameters that indirectly measures the degree of inflammation in the blood (14). We compared ESR and CRP levels at admission of patients to turn a positive rtPCR result to negative within 2 weeks with patients that needed more than 2 weeks to turn a positive rtPCR result to negative. Patients were first divided into non-severe and severe cases. We found that severe patients had significantly higher levels of ESR and CRP than non-severe patients. Patients who recovered from a positive rtPCR result to negative within 2 weeks had lower ESR levels and higher CRP levels than those who recovered over 2 weeks (**Figures 3A,B**). Patients were then divided by the presence of comorbidities. We found that patients who recovered from a positive rtPCR result to negative within 2 weeks had significantly lower ESR levels than those who recovered over 2 weeks in both groups. Moreover, we found

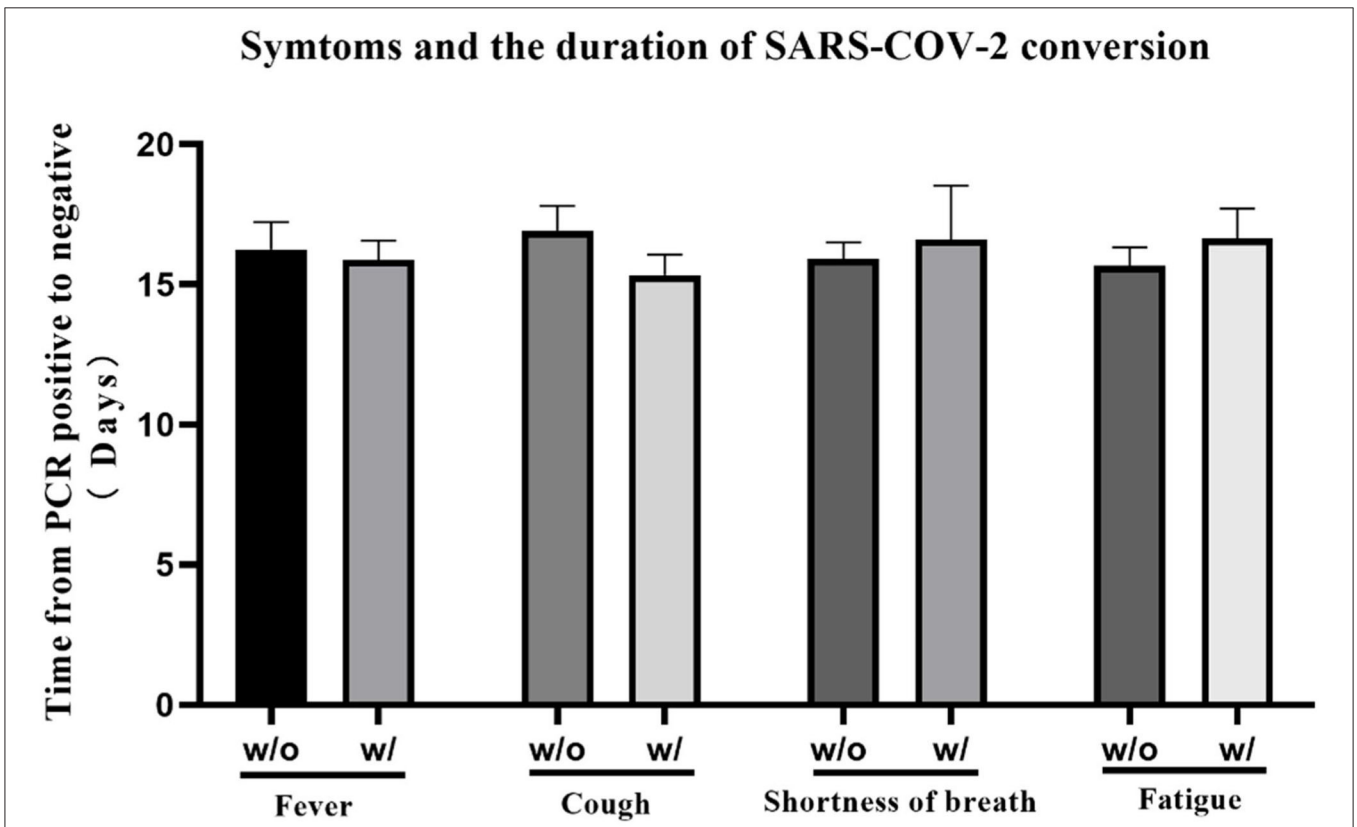

FIGURE 1 | The time for real-time PCR (rtPCR) result transition from positive to negative was compared between patients with or without specific symptom at the onset. The average time of patients with/without fever was 16.24 ± 0.985/15.88 ± 0.696, $p = 0.545$; the average time of patients with/without cough was 16.92 ± 0.882/15.33 ± 0.738, $p = 0.064$; the average time of patients with/without shortness of breath was 15.91 ± 0.589/16.60 ± 1.925, $p = 0.682$; the average time of patients with/without fatigue was 15.67 ± 0.659/16.63 ± 1.074, $p = 0.770$. No significant differences were observed among the different study groups.

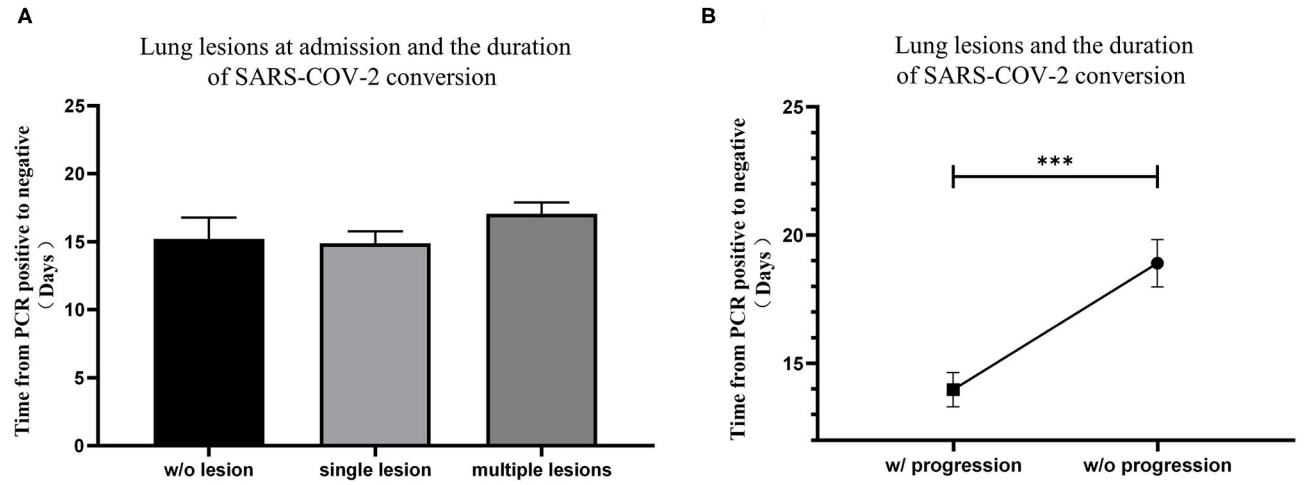

FIGURE 2 | The relationship between lung lesions measured by CT and the conversion time from real-time PCR (rtPCR) positive to negative. **(A)** The conversion time from rtPCR positive to negative of patients with no lung lesion, single lung lesion, and multiple lung lesions was compared. **(B)** The conversion time from rtPCR positive to negative of patients with and without progression of lung lesions was compared. Data are represented the mean ± SEM. The significance was determined as $^{***}p < 0.001$.

that patients with comorbidities had higher CRP levels than patients without comorbidities, and patients who recovered from a positive rtPCR result to negative within 2 weeks had slightly higher CPR levels than those who recovered over 2 weeks in both groups (**Figures 3C,D**).

COVID-19 Patients With Age Above 50, Comorbidities, and Corticosteroid Treatment Needed Longer Time to Recover From a Positive rtPCR Result to a Negative One Under Antiviral Drug Treatments

There is still an interesting debate as to which treatment strategy would be best for COVID-19 patients. It is now widely accepted that antiviral drugs with or without corticosteroids such as dexamethasone could improve clinical outcome of these patients. Four antiviral drugs (Lopinavir/Ritonavir, α-interferon, Arbidol, and Novaferon) were applied to patients in this study. Every study patient received at least one antiviral drug during hospitalization. Since there was no clear application strategy of antiviral drugs to COVID-19 patients at the beginning of this study, the clinical outcome (symptoms, drug side effects, and nucleic acid PCR result) of the initial given antiviral drug determined further drug administration. We summarized the antiviral drugs (used for patients at least once during hospitalization) applied to patients and their relationship to age, comorbidities, and corticosteroids use to see if that were relevant to the time from rtPCR positive to negative (**Table 4**). Ninety-four percent of fatalities are uniformly concentrated in the population over 60 years of age; thus, we speculate that age would determine the time of conversion from rtPCR positive to negative. We first analyzed the conversion time using the cutoff age of 30, 50, and 70 and found that patients over 50 years old needed a significantly longer time for SARS-COV-2 conversion (**Figure 4A**). Therefore, we then compared the duration of rtPRC conversion for patients under and over the age of 50 years among different antiviral strategies (**Figure 4B**). Comorbidities could be a risk factor for COVID-19 patients (15); therefore, we compared the duration of rtPCR conversion for patients with or without comorbidities (**Figure 5**). Hypertension and diabetes were the most common comorbidities for patients in our study, so further comparison for patients with hypertension, diabetes, multiple comorbidities, and no comorbidities was made (**Figure 6**). Moreover, the time of rtPCR conversion was compared for patients with or without corticosteroid used in combination with the antiviral drug (**Figure 7**). Our data showed that patients over the age of 50, with comorbidities and treated with corticosteroids, needed significantly longer conversion time from rtPCR positive to negative. It is worth mentioning that <10% of patients had diabetes but showed a crucial impact on the duration of rtPCR conversion in the Arbidol group.

DISCUSSION

SARS-COV-2 is a single-stranded RNA virus (16). Respiratory specimens (pharyngeal swabs, bronchial/alveolar lavage fluid) or blood specimens can be used for rtPCR nucleic acid testing (9). As an objective marker, PCR detection after SARS-COV-2 infection is of great significance for early diagnosis, treatment, and prevention from virus expansion (17). Moreover, the nucleic acid result is a vital reference for recovery if it converses from positive to negative. In the present study, all 239 COVID-19 patients have recovered after treatment, 55.2% of the patients converted from an rtPCR-positive to rtPCR-negative result within 2 weeks, while 44.8% of the patients needed more than 2 weeks. For a severe virus pandemic situation, medical resources (hospitals, supplies, physicians, nurses, and intensive care unit)

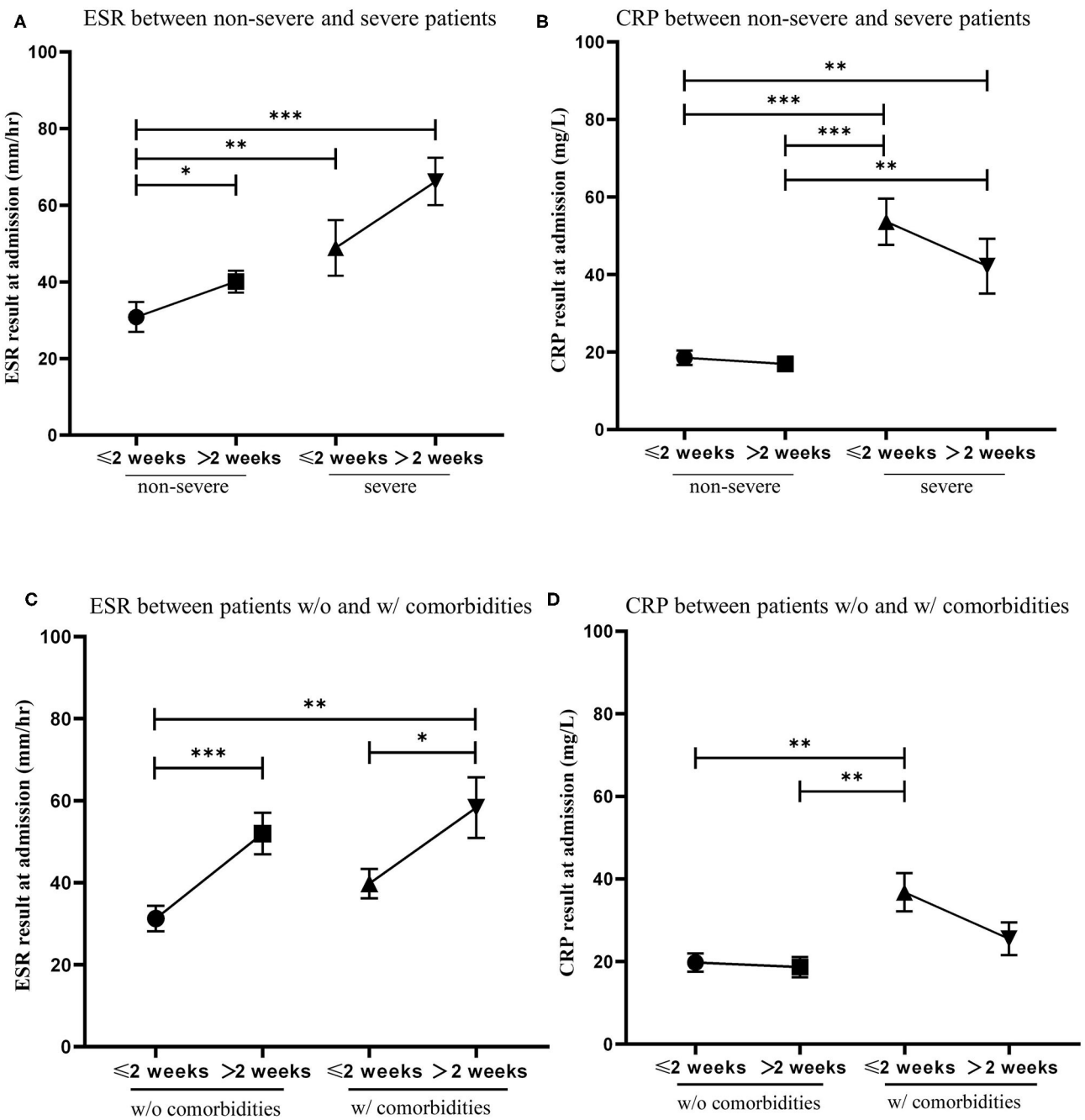

FIGURE 3 | The relationship of the level of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) at admission and the conversion time from real-time PCR (rtPCR) positive to negative. **(A)** Patients were divided into two groups by non-severe and severe cases. Blood test results of ESR was compared between patients recovered from rtPCR positive to negative within 2 weeks and those recovered over 2 weeks. **(B)** Patients were divided into two groups by non-severe and severe cases. Blood test results of CRP was compared between patients recovered from rtPCR positive to negative within 2 weeks and those recovered over 2 weeks. **(C)** Patients were divided into two groups by the presence of comorbidities. Blood test results of ESR was compared between patients recovered from rtPCR positive to negative within 2 weeks and those recovered over 2 weeks. **(D)** Patients were divided into two groups by the presence of comorbidities. Blood test results of CRP was compared between patients recovered from rtPCR positive to negative within 2 weeks and those recovered over 2 weeks. Data are represented the mean ± SEM. The significance was determined as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

are always in high demand. Shortening of the hospital stay could play a crucial role during clinical management of COVID-19; therefore, it is beneficial to determine factors that influence the time from rtPCR-positive to an rtPCR-negative result. As a clinical center for the treatment of COVID-19 in Germany, we have facilitated the cooperative study with a designated hospital

for COVID-19 in China in order to analyze treatments carried out at the beginning of the pandemic and learn from them. We believe that this study will provide valuable suggestions for current and future COVID-19 treatments in Europe, China, and the world. In the present study, age, symptoms at onset, inflammation makers, and various treatments of COVID-19 patients during hospitalization were investigated.

The most commonly reported symptoms of COVID-19 are fever, cough, myalgia, and fatigue, whereas less common reported symptoms include headache, diarrhea, hemoptysis, and runny nose (18). Most patients have more than one symptom at admission (10). Similar results were found in our study that fever, cough, and fatigue were the most common symptoms, and the majority of patients experienced at least two symptoms at admission. Moreover, no significant differences were found between the time from rtPCR conversion from positive to negative and the four main symptoms. CT examination is of great importance in the current diagnosis and treatment of COVID-19, which has become one of the most valuable methods for the evaluation of COVID-19 patients (19). Most previous studies demonstrated that COVID-19 patients have typical CT-detectable lung lesions (20, 21), which was also confirmed in our patient cohort and our results (40.2% had single lung lesion and 49.8% had multiple lung lesions). Although the condition of the lung lesions at admission did not affect the timing of rtPCR conversion, patients who progressed lung lesions during hospitalization experienced a significantly longer conversion time than patients without lung lesion progression. This correlation suggests that CT monitoring is important for COVID-19 patients and that lung lesion change is a potential indicator for assessing SARS-COV-2 evolution. Interestingly, two classical inflammation markers (ESR and CRP) in circulating blood were expressed oppositely in COVID-19 patients. Patients with high ESR levels at hospital admission needed more time (>2 weeks) to convert rtPCR result from positive to negative. In comparison, patients with low levels at hospital admission converted from a positive to a negative rtPCR result within 2 weeks. In contrast, CRP showed an opposite tendency, indicating that ESR might be a practical reference indicator for dynamic monitoring of the virus activity in COVID-19 patients. This could be explained by CRP levels falling more quickly than the ESR, normalizing 3–7 days after resolution of tissue injury, whereas ESR can take up weeks to normalize (22). CRP may respond rapidly during the incubation period of SARS-COV-2, while ESR keeps increasing along with the progression of the

TABLE 4 | The overview of used antiviral drugs during hospitalization.

Antiviral drugs	Lopinavir/ Ritonavir	Arbidol	α-Interferon	Novaferon
Patients (n)	176	130	117	134
Age (mean)	43.59	45.6	43.7	44.3
Comorbidity (%)	27.8%	26.9%	30.8%	27.6%
Hypertension (%)	13.1%	12.3%	14.5%	11.9%
Diabetes (%)	6.3%	5.4%	5.1%	9.0%
With multiple comorbidities	10.2%	8.4%	12.0%	11.9%
Combination with corticosteroid (%)	37%	35.4%	37.6%	26.1%

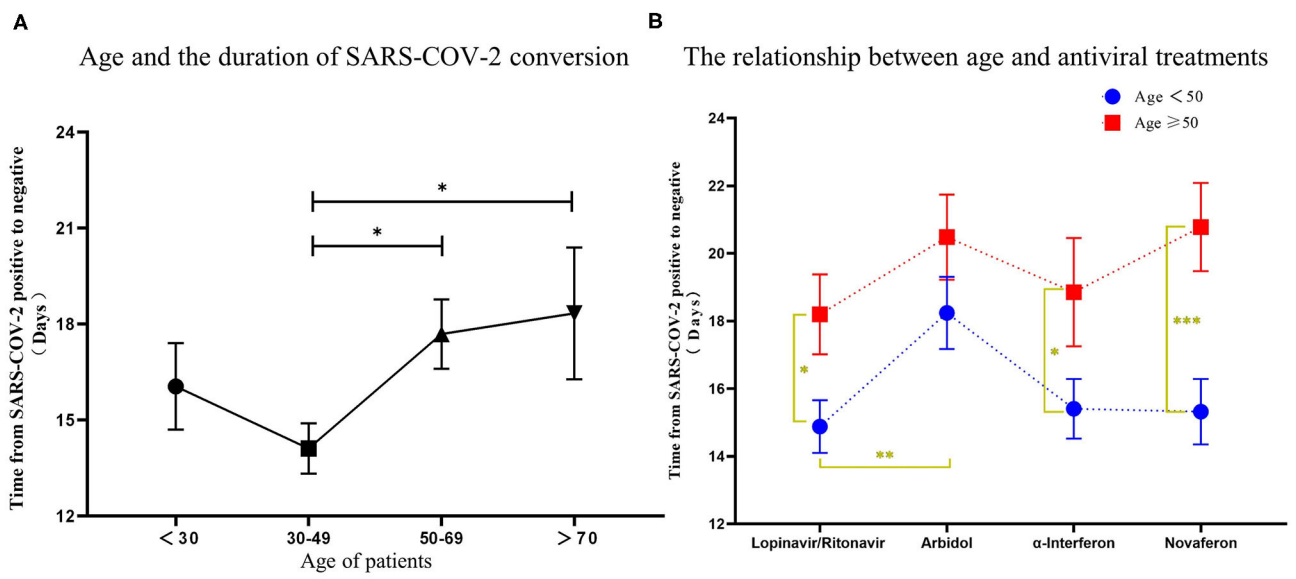

FIGURE 4 | The relationship between age and the conversion time from real-time PCR (rtPCR) positive to negative during antiviral treatment. **(A)** Patients were grouped by age <30 years, 30–50 years, 50–70 years, and over 70 years, and the conversion time from rtPCR positive to negative was compared. **(B)** Patients were divided by different antiviral drugs used, and the time was compared for rtPCR positive to negative results in patients under and over the age of 50. Data are represented the mean ± SEM. The significance was determined as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

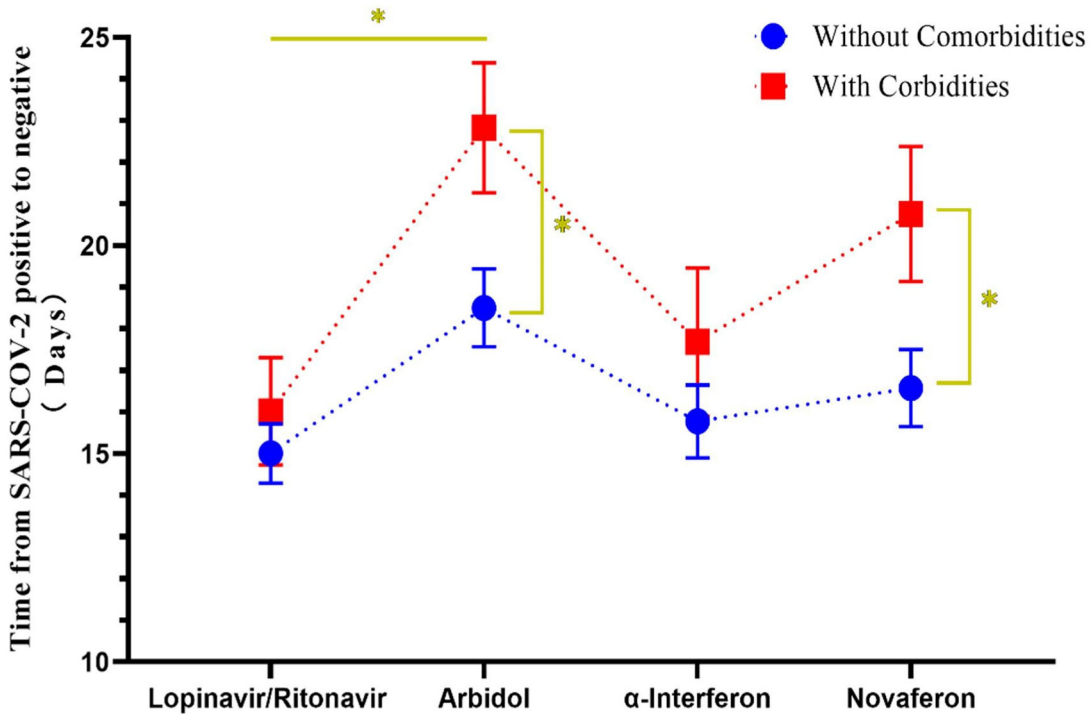

FIGURE 5 | The relationship between comorbidities and the conversion time from real-time PCR (rtPCR) positive to negative during antiviral treatment. Patients were divided by different used antiviral drugs, and the conversion time from rtPCR positive to negative of patients with or without comorbidities was compared. Patients with comorbidities had a longer time of rtPCR conversion than patients without comorbidities throughout antiviral drug treatment. Data are presented as mean ± SEM. The significance was determined as $^{*}p < 0.05$.

virus. Further studies are needed to confirm our results and explain the underlying mechanism.

SARS-COV-2 keeps spreading; no designated drug or vaccine has yet been approved to treat human coronaviruses. Lopinavir/Ritonavir, α-interferon, and Arbidol are commonly used to treat SARS-COV-2 infections (23, 24). Novaferon, a novel drug approved for the treatment of chronic hepatitis B in China, exhibits potent antiviral activities and promising antiviral effects on COVID-19 patients (25). A randomized, controlled, open-label trial of 199 hospitalized COVID-19 patients showed no benefit of Lopinavir–Ritonavir to clinical improvement beyond the standard of care, although Lopinavir–Ritonavir was found to have a benefit for some secondary endpoints, and the safety of the treatment was already confirmed (26). α-Interferon and Arbidol are recommended by different research groups to treat COVID-19 (27, 28). All study patients received antiviral drug treatment and converted from SARS-COV-2 positive to negative in our study, confirming the safety of the mentioned antiviral drugs. However, the exact efficacy, combination, and mechanisms of these drugs for SARS-COV-2 still need to be explored. Furthermore, it is noteworthy that patients responded to antiviral drugs differently. We found that patients above 50 years of age, with comorbidities, and the supplementation of corticosteroid needed a significantly longer time to convert from SARS-COV-2-positive to SARS-COV-2-negative result during antiviral treatments.

An analysis of 72,314 diagnosed patients in China showed that people of all ages are generally susceptible. However, the age group 30–79 years accounts for 87%, while children under the age of nine accounts for only 1% (29). Older adults and people with chronic diseases such as asthma, diabetes, and heart disease may be at increased risk of SARS-COV-2 infection (30, 31). Older age has been proven to be a potential risk factor to poor prognosis of COVID-19 patients (31). At the beginning of the epidemic, patients over 70 years with severe symptoms of COVID-19 were classified being at risk and isolated (32). After a few months, the most vulnerable to a COVID-19 infection age group was decreased to 60 years (33, 34). According to the results of our study, the vulnerable age for COVID-19 patients could be even further decreased to 50 years. We found that the rtPCR results in patients over the age of 50 years took longer to convert from positive to negative than in the patient group 50 years and younger. Thus, COVID-19 patients over the age of 50 should practice strict public health measures due to the increased risk of

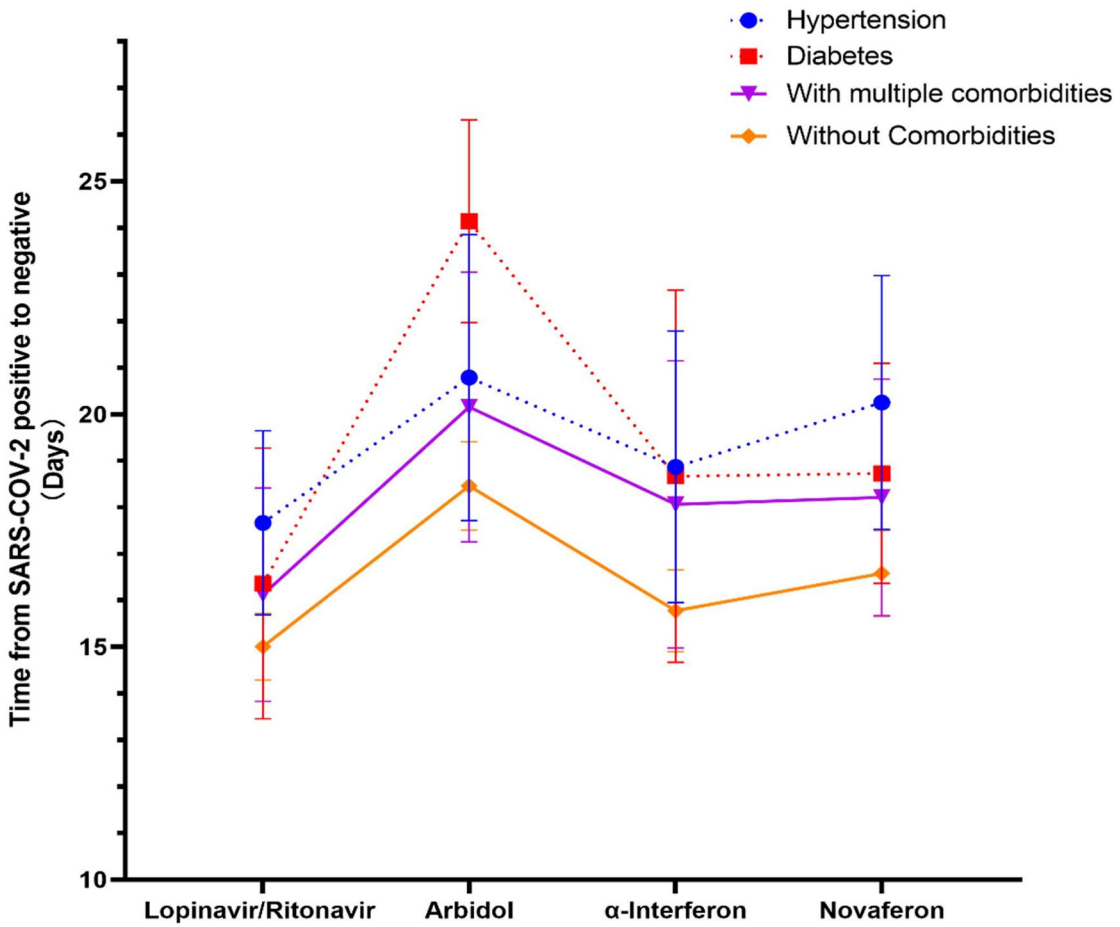

FIGURE 6 | The relationship between specific comorbidities and the conversion time from real-time PRC (rtPCR) positive to negative during antiviral treatment. The time to conversion from rtPCR positive to negative was compared for patients with hypertension, diabetes, multiple comorbidities, and no comorbidities treated with different antiviral drugs. Patients with diabetes had a longer duration of rtPCR conversion than patients without comorbidities in the Arbidol group during antiviral drug treatment ($p = 0.11$). Data are represented the mean ± SEM.

prolonged time to carry the SARS-COV-2 virus and consequently slower conversion from a positive to a negative rtPCR result.

A multitude of previous studies has documented that patients with comorbidities have escalated risks of poorer clinical outcomes with avian influenza, SARS-CoV, and Middle East respiratory syndrome (MERS)-CoV infections. A nationwide analysis of 1,590 COVID-19 patients in China showed that patients with any comorbidity yielded poorer clinical outcomes than those without among laboratory-confirmed cases of COVID-19 (15). A recent meta-analysis found that underlying diseases could be risk factors for severe patients compared with non-severe patients (15). The present study confirms this result, demonstrating that patients with comorbidities needed longer times to convert from a positive PCR result to a negative one. A previous study reported that diabetes could be a significant predictor of morbidity and mortality of COVID-19 (35). Our results showed that diabetic patients treated with Arbidol had a prolonged SARS-CoV-2 conversion, suggesting that the effects of antiviral drugs differ from patients with specific comorbidities. This may be related to the pathological changes in the comorbidities or to the medications for treating comorbidities. One study has shown that the use of antagonists of the renin–angiotensin–aldosterone system in diabetes contributes to a poor prognosis for COVID-19, and treatment of COVID-19 such as antivirals and corticosteroids may worsen glucose control in diabetics (36). Therefore, we suggest that the relationship between the comorbidity treatments and treatments of COVID-19, as well as drug–drug interactions during the treatment of COVID-19, are well worth being further studied.

In the past, corticosteroids were widely used during SARS-CoV1 and the MERS-CoV pandemic (37, 38). A meta-analysis of corticosteroid uses in SARS patients provided conclusive results that may delay viral clearance if given before viral replication is controlled (39). In a retrospective

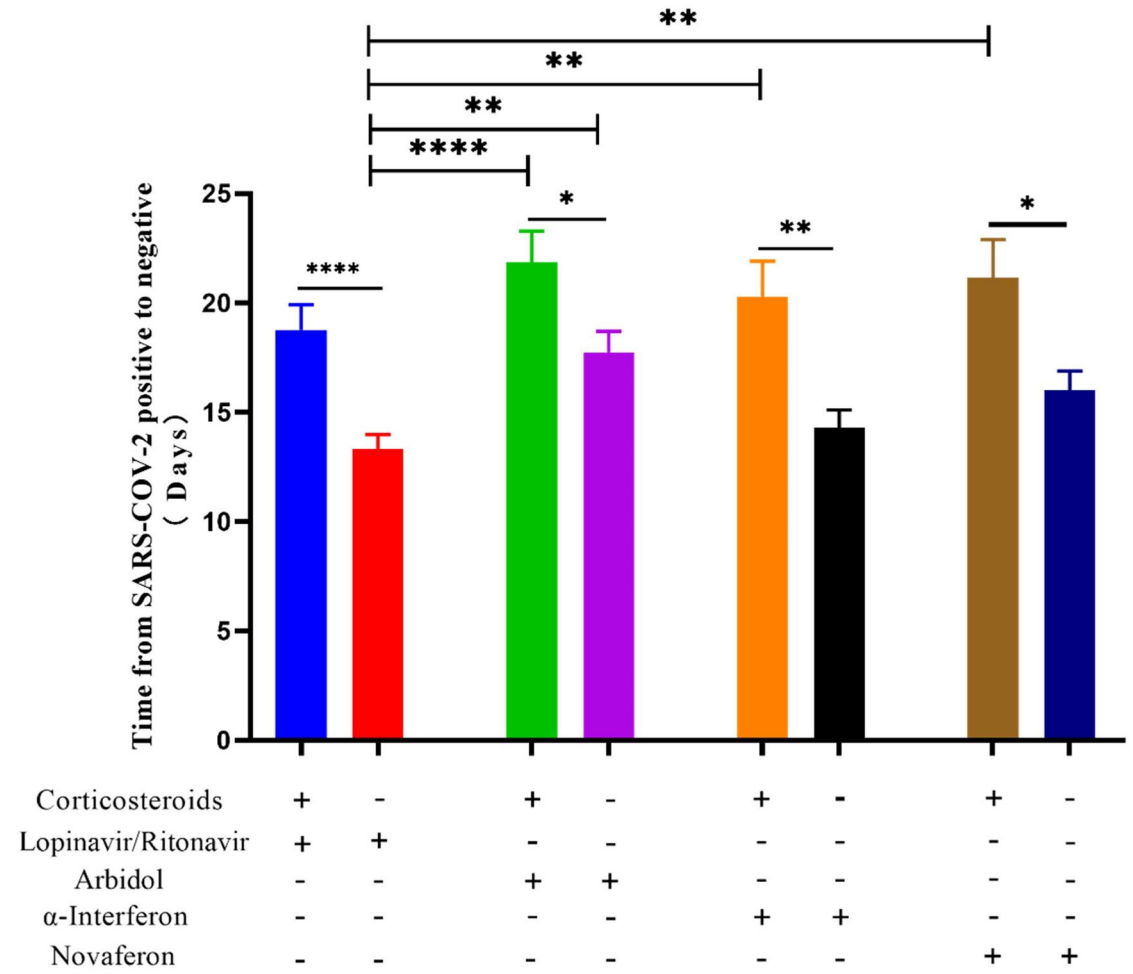

FIGURE 7 | The relationship between the use of corticosteroids and the conversion time from real-time PCR (rtPCR) positive to negative during antiviral treatment. Patients were divided by used antiviral drugs and corticosteroids use. Patients treated with corticosteroids showed in all groups a significantly longer conversion time from rtPCR positive to negative. Data are presented as mean ± SEM. The significance was determined as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$.

study of 309 critical patients with MERS, nearly half of the patients (49%) were treated with corticosteroids, and these patients were more likely to require mechanical ventilation, vasopressors, and renal replacement therapy (40). In addition, influenza respiratory syncytial virus (RSV) indicated that patients treated with corticosteroids had impaired antibody responses. For COVID-19 patients, corticosteroids are also widely used in septic shock despite uncertainty over their efficacy (41). Dexamethasone is the latest drug touted by experts in the UK as a possible treatment for COVID-19, with evidence suggesting that it can successfully reduce deaths from the virus by up to one-third in critically ill patients (42). According to the latest version of the WHO guidelines for COVID-19, severe and critical COVID-19 patients are recommended to use systemic corticosteroids, while non-severe patients are not recommended to use corticosteroids (43). In the present study, all patients had recovery from COVID-19, and no deaths occurred. Eighty-four percent of patients in our study were non-severed cases. It is shown for recovered patients that it took significantly longer time to reverse SARS-COV-2 from positive to negative in patients who have been treated with corticosteroids than those without during antiviral treatments, indicating that corticosteroids have a delaying effect on the clearance of SARS-COV-2 pathogens. In theory, corticosteroid therapy may bring direct benefit to symptom recovery. However, it may act as an immunosuppressive agent and not only inhibits inflammation of the lung but also suppress the immune response and pathogen clearance, therefore delaying the recovery. Overall, corticosteroids are proven to reduce the mortality of inpatients with critical COVID-19; however, corticosteroids use is likely to prolong the duration from SARS-COV-2 positive to negative, especially for non-severe COVID-19

patients. Therefore, corticosteroids should be used with caution for non-severe COVID-19 patients.

CONCLUSION

rtPCR test is of great importance to evaluate COVID-19 patients, serving as a reliable indicator of SARS-COV-2 clearance in patients. It is of great benefit to reduce the time of SARS-COV-2 clearance and thus shorten the hospital stay of COVID-19 patients to save medical resources. ESR could be an indirect indicator to monitor SARS-COV-2 activity in patients over time. During antiviral treatment, COVID-19 patients at the age of over 50, having comorbidities, and/or being exposed to a corticosteroid due to respiratory dysfunction will most likely have a prolonged conversion from rtPCR positive to negative.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The First Hospital of Changsha Hospital Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SZ: writing—original draft and review and editing. YH: writing—original draft and data curation. WT: data curation. AN: writing—review and editing, and conceptualization. FZ: data curation and conceptualization. All authors contributed to the article and approved the submitted version.

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Identification of COVID-19 Clinical Phenotypes by Principal Component Analysis-Based Cluster Analysis

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Background: COVID-19 has been quickly spreading, making it a serious public health threat. It is important to identify phenotypes to predict the severity of disease and design an individualized treatment.

Methods: We collected data from 213 COVID-19 patients in Wuhan Pulmonary Hospital from January 1 to March 30, 2020. Principal component analysis (PCA) and cluster analysis were used to classify patients.

Results: We identified three distinct subgroups of COVID-19. Cluster 1 was the largest group (52.6%) and characterized by oldest age, lowest cellular immune function, and albumin levels. 38.5% of subjects were grouped into Cluster 2. Most of the lab results in Cluster 2 fell between those of Clusters 1 and 3. Cluster 3 was the smallest cluster (8.9%), characterized by youngest age and highest cellular immune function. The incidence of respiratory failure, acute respiratory distress syndrome (ARDS), heart failure, and usage of non-invasive mechanical ventilation in Cluster 1 was significantly higher than others ($P < 0.05$). Cluster 1 had the highest death rate of 30.4% ($P = 0.005$). Although there were significant differences in age between Clusters 2 and 3 ($P < 0.001$), we found that there was no difference in demand for medical resources.

Conclusions: We identified three distinct clusters of the COVID-19 patients. The results show that age alone could not be used to assess a patient's condition. Specifically, management of albumin, and immune function are important in reducing the severity of disease.

Keywords: COVID-19, phenotype, treatment, principal component analysis, cluster analysis

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INTRODUCTION

Since December 2019, pneumonia cases with unknown cause have been reported in Wuhan (1). It has been identified as an acute respiratory infection caused by a novel coronavirus, later named COVID-19 by the World Health Organization (2). Since that time, COVID-19 has been quickly spreading in China and other countries, making it a serious global public health threat (3). It is important for health professionals to take coordinated, timely, and effective actions to help prevent additional cases or poor health outcomes.

The entire population is generally susceptible to the virus. Confirmed cases need to be treated in designated hospitals with effective isolation and protection conditions. Critical cases should be admitted to the ICU as soon as possible (3). Mechanical ventilation, blood purification, and extracorporeal membrane oxygenation (EMCO) should be applied cautiously in severe COVID-19 patients (2). Beyond these invasive rescue methods, doctors hope to find ways to prevent disease progress from the early stage in the clinic.

Cluster analysis is one of the unsupervised learning methods which has been successfully applied in medical research (4). Cluster generation involves merging samples into larger clusters to minimize the within-cluster variations amongst patients and to maximize the between-cluster variations. Using cluster analysis, we can take advantage of in-depth phenotyping to reveal unique patterns of association among phenotypic variables (5), which may allow health professionals to develop specialized and more effective therapeutic strategies for the treatment of COVID-19 patients.

We hypothesized that COVID-19 comprises discrete clusters of patients with different clinical characteristics associated with different outcomes. To test this hypothesis, we used cluster analysis to identify COVID-19 subgroups and then determined the disease severity among subgroups. We demonstrate that this unbiased clustering approach could predict the severity of disease in patients and thus reveal the key variables clinicians could consider when evaluating patients.

MATERIALS AND METHODS

Study Design and Participants

We conducted a retrospective, single centered and observational study in Wuhan Pulmonary Hospital, Hubei Province, China (a COVID-19-designated hospital in the epidemic outbreak) and collected clinical data from the patients diagnosed with COVID-19 between January 1 and March 30, 2020. Patients with missing clinical data were excluded.

The diagnosis and treatment of COVID-19 complied with the "new coronary pneumonia diagnosis and treatment plan" issued by the health commission of the People's Republic of China. Laboratory diagnosis of COVID-19 was confirmed by viral nucleic acid test (NAT) using high-throughput sequencing or real-time reverse-transcriptase–polymerase-chain-reaction (RT-PCR), which can amplify the open reading frame 1ab (ORF1ab) and nucleocapsid protein (NP) gene fragments of COVID-19 virus from the sputum, pharyngeal swab, or lower respiratory tract samples.

The National Health Commission of the People's Republic of China affirmed that data collection and analysis of cases and close contacts are part of ongoing investigations into outbreaks of public health events and are therefore exempt from the approval requirements of the institutional review board.

Data Collection

Clinical data include demographic information (gender, age, comorbidities), laboratory tests (routine blood test, coagulation test, infection markers, liver and kidney function, and markers of myocardial injury), and outcomes (survival or death at hospital discharge).

Statistical Analysis

The main factors with the highest loading in 18 variables (including all the laboratory tests) were selected using principal component analysis (PCA) at baseline. K-means cluster analysis (6), one of the most widely adopted clustering algorithms, was carried out to classify COVID-19 patients into different groups using clinical data based on the PCA results.

PCA analysis was performed using the following variables: D-Dimer, fibrinogen (FIB), activated partial thromboplastin time (APTT), prothrombin time (PT), c-reactive protein (CRP), procalcitonin (PCT), white blood cell (WBC), neutrophil count, lymphocyte count, monocyte count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (Alb), helper T lymphocyte count, cytotoxic T lymphocyte count, creatinine (Cr), troponin I (TNI), and N-terminal pro-Brain Natriuretic Peptide (NT-proBNP). In order to select the number of important principal components, we chose values with an eigenvalue >1. The Oblimin method was used in the square rotation. The similarity of data was calculated using the principal factors that were identified by PCA-transformed data. Kaiser–Meyer–Olkin (KMO) and the Bartlett's test of Sphericity assessed the adaptive validity of PCA analysis. The representative variables of principal components were chosen based on their factor loading.

We performed a K-means cluster analysis in this study. The main steps were as follows: First, the initial cluster center was selected with the number of K. Second, cluster steps were repeated until cluster membership stabilized. Third, each point was assigned to its closest cluster center. Finally, the new cluster centers were computed.

SPSS version 24.0 (IBM Corp, Armonk, NY) was used for statistical analysis. Qualitative and quantitative variables were summarized using mean and standard deviation (SD), median and inter-quartile range (IQR), and number and percentage, respectively. Differences between clusters in qualitative variables were analyzed using the Chi-squared test. Differences in the quantitative variables were analyzed using the *t*-test. In the case of non-normally distributed variables, the non-parametric Mann–Whitney test was used. A $P < 0.05$ was considered statistically significant.

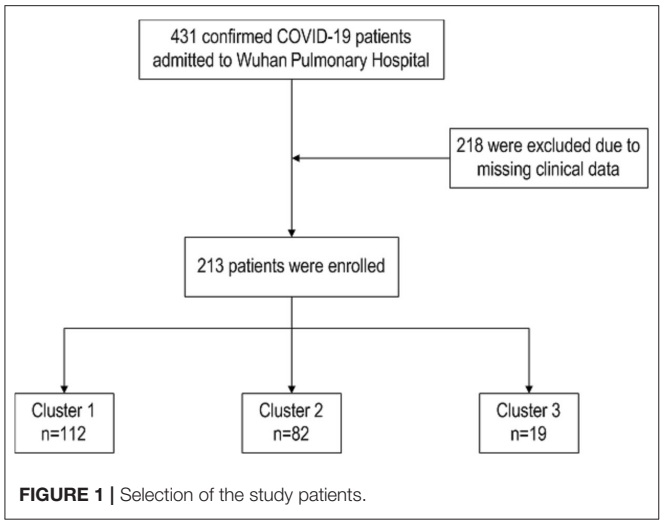

FIGURE 1 | Selection of the study patients.

RESULTS

Demographics and Baseline Characteristics of Patients With COVID-19

There were 431 confirmed COVID-19 patients admitted to Wuhan Pulmonary Hospital between January 1 and March 30, 2020 and 218 (52.8%) were excluded due to missing clinical data (**Figure 1**). Two hundred and thirteen patients were ultimately enrolled with a mean age of 61.85 ± 14.72 years, and 116 (54.50%) of them were males. 167 (78.40%) patients survived, while 46 (21.60%) died. Demographic characteristics, laboratory tests, and comorbidities of all patients are shown in **Table 1**.

Principal Component Analysis and Cluster Analysis for the Identification of COVID-19 Clusters

The KMO value was 0.676, and the *p*-value of Bartlett's test of sphericity was <0.001. Six components were retained using the PCA analysis. These six components significantly contributed to explaining the relationships among the 18 variables and accounted for 73.18% of the information. The following representative variables were chosen based on relatively high factor loading: factor 1, CRP and neutrophil counts; factor 2, WBC and monocyte counts; factor 3, ALT and AST; factor 4, PCT and Fib; factor 5, TNI and D-Dimer; and factor 6, Alb and NT-proBNP (**Table 2**).

Baseline Characteristics of COVID-19 Clusters

Three distinct subgroups were identified using the cluster analysis (**Table 3**). Differences between Clusters 2 and 3 are shown in **Supplementary Table 1**.

In total, 52.6% of subjects ($n = 112$) were grouped into Cluster 1. This cluster was characterized by the oldest age with mean age of 72.7 ± 6.7 years, most obvious inflammatory reaction with the highest CRP and neutrophil count, the lowest lymphocyte count and cellular immune function and albumin level, and the highest NT-proBNP.

38.5% of subjects ($n = 82$) were grouped into Cluster 2. This cluster had the middle age with mean age of 54.1 ± 5.8 years. NT-proBNP, cytotoxic T lymphocyte count, helper T lymphocyte count, AST, and lymphocyte count fell between those of Clusters 1 and 3. CRP, Alb, and D-Dimer of Cluster 2 had a significant difference between Cluster 1. Clusters 2 was characterized by middle age and general basic situation.

Cluster 3 was the smallest cluster ($n = 19$; 8.9% of subjects). It was characterized by youngest age with mean (SD) age of 31.4 (12.2) years and highest cytotoxic T lymphocyte count.

There was no significant difference in fibrinogen, activated APTT, PT, WBC, monocyte count, ALT, creatinine, and PCT among the three clusters.

TABLE 1 | Baseline characteristics and laboratory tests of 213 patients.

Characteristics		Count (%) or Mean (SD) or Median (IQR)
Gender (Male, %)		116 (54.5%)
Age (years)		61.7 (14.7)
D-Dimer (mg/L)		0.5 (0.2–1.7)
FIB (g/L)		4.2 (1.4)
APTT (s)		35.8 (32.5–39.7)
PT (s)		13.1 (12.5–14.3)
WBC ($\times 10^9$/L)		6.7 (5.1–9.2)
Neutrophil count ($\times 10^9$/L)		5.0 (3.2–7.6)
Lymphocyte count ($\times 10^9$/L)		0.9 (0.6–1.5)
Monocyte coun t ($\times 10^9$/L)		0.4 (0.2)
Alanine aminotransferase (μ/L)		27 (16–41)
Aspartate aminotransferase (μ/L)		25 (17.5–42)
Albumin (g/L)		36.0 (5.4)
Creatinine (μmol/L)		68 (56–83)
Helper T lymphocyte count (n/μl)		258.3 (23.1–525.6)
Cytotoxic T lymphocyte count (n/μl)		145.4 (72.9–313.0)
CRP (mg/L)		32.4 (3.4–81.7)
PCT (ng/ml)		0.0 (0.0–0.1)
TNI (ng/ml)		0.0 (0.0–0.0)
NT-proBNP (pg/ml)		144 (34–558)
Death (*n*, %)		46 (21.6%)
Ventilator (*n*, %)	Invasive mechanical ventilation	33 (15.5%)
	Non-invasive mechanical ventilation	49 (23%)
Comorbidity (*n*, %)	Respiratory failure	37 (17.4%)
	ARDS	34 (16%)
	Heart failure	50 (23.5%)
	AKI	12 (5.6%)
	Diabetes mellitus	42 (19.7%)

ARDS, acute respiratory dyspnea syndrome; AKI, acute kidney injury; CRP, C-reactive protein; PCT, procalcitonin; NT-proBNP, N-terminal pro brain natriuretic peptide; TNI, troponinI; FIB, fibrinogen; APTT, anginal partial thromboplastin time; PT, prothrombin time; WBC, white blood cell.

TABLE 2 | Correlations of the 18 original variables with the six main factors derived from the principal component analysis.

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6
Eigenvalue	4.388	2.539	1.673	1.629	1.136	1.076
% variance explained	25.812	14.934	9.840	9.581	6.683	6.328
APTT	0.139	0.304	0.409	−0.751	−0.119	0.077
PT	0.197	0.280	0.201	−0.815	−0.113	−0.130
WBC	0.423	0.743	−0.154	0.203	−0.315	−0.043
Monocyte count	−0.254	0.646	0.033	0.242	−0.285	−0.309
Lymphocyte count	−0.696	0.514	0.261	0.028	0.058	0.072
Neutrophil count	0.603	0.592	−0.196	0.175	−0.293	−0.046
Alb	−0.707	0.068	0.146	0.018	0.017	0.174
CRP	0.747	0.014	0.232	−0.012	−0.212	0.310
ALT	0.232	−0.098	0.709	0.315	0.099	−0.369
AST	0.485	−0.047	0.705	0.218	0.295	−0.119
Cr	0.275	0.038	0.003	−0.082	0.257	−0.288
TNI	0.401	0.418	−0.084	−0.009	0.416	−0.146
PCT	0.379	0.139	0.313	0.232	0.029	0.712
Helper T lymphocyte count	−0.768	0.418	0.174	0.080	0.147	0.075
Cytotoxic T lymphocyte count	−0.761	0.425	0.182	0.105	0.039	0.065
NT-proBNP	0.473	0.368	−0.105	0.039	0.295	0.186
Fib	0.283	−0.223	0.201	0.205	−0.511	−0.130
D-Dimer	0.426	0.323	−0.282	−0.002	0.517	−0.021

CRP, C-reactive protein; PCT, procalcitonin; NT-pro BNP, N-terminal pro brain natriuretic peptide; TNI, troponinI; Flb, fibrinogen; APTT, anginal partial thromboplastin time; PT, prothrombin time; WBC, white blood cell; Cr, creatinine; Alb, albumin; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase.

TABLE 3 | Baseline characteristics of three clusters.

	Cluster 1 ($n = 112$)	Cluster 2 ($n = 82$)	Cluster 3 ($n = 19$)	*P*
Gender (Male, %)	63 (56.3%)	43 (52.4%)	10 (52.6)	0.620
Age (years)	72.7 (6.7)	54.1 (5.8)	31.4 (12.2)	<0.001
D-Dimer (mg/L)	0.9 (0.4–3.5)	0.3 (0.2–0.6)	0.3 (0.1–0.6)	<0.001
FIB (g/L)	4.1 (1.4)	4.3 (1.6)	4.1 (1.2)	0.773
APTT (s)	36.3 (23.6–40.6)	34.5 (31.8–37.2)	35.6 (33.4–41.8)	0.082
PT (s)	13.2 (12.5–14.4)	13.0 (12.4–13.9)	12.9 (12.5–13.7)	0.220
WBC ($\times 10^9$/L)	6.7 (5.2–9.3)	6.8 (4.8–9.3)	6.3 (5.2–9.1)	0.771
Neutrophil count ($\times 10^9$/L)	5.1 (3.7–8.0)	5.0 (3.0–7.8)	3.7 (2.8–4.9)	0.029
Lymphocyte count ($\times 10^9$/L)	0.8 (0.5–1.3)	1.0 (0.6–1.7)	1.5 (0.9–2.3)	0.001
Monocyte count ($\times 10^9$/L)	0.4 (0.2)	0.4 (0.2)	0.4 (0.1)	0.293
Alanine aminotransferase (μ/L)	26.5 (16.3–43.8)	28.5 (17–40.5)	19 (11–32)	0.16
Aspartate aminotransferase (μ/L)	29 (20–44.8)	23.5 (16–40.2)	19 (14–32)	0.009
Albumin (g/L)	34.8 (5.2)	37.3 (5.1)	37.9 (6.6)	0.002
Creatinine (μmol/L)	70 (58–89)	66.5 (53.8–78)	73 (52–78)	0.205
Helper T lymphocyte count (n/μl)	237.0 (85.2–422.5)	262.4 (142.7–652.7)	366.0 (274.4–696.8)	0.003
Cytotoxic T lymphocyte count (n/μl)	115.4 (51.0–239.8)	189.9 (97.8–387.8)	316.4 (164.3–498.8)	<0.001
CRP (mg/L)	44.4 (15.0–85.1)	22.6 (1.0–83.0)	19.5 (1.0–31)	0.002
PCT (ng/ml)	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.065
TNI (ng/ml)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.015
NT-proBNP (pg/ml)	390 (94.8–875.6)	48.5 (15–188)	15 (15–292)	<0.001

CRP, C-reactive protein; PCT, procalcitonin; NT-pro BNP, N-terminal pro brain natriuretic peptide; TNI, troponinI; FIB, fibrinogen; APTT, anginal partial thromboplastin time; PT, prothrombin time; WBC, white blood cell.

TABLE 4 | Disease severity of three clusters.

	Cluster 1 ($n = 112$)	Cluster 2 ($n = 82$)	Cluster 3 ($n = 19$)	*P*
Invasive mechanical ventilation	22 (19.6%)	10 (12.2%)	1 (5.3%)	0.056
Non-invasive mechanical ventilation	31 (27.7%)	18 (22%)	0 (0%)	0.017
Respiratory failure	30 (26.8%)	6 (7.3%)	1 (5.3%)	<0.001
ARDS	24 (21.4%)	9 (11%)	1 (5.3%)	0.019
Heart failure	36 (32.6%)	13 (15.9%)	1 (5.3%)	<0.001
AKI	9 (8%)	3 (3.7%)	0 (0%)	0.087
Death	34 (30.4%)	9 (11%)	3 (15.8%)	0.005

ARDS, acute respiratory dyspnea syndrome; AKI, acute kidney injury.

COVID-19 Clusters and Disease Severity

The disease severity of COVID-19 in the current patient population was compared across the clusters (**Table 4**). Differences between Clusters 2 and 3 are shown in **Supplementary Table 2**. The incidence of respiratory failure, acute respiratory distress syndrome (ARDS), and heart failure in Cluster 1 was significantly higher than the other two clusters ($P < 0.05$). The proportion of non-invasive mechanical ventilation usage in Cluster 1 was 27.7%, which was significantly higher than other clusters ($P = 0.017$). Cluster 1 also had the highest death rate of 30.4% ($P = 0.005$).

DISCUSSION

COVID-19 is a novel, rapidly spreading, viral illness that represents an emergent global health threat. Mortality rate is higher in elderly and intensive care unit (ICU) COVID-19 patients, reaching 17–38% in recent reports (7, 8). Progressive lymphocytopenia was often found in severe cases (9–11). In this study, we identified three distinct subgroups of COVID-19 through a cluster analysis of 213 patients. Cluster 1 was characterized by oldest age, highest mortality rate (30.36%), and significantly lower lymphocyte count. This result was consistent with previous reports (7, 8).

The immune system of a host controls invading pathogens and thereby determines the prognosis of patients with any infectious disease, including pneumonia (12). As immune deficiency is closely tied to mortality, evaluating the immune condition could be an important companion to monitoring a patient's general condition in order to estimate prognosis (13). We found that helper T lymphocyte count and cytotoxic T lymphocyte count in Cluster 1 were significantly lower than those of the other two clusters. This suggested more impaired immune function in the Cluster 1 patients. Treating the immune deficiency at the early stage of disease may reduce the risk of disease deterioration and improve patient prognosis. Therefore, more attention to immune function is required in the elderly, severely ill patients instead of focusing on invasive treatment only.

Low albumin can lead to hypoproteinemia, and it can cause a range of diseases, such as serous effusion, pulmonary edema, heart failure, and more. Timely correction of hypoproteinemia could effectively prevent the incidence of complications (14). Therefore we compared the albumin differences between three clusters. Albumin of Cluster 1 was significantly lower than the other two clusters in our study. Therefore, it is also important to pay attention to the albumin level in elderly patients.

Our cluster analysis suggests that immunological parameters (helper T lymphocyte count and cytotoxic T lymphocyte count) and serum albumin level are important in determining prognosis and the vulnerability to developing comorbidities, including respiratory failure, ARDS, and heart failure. Improving the immune status and albumin level of patients may be a potential measures to prevent disease progression.

The mortality rate was higher in elderly patients (7, 8). We found that the mortality rate of Cluster 3, which was characterized by the youngest mean age, was not significantly different from middle-aged patients who grouped in Cluster 2. This result aroused our attention. In previous studies, it was mentioned that some COVID-19 patients showed immune imbalance and a cytokine storm, which could be responsible for further lung injury (15–17). Young patients in Cluster 3 had the highest T lymphocyte count, and most likely had a cytokine storm. Thus, is the implication to clinicians that if a younger patient presents with COVID-19, they should check T lymphocyte counts because those with very high levels may be at risk of developing severe disease despite a younger age. This needs further pathological research to validate.

D-Dimer is a degradation product that is produced in hydrolysis of fibrin (18). Studies have reported increase in D-Dimer levels in patients with pneumonia, has an indication of the presence of thrombosis and the blood hypercoagulable state (19, 20). High D-Dimer is likely to be associated with persistent clotting disorders, microthrombotic formation, pulmonary embolism and acute myocardial infarction in long-stay patients or death patients, which may cause refractory hypoxemia, respiratory failure, disseminated intravascular coagulation or even death. Our previous study found that COVID-19 patients with higher initial and peak D-Dimer value tended to have a higher risk of death (21). In this study, we found that D-Dimer of Cluster 1 was significantly higher than other two clusters. Cluster 1 also had the highest death rate of 30.4%, which was consistent with previous studies. These patients were likely to have myocardial infarction and/or pulmonary embolism, and it might also explain the difference of myocardial enzymes (TNI and AST) among the three clusters. This might suggest the importance of early anticoagulant intervention.

Neutrophil count and lymphocyte count were found to have great prognostic power in community-acquired pneumonia. The

increase of neutrophils often indicates that the patients have bacterial infection and the infection is aggravated. The decrease of lymphocyte means that the immune function is poor (22, 23). At the early stage of COVID-19, the total number of leukocytes is normal or decreases, while the lymphocyte count decreases (3). We found that Cluster 1 had the lowest lymphocyte count and the highest neutrophil count. There was no difference in Neutrophil count and lymphocyte count between Cluster 2 and 3. Our previous study found that COVID-19 patients with high neutrophil-lymphocyte Count Ratio might have a poor prognosis, even a risk of death (21). Those might suggest that the aggravated condition and the infection is difficult to control in Cluster 1.

According to our clustering results in disease severity, patients in Cluster 1 had a high incidence of respiratory failure, ARDS, heart failure, and high utilization rate of non-invasive mechanical ventilation. The demand for medical resources of these patients is significantly higher than other clusters. Thus, we suggest that Cluster 1 needs a comprehensive treatment plan, or may even need to stay in the intensive care unit. Although there were significant differences in age between Clusters 2 and 3, we also found that there was no significant difference in demand for medical resources between these two clusters. It could be interpreted that doctors should pay the same clinical attention to middle-aged and young patients. Age alone could not be used to assess a patient's condition, we must correct the misunderstanding that young patients should always be assumed to have relatively mild disease in COVID-19.

There are some potential limitations in our study. First, this was a single center retrospective study. All of the data were collected from patients in Wuhan Pulmonary Hospital. Most of the patients in this hospital were symptomatic, severe or even critical. As a result, the proportion of young and mild disease patients in the study was relatively low. Second, only 213 out of 413 patients were enrolled in our study. The exclusion of patients with missing clinical data might cause some bias in our analysis. Our results could be more representative if we are able to collect these data in the future. Finally, our data may be subjected to recall bias and selection bias due to the nature of our study. For example, the record of patients' comorbidities might not be accurate and complete, considering the unprecedented pressure during admission and treatment.

Further studies with more detailed and representative data are needed. In particular, a long-term follow up of the patients will allow us to further explore the differences between phenotypes.

CONCLUSIONS

We identified three distinct subclasses of COVID-19 patients in Wuhan Pulmonary Hospital. It might be necessary to improve the immune function and pay attention to the underlying health conditions in the elderly patients. D-Dimer, lymphocyte count, neutrophil count, NT-proBNP, T lymphocyte count, and serum albumin should be paid attention to. This might remind us that correction of these abnormal lab results in time can be useful in preventing the corresponding complications and reducing the mortality rate. Age alone could not be used to assess a patient's condition; cluster assessment may be more reliable.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The National Health Commission of the People's Republic of China. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article. Informed consent was exempted with the approval of Medical Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China (No. XHEC-D-2020-052).

AUTHOR CONTRIBUTIONS

YC, HH, and LY designed the current study and revised the manuscript. YT, GC, XLi, CJ, MH, GZ, XLa, YW, and XD collected data. WY and WL wrote the manuscript and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.570614/full#supplementary-material

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Women's Knowledge, Attitude, and Perceptions Toward COVID-19 in Lower-Middle-Income Countries: A Representative Cross-Sectional Study in Bangladesh

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The coronavirus disease 2019 (COVID-19) is a global health emergency of unprecedented proportions. Countries around the world have taken extraordinary steps to control the disease. The preventive measures face challenges in low and lower middle income countries (LICs and LMICs). Especially the marginalized communities, e.g., women are the hardest hit of the virus. This study took Bangladesh as a representative LMIC and aimed to determine the level of knowledge, perception, attitude, and preparedness related to COVID-19 among the adult women in the country. Using a comprehensive questionnaire, we channeled a cross-sectional study among adult women in Bangladesh. Participant's self-reported data on the knowledge, attitude, and preparedness were tabulated and analyzed using suitable statistical tools. A total of 1,869 adults from 61 districts of Bangladesh took part in this study. Ninety seven percentage of the participants claimed to have heard of COVID-19 before it arrived in Bangladesh. Regarding the general knowledge related to COVID-19's causal agent, symptoms, and treatment, the positive response rate was nearly 80%, with a mean of 10.68 ± 1.72. Younger and educated women had better knowledge levels compared to the older and lower-educated participants ($p < 0.01$). More efforts are required to educate women with older age and lower socioeconomic status. An overall positive attitude and perception were observed, although a significant proportion of the participants opined that the Government's efforts in controlling the outbreak were not adequate. Although the participants had a satisfactory level of knowledge and a positive attitude in adopting preventive measures against COVID-19, greater efforts are needed from the healthcare authorities and Government.

Keywords: COVID-19, SARS-CoV-2, women, health literacy, knowledge, awareness, preparedness, attitude

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a new member of the coronaviridae family of RNA viruses (1). Infection with SARS-COV-2, leading to coronavirus disease 2019 (COVID-19) in humans, can result in respiratory syndromes ranging from an uncomplicated upper respiratory tract distress to severe viral pneumonia with multiorgan failure and death (2). This new virus transmits by droplets from asymptomatic or oligosymptomatic patients and proximally through aerosols in health care environments (3, 4). Within months after the first infection detected in humans late in 2019, this highly contagious virus with the ability to cause severe respiratory disease has hit the health systems across the world (5). In around 6 months after the first emergence of the virus, nearly 4.5 million confirmed cases have been identified in 185 countries around the globe, and over 300 thousand people have died of the disease (6).

The disease has evolved into a pandemic, and the World Health Organization (WHO) has declared it a global health emergency of unprecedented proportions (7). The outbreak substantially impacted millions of people around the world. As there is yet a vaccine or treatment strategy to be approved for COVID-19, only strong infection control measures can help minimize the spread of the virus in the community and health care facilities. Countries worldwide adopted extraordinary control measures soon after the virus's emergence, and a multi-level stress-coping-adjustment procedure is in progress (8). In order to execute the control measures effectively, every individual across the world requires to pay attention to the dramatically changing messages about public health and take prompt actions to limit the virus spread and individual risk (7, 8). Lessons learned from previous outbreaks indicate that poor knowledge, attitudes, and perception (KAP) toward infectious diseases and health literacy may challenge the efforts to prevent the spread of disease (9). Besides, under-estimating potential risk, stigmatization, panic emotions, and wrong measures to avoid the infection may affect combating such a situation (10).

However, the prevailing rhetoric related to the pandemic is often not intelligible and evenly disseminated to the mass people (11). Consequently, the messaging regarding the strategic measures and imminent threat of COVID-19 pandemic is at odds, leading to miscommunication, public confusion, and inaction (12). The situation is far more complicated in low and lower-middle-income countries (LICs and LMICs), like in Bangladesh, where significant portions of the population have minimal health literacy (13, 14). Among the people in these underprivileged communities, women are even more disadvantaged due to cultural norms and values. A large proportion of women in these countries have negligible access to information (15). As a result, women in LICs and LMICs supposedly have lower knowledge-index. In the situation of a health emergency, when knowing and understanding the critical and rapidly changing health messages come with foremost importance, people living in marginalized communities, like the women in LICs and LMICs with limited access to information, may experience extremely inadequate health communication (16, 17). The limitations of health communication can potentially lead these people to be further marginalized and exposed to elevated risks (17, 18).

We did a time-sensitive study among the adult women representing different social groups of Bangladesh, an LMIC in Southeast Asia. Non-therapeutic preventive measures to limit the spread of SARS-CoV-2, e.g., social distancing, faced an enormous challenge during the early stage of the outbreak in Bangladesh. With a huge lack of diagnostic and hospital facilities and poor coordination of management strategies, the country was walloped by the emergence of the virus (19). Assumably, health literacy, and awareness among the mass people, especially among the marginalized population like the women, would be vital to control the virus spread and mitigate the pandemic's impact. This study aimed to assess knowledge, attitudes, and perceptions about COVID-19 among the female population in a resource-limited LMIC.

METHODS

Study Design and Population

This cross-sectional survey took place during the initial weeks of lockdown enforced in Bangladesh. Only adult women of Bangladeshi nationality, who are not working in the medical field, were recruited in the study using convenience and snowball sampling methods. Given the current situation, a thorough community-based survey was not feasible. We collected the data through telephone, online, or in-person interviews, when possible. A team comprised of graduate students who majored in health and life sciences, clinicians, and statisticians conducted the questionnaire-based interviews.

Measurements

The newly prepared questionnaire consisted of five parts concerning the demographic backgrounds of the participants, and their knowledge, attitude, and preparedness (KAP) related to COVID-19. Following the guidelines for clinical and community management of COVID-19 by authorized bodies, we developed this questionnaire to assess the self-reported KAP of the participants (7, 20–22). The questionnaire included 15, 25, and 17 items related to the knowledge, attitude/perception, and preparedness of the women related to COVID-19. Each positive response (correct answer, where applicable) in the knowledge section was given 1 point, whereas a negative response received a 0 point.

Validation of the Questionnaire

We evaluated the newly prepared questionnaire in a preliminary study. Initially, we asked a group of Bangladeshi researchers in the field of epidemiology to independently assess the degree to which the questionnaire is relevant and is able to measure women's KAP regarding COVID-19 correctly. In terms of language, formatting, and contents, we made essential modifications to the questionnaire to address their comments. Later on, to pre-test the questionnaire, we interviewed 35 participants twice 15 days apart using the modified questionnaire.

Obtained data were used to assess internal consistency and test-retest reliabilities using Cronbach's α and intra-class correlation analysis. Both assessments indicated satisfactory levels of reliability of the questionnaire (Cronbach's α = 0.79, intra-class correlation coefficient = 0.96). No data from the above mentioned 35 participants were included in the final analyses.

Statistical Analysis

We evaluated all data using the Statistical Package for the Social Sciences (IBM SPSS, v 22.0; Chicago, IL) software (23). Associations between participant characteristics and survey responses were then examined in bivariate analyses using Student's *t*-tests, z-statistic, $\chi 2$ tests, or analysis of variance (ANOVA), as suitable. For the continuous outcome of a perceived concern, we used multivariable linear regression models to estimate least-squares means (with 95% confidence intervals). The quantitative variables were reported either as mean ± standard deviation or frequency (%). All computations included the KAP variables as primary covariates of interest. Other variables affecting the KAP were also analyzed using appropriate statistical tools. All analyses were performed at α-levels of 0.05 and 0.01 ($p = 0.05$ and $p = 0.01$, respectively).

Ethics Statement

The internal Ethical Review Board (ERB) at the Department of Biochemistry and Molecular Biology, Shahjalal University of Science and Technology approved the study protocol (Reference ID: 02/BMB/2020). All participants in this study provided informed consent as per the World Medical Association (Helsingin julistus). No participants received any monetary rewards for participating in the study.

RESULTS

Participants' Characteristics

In total, our interviewers invited 3,150 adult women to participate in this study, of which 1,246 declined to participate (**Supplementary Table 1**). A total of 1,904 women took part in the interviews; however, we excluded questionnaires of 35 participants due to incompleteness. Henceforth, the final analysis of this study consisted of data obtained from 1869 women, giving an overall response rate of 59.33%.

The mean age of the participants was 29.545 ± 12.009 years (range: 18–86 years), with over 40% (43.23%) of them aged 18 to 30 years and only 5.67% aged 60 years and above (**Table 1**, **Supplementary Figure 1**). The sample pool consisted of diverse socioeconomic backgrounds, originating from 61 of 64 districts of Bangladesh (**Table 1**, **Supplementary Figures 1, 2**). Over 50% (51.20%) of the participants had higher secondary or more education, with 17.39% of them being university or college graduates. Nearly one-third of the participants (32.69%) were students, another one-third (37.72%) were either unemployed, retired, or housewives (not involved in earning). Besides, ∼60% (61.10%) of participants were either single or ever married at the time of the study. In terms of religious background, over 86% (86.35%) of participants were Muslims (**Table 1**). Over 10% (12.09%) of all participants had multiple clinical conditions (>1 clinical conditions), including coronary complications, respiratory, and pulmonological complications (**Supplementary Table 2**). Nearly 10% (9.84%) of the participants reported that they frequently experienced feverish symptoms.

TABLE 1 | Sociodemographic characteristics of participants.

Variables	Value (n = 1,869)
Age (years)	29.545 ± 12.009
Marital status (*n*, %)	
Single	801, 42.86
Married	727, 38.90
Ever married	341, 18.24
Education level (*n*, %)	
No schooling	88, 4.71
Primary	240, 18.84
Secondary	508, 27.18
Higher secondary	632, 33.81
University/college	325, 17.39
Other	76, 4.07
Occupation (*n*, %)	
Unemployed/retired	294, 15.73
Student	611, 32.69
Self-employed	43, 2.30
Business	102, 5.45
Maidservants/household helping hands	146, 7.81
Service holder in a Government organization	105, 5.61
Service holder in a non-government/private organization	157, 8.40
Housewives (not involved in earning)	411, 21.99
Religion (*n*, %)	
Islam	1,614, 86.35
Hindu	214, 11.44
Buddhist	27, 1.44
Christian	05, 0.27
Prefer not to say	09, 0.48
Ethnicity (*n*, %)	
Bengali	1,848, 98.88
First nations/tribal	21, 1.12

Knowledge Related to COVID-19

97% (n = 1,813) of all participants reported having heard of the COVID-19 outbreak. Out of the 1,813 participants who had heard of the outbreak, 1,759 (97.02%) knew that the virus had arrived in Bangladesh (**Table 2**). The source of knowledge for most the participant was internet (n = 1,173, 64.7%) and the TV (n = 919, 50.69%) (**Supplementary Table 3**). Over two-thirds (67.84%) of the participants claimed that they knew about COVID-19 after its emergence in China and before it arrived in Bangladesh (**Supplementary Figure 3**). Overall, the positive response rate was nearly 80% (**Table 2**). The total knowledge score ranged from 4 to 14, with a mean of 10.68 ± 1.72.

Table 2, Supplementary Figure 4 presents the outcomes of knowledge assessment of the women regarding COVID-19's

TABLE 2 | Women's knowledge related to COVID-19.

	Response		
	Yes (*n*, %)	**No (*n*, %)**	**Maybe (*n*, %)**
Knows about contagious diseases	1,616, 86.46	178, 9.52	75, 4.01
Knows about viral flus	1,573, 84.16	231, 12.36	65, 3.48
Has idea about the general flu protocol of WHO	1,248, 66.77	450, 24.08	171, 9.15
Knows what causes (causal agent) COVID-19	1,529, 81.81	256, 13.7	84, 4.49
Knows that COVID-19 is a contagious disease	1,424, 76.19	331, 17.71	114, 6.1
Knows about the mode of transmission of COVID-19	1,506, 80.58	265, 14.18	98, 5.24
Knows about the symptoms of COVID-19	1,440, 77.05	315, 16.85	114, 6.1
Knows about the unavailability of COVID-19 treatments	1,501, 80.31	267, 14.29	101, 5.4
Knows who are the vulnerable group to COVID-19	1,488, 79.61	276, 14.77	105, 5.62
Knows what quarantine means	1,392, 74.48	359, 19.21	118, 6.31
Knows what social distancing means	1,424, 76.2	332, 17.76	113, 6.04
Overall response rate (mean ± SD)	78.51 ± 5.06	15.86 ± 3.7	5.63 ± 1.42

mode of transmission, common symptoms, vulnerable groups, and rhetoric related to the pandemic.

Perceptions and Attitudes Related to COVID-19

Nearly 4 out of 5 (83.28%) women acknowledged that they fear COVID-19 (**Table 3**). Over 50% (52.56%) of the participants were concerned because they have older family members. Around 3 out of 4 participants perceived COVID-19 as a dangerous public health threat (75.46%). However, they thought it to be common cases of flu (72.59%) (**Table 3**). One-fourth of the women (25.32%) assumed that COVID-19 is a curse from the GOD. Only ∼40% of women thought that people around them are aware of the COVID-19 situation. The majority of the participants responded that Bangladesh's efforts and preparations in COVID-19 management was not enough and satisfying (**Table 3**). 46.8% thought that the media coverage about this disease is exaggerated. Two out of five women (39.33%) thought that the Bangladesh Government's timely measures could help reduce the spread of the virus, while only one-third (34.80%) thought that the doctors and nurses in Bangladesh are trained to handle COVID-19 patients. Over 85% (87.42%) opined that the Government should subsidize for treatment of COVID-19 patients. More than a half (53.94%) thought that the Government was not transparent on COVID-19 related information. Nearly half (46.39%) of the women feared that COVID-19 would result in a devastating fatality in the country (**Table 3**).

Preparedness Related to COVID-19

Figure 1 presents the preparedness of the women to limit the spread of COVID-19 and their responses. Seventy one percentage of participants reported that they are washing their hands more frequently than ever and for an extended period (**Figure 1**). They also reported that they managed to buy extra foods (50.27%), medicines (68.16%), daily goods (51.16%) for an extended period, and also bought disinfectants and hand soaps/sanitizers as precautions (48.66% and 61%, respectively) (**Figure 1**). Two out of five of every woman who participated claimed that they were avoiding to meet with their friends even if they have no symptoms (46.51%), to attend any public gatherings (e.g., political or religious gatherings) (38.82%) and places where many people used to gather (42.21%). Over 70% (74.59%) of the participants also claimed to abstain from meeting with anyone who has recently come from abroad. Nearly 60% (58.49%) of the women reported that they were trying to follow the guidelines of WHO regarding COVID-19. Around 40% of the participants (40.07%) women were using either a facemask or a KN95 mask/respirator (**Figure 1**).

Sociodemographic Characteristics and COVID-19 Related Knowledge

We observed a significantly different mean knowledge scores between different age groups ($p < 0.01$) (**Table 4**). Compared to the younger age groups, participants aged 51–60 years and >60 years had a significantly lower mean knowledge score (9.70 ± 1.6 and 9.95 ± 1.99, respectively). Also, women from urban areas had a significantly higher mean knowledge score (10.87 ± 1.65) compared to residents from rural areas (10.01 ± 1.79) ($p < 0.01$). The knowledge mean scores did not correlate significantly to the education-levels of the women ($p = 0.001$). Also, women who did not participate in active earning (unemployed/retired and housewives) had a significantly lower knowledge level ($p = 0.0018$).

DISCUSSION

With one of the world's densest populations, Bangladesh is an LMIC in Southeast Asia. Like most other LICs and LMICs, it has limited health facilities, and its citizens' health literacy level is not satisfactory (24, 25). As a result, it supposedly faces a significant challenge in implementing any health measures. Besides, many senior citizens and mid-aged people in the country have non-communicable disorders, including chronic obstructive pulmonary disease (11.9%), cardiac disorders (4.5%),

TABLE 3 | Perceptions of the women about COVID-19.

Perceptions and attitudes about COVID-19	Response (n = 1,813)		
	Yes (n, %)	No (n, %)	Maybe (n, %)
I fear COVID-19	1,510, 83.29	179, 9.87	124, 6.84
I am scared because my family have older (>60 yrs) adults (including me)	953, 52.56	261, 14.4	599, 33.0
COVID-19 is like the common-flus	1,316, 72.58	348, 19.19	149, 8.22
COVID-19 is a dangerous public health threat	1,368, 75.46	249, 13.73	196, 10.81
I am satisfied with Bangladesh's efforts to tackle the pandemic	849, 46.83	760, 41.92	204, 11.25
COVID-19 arrived in Bangladesh by people coming from abroad	1,227, 67.68	311, 17.15	275, 15.17
COVID-19 is a religious curse	459, 25.32	1090, 60.12	264, 14.56
Aggressive screening would help the management of COVID-19	1,556, 85.82	158, 8.71	99, 5.46
Bangladesh have enough facilities for screening COVID-19	683, 37.67	743, 40.98	387, 21.35
People around you are aware of the current situation	726, 40.04	928, 51.19	159, 8.77
Bangladesh have enough ventilation facilities to help critical patients	629, 34.69	853, 47.05	331, 18.26
Timely measures by the Government could help reduce the spread of COVID-19 in Bangladesh	771, 42.53	713, 39.33	329, 18.15
The government should subsidize for treatment of COVID-19	1,585, 87.42	129, 7.16	99, 5.46
Bangladeshi doctors and nurses are trained to treat COVID-19 patients	631, 34.80	826, 45.56	356, 19.64
The mosques and religious congregations should remain discontinued	845, 46.61	742, 40.93	226, 12.47
Bangladesh is economically able to tackle COVID-19 challenge	507, 27.96	1193, 65.80	113, 6.23
COVID-19 pandemic may cause a food crisis in the country	852, 46.99	813, 44.84	148, 8.16
Hand sanitizers, hand soaps, and masks should be available freely	1,127, 62.16	556, 30.67	130, 7.17
Bangladeshi doctors have enough personal protective equipment	919, 50.69	771, 42.53	123, 6.78
The pandemic will severely hamper the education system	1,316, 72.59	354, 19.58	142, 7.83
COVID-19 will cause devastating fatality in Bangladesh	841, 46.39	631, 34.80	341, 18.81
Media coverage about this disease is exaggerated	848, 46.77	649, 35.8	316, 17.43
The government is transparent on COVID-19 information in Bangladesh	609, 33.59	978, 53.94	226, 12.47
Bangladesh is dependent on foreign grants for controlling COVID-19	763, 42.08	825, 45.5	225, 12.41
Bangladesh will collapse due to COVID-19 pandemic	786, 43.35	753, 41.53	274, 15.11

diabetes (9.7%), and asthma (5.2%) (26–29). These people with multiple comorbidities are also especially vulnerable to emerging infectious diseases, e.g., the COVID-19.

As the COVID-19 outbreak quickly surges around the globe, every country is taking extraordinary measures to control its spread. Since there is yet a vaccination or effective treatment strategy against the disease, the control measures basically include non-therapeutic preventive strategies. Effective implementation of these measures requires wholehearted efforts by the government bodies, together with personal understanding and practices, and it depends on the KAP of the general public about the disease. Usually, in the LICs and LMICs, general people, especially the marginalized communities like the females, are less aware of health-related issues. The health-related marginalization of women is due to the availability of few recourses to access health service and literacy. As a result, they show poor perception and attitude toward health issues. While combating a global health crisis like the COVID-19 pandemic, the poor KAP of women may hamper the implementation of control and mitigation strategies. They remain at the core of the fight against the COVID-19 pandemic (30). It is also reported that women, especially the women workers, are among the hardest-hit groups by COVID-19 in Bangladesh (31). Here we present the results of a survey about the KAP of the Bangladeshi women toward the COVID-19 disease. It represents the first study to evaluate the KAP-level among women in LICs and LMICs, including Bangladesh.

The educated women, having an equivalent or higher education than higher-secondary level (51.2%), dominated the study population (**Table 1**). Also, a significant proportion of the women were students, preferably of college or university levels (**Table 1**). From the perspective of religious background, ethnicity, the structure of the studied population was comparable with the general female population of Bangladesh (**Table 1**) (32). Interestingly, the prevalence of comorbid conditions was relatively high compared to the country's overall frequency (26–29). Nearly 40% of the women (40.67%), among who were invited to participate, declined to participate (**Supplementary Table 1**). The reason for declining to participate included no time for participating (60.67%), lack of interest (29.13%), and fear of forgery (11.56%). The final sample included data from 1,869 women, representing an ideal survey sample size (at a 99% confidence interval, the limit of precision of 1%) (**Supplementary Table 1**). Overall, though educated women dominated the sample population, it was diverse and corresponded to the general trends of Bangladesh's population. However, as we distributed the survey using convenience and snowball methods, it may have influenced the general

FIGURE 1 | Womens preparedness related to COVID-19.

TABLE 4 | Factors affecting the knowledge score of women.

Predictor	Knowledge score (mean, SD)	*p*-value
Age (years)		
18–30	10.89 ± 1.75	< 0.001
31–40	10.8 ± 1.64	
41–50	10.82 ± 1.76	
51–60	9.7 ± 1.6	
>60	9.95 ± 1.99	
Education		
High school or below	10.61 ± 1.71	0.001
College/university or above	10.93 ± 1.74	
Marital status		
Single	10.77 ± 1.7	
Married	10.62 ± 1.78	
Ever married	10.59 ± 1.63	
Area of residence		
Urban	10.87 ± 1.65	<0.001
Rural	10.01 ± 1.79	
Slum	9.68 ± 1.7	
Occupation		
Student	11.01 ± 1.8	0.1249
Involved in active earning	10.86 ± 1.5	
Unemployed/retired and housewives	10.56 ± 1.82	0.0018

**SD, standard deviation.*

characteristics of the sample population. Given the overall situation due to COVID-19, we were unable to conduct a more rigorous survey, and this represents a major limitation of this study.

In general, women who participated in our study had good general knowledge about the disease, its mode of transmission, and prevention (**Table 2**). The primary sources of knowledge on COVID-19 among women included the internet (64.7%) and TV (50.69%) (**Supplementary Table 2**). A possible reason for the internet being the most favored source of knowledge could be the inclusion of more young women, e.g., students who used to surf the internet more than the mid-aged and older women. These channels of knowledge, e.g., the internet and TV, provide an uncomplicated and accessible way to receive information related to COVID-19; these can also provide misinformation, fabricated data, and rumors (33, 34). Henceforth, caution about the use of these channels must be in place (34–36). The overall response rate was nearing 80% (78.51 ± 5.06)%, and the average knowledge score was 10.68/16 (±1.72), denoting a satisfactory level of knowledge on COVID-19's causal agent, mode of transmission, symptoms, vulnerability group, relevant rhetorics, and treatment. Women's COVID-19 related knowledge level observed in this study exceeded the knowledge levels of both general (includes both men and women) and female sub-groups reported in two other recent studies held in Bangladesh (37, 38). Given that both of these studies report the outcomes based on sample populations dominated by young and educated individuals, it indicates that women in Bangladesh are more knowledgeable about COVID-19 than men.

We observed a comparatively lower COVID-19 related knowledge level among older women (**Supplementary Figure 1**). These results are similar to the outcomes reported for the general population of Bangladesh, Egypt, and China, where participants with high socioeconomic status were more knowledgeable than participants coming with lower status (10, 35, 37, 38). It indicates that extensive efforts are required to deliver messages to the older group of women, who may have difficulties accessing the most favorable sources of COVID-19 related knowledge.

When analyzing women's perception and attitude toward COVID-19, we found that over four of five women were scared of the infection, and the primary reason (52.56%) for their fear was due to the presence of older individuals in their family (**Table 3**). A similar response was reported from other studies held on general populations as well (10, 35). Unlike previous reports in other LMICs, around half of the participants assumed that the media was exaggerating the risk (**Table 3**) (35, 39).

The majority of the women opined that the Bangladeshi administration and health professionals had inadequate preparations to combat the outbreak. Many of the women were also concerned about the awareness of the people around them. It is apparent in the participants' opinion that Bangladeshi authorities failed to manage the COVID-19 outbreak better, which could lead to a devastating fatality in the country. Bangladesh's poor preparation for tackling the COVID-19 situation was also reported in a recent interagency memo of the United Nations, led by the WHO (40). Although most of the participants were concerned about a possible economic crisis, they opined that the Government should subsidize the treatment of COVID-19. Concerns over the Government's transparency regarding COVID-19 related information, suppressing unrestricted dissents, the possibility of food unavailability in the future, and education of children (and themselves) were also in place. Many were scared that the country would collapse in tackling the pandemic. However, the authorities of Bangladesh have repeatedly claimed that their prompt measures kept the outbreak of COVID-19 well under control in the country, and the Government is fully transparent about its policies to mitigate the situation (41–44).

One-fourth of our participants assumed the COVID-19 as a religious curse. In response to the extraordinary situations due to COVID-19, many countries curbed the religious congregations, and some Muslim-dominant countries even temporarily amended the adhan (call for prayer) to urge followers to pray in their homes than to come to the mosques (45). Bangladesh also suspended regular and special prayers in the mosques and applied restrictions to all other religious groups (46). Interestingly, a mixed opinion was observed regarding public religious congregations among the women in Bangladesh (**Table 3**). A possible reason for this mixed opinion could be the strong influence of religious beliefs among the population of semi-conservative societal structure in Bangladesh- where people used to face dilemmas in amending religious practices even when there are logical grounds (47).

Participants in our study showed a sort of good personal preparedness in response to COVID-19 (**Figure 1**). They considered the value of frequent handwashing for an extended time, avoiding to touch eyes, nose, and mouth, and limited

personal contact. However, the proportion of women was low compared to the general population (internet users), as described by previous studies held in Bangladesh, where ∼95% of participants were practicing social distancing (37). In our study, ∼75% of the participants avoided social gatherings, meeting with people coming from abroad, and canceled preplanned visits during the summer vacations. Nearly half of the women stocked more foods, daily goods, and regularly needed medicines for a longer time. Also, many of them bought hand soaps, sanitizers, and disinfectants. Around 40% of women were using a facemask or KN95 mask or a respirator. The percentage of participants practicing the use of masks was comparable with the reports from Egypt, but not with China, where almost all participants reported putting face masks when they go out (10, 35). It is apparent that the use of masks was substantially low among women in Bangladesh as compared to the internet users (men and women) in the country, >91% of whom used to put a mask when going outside the home (37). Regarding the use of masks, the Center for Disease Control and Prevention (CDC) emphasized putting face coverings in areas where the transmission is at the community level, while WHO recommends using it for those who have respiratory symptoms or are caring for another person with symptoms (48, 49). It is crucial to develop local guidelines for mask-use in Bangladesh by the health experts and Government bodies. Unnecessary use of masks needs to be prevented while confirming that the health risk is not hampered during this unprecedented time. Similar guidelines are also needed for the use of hand soaps, sanitizers, and disinfectants, as overuse of these chemical substances may harm the dermatological aspects of men.

Bangladesh's efforts in controlling the COVID-19 pandemic had numerous limitations (19). As the country had, like almost all other countries, no experience in facing a coronavirus outbreak, weakness in its preparation is relatable. Besides, as a small country with limited resources but a huge population, its efforts faced enormous challenges (19). The KAP of its citizens, including marginalized communities like women, was satisfactory at individual levels, which is essential to control the infection (9, 10, 50). However, the country's efforts seem to fail in controlling the spread (37, 38). As of early October, 6 months after the detection of the first COVID-19 case in Bangladesh, the number of active and new cases is increasing by leaps and bounds (51). A similar situation is observed in other LMICs in Bangladesh's neighborhood and beyond (39, 52–54). Although the Governments of the countries have taken significant measures to limit the spread of the disease, more effort is needed to support the most affected groups.

This study indicates that the healthcare authorities, media, and Governments in LICs and LMICs should be more careful and transparent in spreading knowledge to its people, especially to those who are marginalized, e.g., women and older individuals with low socioeconomic status, when fighting a pandemic like the COVID-19.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Review Board (ERB), Department of Biochemistry and Molecular Biology, School of Life Sciences, Shahjalal University of Science and Technology, Sylhet - 3114, Bangladesh (Reference ID: ERB/02/BMB/2020). The patients/participants provided their informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MH conceived the study. MH, SA, YA, and NA designed the study. MU, YA, BS, RR, RI, and NA conducted the surveys. SA analyzed and interpreted the data. AN and NH helped the interpretation of the data. SA and MU wrote the draft manuscript. SA and NA carried out the revisions. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.571689/full#supplementary-material

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Prediction of COVID-19 Patients at High Risk of Progression to Severe Disease

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In order to develop a novel scoring model for the prediction of coronavirus disease-19 (COVID-19) patients at high risk of severe disease, we retrospectively studied 419 patients from five hospitals in Shanghai, Hubei, and Jiangsu Provinces from January 22 to March 30, 2020. Multivariate Cox regression and orthogonal projections to latent structures discriminant analysis (OPLS-DA) were both used to identify high-risk factors for disease severity in COVID-19 patients. The prediction model was developed based on four high-risk factors. Multivariate analysis showed that comorbidity [hazard ratio (HR) 3.17, 95% confidence interval (CI) 1.96–5.11], albumin (ALB) level (HR 3.67, 95% CI 1.91–7.02), C-reactive protein (CRP) level (HR 3.16, 95% CI 1.68–5.96), and age ≥60 years (HR 2.31, 95% CI 1.43–3.73) were independent risk factors for disease severity in COVID-19 patients. OPLS-DA identified that the top five influencing parameters for COVID-19 severity were CRP, ALB, age ≥60 years, comorbidity, and lactate dehydrogenase (LDH) level. When incorporating the above four factors, the nomogram had a good concordance index of 0.86 (95% CI 0.83–0.89) and had an optimal agreement between the predictive nomogram and the actual observation with a slope of 0.95 (R^2 = 0.89) in the 7-day prediction and 0.96 (R^2 = 0.92) in the 14-day prediction after 1,000 bootstrap sampling. The area under the receiver operating characteristic curve of the COVID-19-American Association for Clinical Chemistry (AACC) model was 0.85 (95% CI 0.81–0.90). According to the probability of severity, the model divided the patients into three groups: low risk, intermediate risk, and high risk. The COVID-19-AACC model is an effective method for clinicians to screen patients at high risk of severe disease.

Keywords: COVID-19, severity, risk factors, scoring model, nomogram

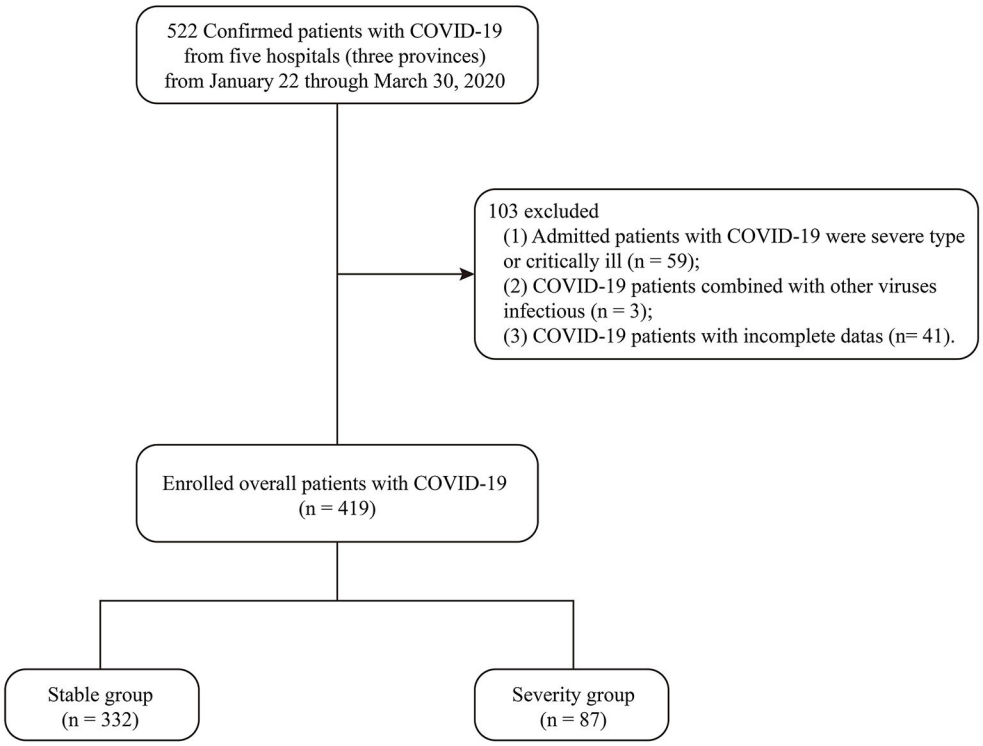

FIGURE 1 | Flowchart of COVID-19 patient enrollment including the stable group and the severe group.

HIGHLIGHTS

- The severity and mortality of COVID-19 patients urgently need to be resolved.
- Comorbidity, ALB, CRP, and age ≥60 years are independent risk factors for severe COVID-19.
- The COVID-19-AACC model is effective for screening patients at risk of severe disease.

INTRODUCTION

In December 2019, an increasing number of patients with pneumonia of unknown cause were found in Wuhan, China (1, 2). A novel coronavirus was identified by gene detection and virus isolation. On January 12, 2020, the World Health Organization (WHO) named the virus "2019-nCoV" (3), and on February 11, 2020, the WHO renamed it severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the disease it caused coronavirus disease 2019 (COVID-19) (4). The epidemic soon spread all over China and 212 other countries and areas around the world, resulting in more than 4.72 million people infected and over 300,000 deaths up to May 17, 2020. It has been shown that COVID-19 is more contagious than SARS-CoV seen in 2003, and that medical staff were also infected during the epidemic (5, 6).

Wu et al. (7) first reported that timely antiviral treatment may slow the progression of COVID-19 caused by SARS-CoV-2 and improve the prognosis. Nahama et al. (8) found that the use of resiniferatoxin could improve patient outcomes in those with advanced COVID-19. Omarjee et al. (9) demonstrated that targeting T-cell senescence and cytokine storm with rapamycin may prevent progression in COVID-19. However, up to the date of submission of this report, there are still no specific drugs for COVID-19 patients worldwide, and the severity and mortality of COVID-19 patients are urgent problems that still need to be resolved (10, 11). Hence, it is extremely important to understand the critical factors associated with the severity of COVID-19 and provide convenient and efficient diagnostic methods. Xiao et al. (12) developed an artificial intelligence-assisted tool using computed tomography (CT) imaging to predict disease severity and further estimate the risk of developing severe disease in patients suffering from COVID-19.

In the present study, we aimed to develop a novel scoring model for predicting patients at high risk of severe COVID-19, which would facilitate clinicians to manage COVID-19 patients.

Abbreviations: COVID-19, coronavirus disease-19; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SARS, severe acute respiratory syndrome; MERS, Middle East respiratory syndrome; WHO, World Health Organization; HR, hazard ratio; CI, confidence interval; OPLS-DA, orthogonal projections to latent structures discriminant analysis; CRP, C-reactive protein; ALB, albumin; LDH, lactate dehydrogenase.

PATIENTS AND METHODS

Patients

In this study, 419 consecutive patients with confirmed COVID-19 were enrolled from the Shanghai Public Health Clinical Center

TABLE 1 | Characteristics of COVID-19 patients in this study.

	Overall (n = 419)	Stable group (n = 332)	Severity group (n = 87)	P value
Age, years	47.1 ± 16.0	44.6 ± 15.2	56.8 ± 15.4	0.000
Gender (n, %)				0.053
Male	207 (49.40)	156 (46.99)	51 (58.62)	
Female	212 (50.60)	176 (53.01)	36 (41.38)	
Comorbidity (n, %)				0.000
Without	280 (66.83)	249 (75.00)	31 (35.63)	
With	139 (33.17)	83 (25.00)	56 (64.37)	
Lymphocyte, $\times10^9$/L	1.2 (0.8–1.6)	1.2 (0.9–1.7)	0.8 (0.6–1.1)	0.000
D-dimer, mg/L	0.28 (0.19–0.54)	0.24 (0.17–0.45)	0.51 (0.30–0.91)	0.000
ALT, U/L	24 (15–38)	23 (14–38)	27 (18–39)	0.072
ALB	40 (37–43)	41 (38–44)	37 (33–39)	0.000
TBIL, μmol/L	10.4 (7.3–15.4)	9.8 (7.0–14.5)	13.4 (8.3–18.8)	0.002
LDH, U/L	213 (172–281)	204 (166–256)	281 (212–346)	0.000
CRP	8.0 (2.6–26.3)	6.2 (1.8–18.4)	39.0 (15.0–85.2)	0.000
PCT, μg/L	0.05 (0.02–0.05)	0.04 (0.02–0.05)	0.05 (0.04–0.09)	0.000
D-dimer, mg/L				0.000
≤0.55	318 (75.89)	269 (81.02)	49 (56.32)	
>0.55	101 (24.11)	63 (18.98)	38 (43.68)	
Lymphocyte, $\times10^9$/L (n, %)				0.000
>1.0	259 (61.81)	230 (69.28)	29 (33.33)	
≤1.0	160 (38.19)	102 (30.72)	58 (66.67)	
Age, years (n, %)				0.000
≤60	331 (79.00)	287 (86.45)	44 (50.57)	
>60	88 (21.00)	45 (13.55)	43 (49.43)	
LDH, U/L (n, %)				0.000
≤250	272 (64.92)	241 (72.59)	31 (35.63)	
250–500	138 (32.94)	86 (25.90)	52 (59.77)	
>500	9 (2.15)	5 (1.51)	4 (4.60)	
CRP, mg/L (n, %)				0.000
<10	229 (54.65)	213 (64.16)	16 (18.39)	
≥10	190 (45.35)	119 (35.84)	71 (85.06)	
ALB, g/L (n, %)				0.000
≥40	215 (51.31)	202 (60.84)	13 (14.94)	
<40	204 (48.69)	130 (39.16)	74 (85.06)	

(208 cases), the Wuhan Central Hospital, Tongji Medical College, Huazhong University of Science and Technology (130 cases), the third People's Hospital of Yancheng City (15 cases), the Fifth People's Hospital of Wuxi (46 cases), and the Second People's Hospital of Yancheng City (20 cases) from January 22 to March 30, 2020, and the follow-up ended on April 30, 2020. All patients admitted with severe COVID-19 to these five hospitals were excluded. This retrospective study was performed in accordance with the Helsinki Declaration and was approved by the Ethics Committee of the Shanghai Public Health Clinical Center (YJ-2020-S089-02).

Definition and Clinical Classification of Cases

All the enrolled COVID-19 patients were diagnosed based on the WHO criteria (13) and the National Health Commission of China criteria. We defined the COVID-19 patients according to epidemiological history consistent with any two clinical manifestations and pathogenic evidence. SARS-CoV-2 RNA was tested with samples from the nose, pharynx, and anus swabs, respectively, by real time-polymerase chain reaction (PCR). We defined the clinical classification and epidemiological history of COVID-19 patients as described previously (14): the first generation (Generation I): patients with a history of exposure to the south China seafood market in Wuhan, China; the second generation (Generation II): patients with Wuhan tourism experience; the third generation (Generation III): imported cases; and the fourth generation (Generation IV): patients infected by Generation III patients. The progression to severe COVID-19 during the observation period was diagnosed based on heart and pulmonary function recovery and lung CT findings. We divided the patients into the severe group and the stable group according to whether the patients had progression to severe COVID-19. In this study, all COVID-19 patients who also had other virus infections were excluded.

Data Collection

We retrospectively collected data from the patients' medical records and attending doctors, including clinical baseline data, laboratory parameters, length of stay, and so on. At the time of admission, all patients underwent laboratory examinations. All data were collected on the first day after admission. Clinical outcomes were followed up till April 30, 2020.

Statistical Analysis

Statistical analyses were performed by SPSS (version 25; IBM SPSS Statistics, United States) and R software, version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). Continuous variables with normal distribution were expressed as mean ± standard deviation and were compared using the independent sample t-test. Data with non-normal distribution were expressed as median (IQR) and were compared using the non-parametric test. The classified variables were tested using the chi-square test. A value of $P < 0.05$ was considered statistically significant. The significance of each variable was assessed using the univariate and multivariate Cox proportional hazards model to investigate the independent high-risk factors for disease severity with their hazard ratio (HR) and 95% confidence interval (CI). The performance of the nomogram was evaluated by calibration with 1,000 bootstrap samples to decrease the overfit bias. The receiver operating characteristic (ROC) package in R software was used to compare the time-dependent area under the ROC curve (td-AUC). Orthogonal projections to latent structures discriminant analysis (OPLS-DA) was performed with SIMCA version 14.1.0.2047.

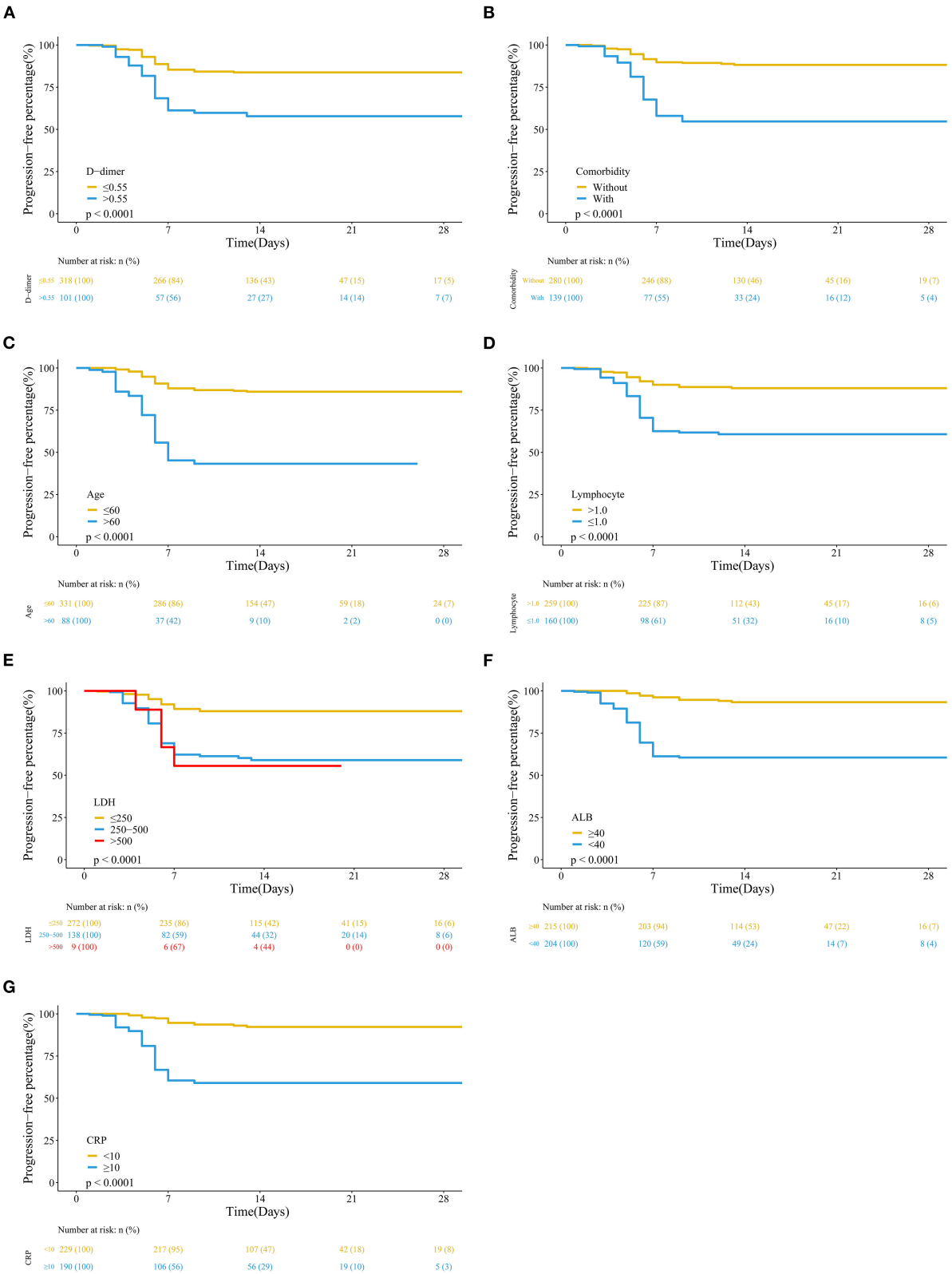

FIGURE 2 | Kaplan–Meier analysis of high-risk factors for severe COVID-19. We defined the time from admission after infection and days to severe disease development or discharge. **(A)** D-dimer; **(B)** comorbidity; **(C)** age; **(D)** lymphocyte count; **(E)** lactate dehydrogenase (LDH); **(F)** albumin (ALB); **(G)** C-reactive protein (CRP).

TABLE 2 | The univariate and multivariate logistic regression analysis independent high-risk factors for severity of COVID-19 patients.

	Un COX analysis		Mul COX analysis	
	HR (95% CI)	***P* value**	**HR (95% CI)**	***P* value**
D-dimer (mg/L)				
≤0.55	1	–	1	–
>0.55	2.961 (1.936–4.529)	0.000	1.070 (0.672–1.702)	0.776
Comorbidity				
Without	1	–	1	–
With	4.617 (2.971–7.173)	0.000	3.166 (1.960–5.114)	0.000
Age (years)				
≤60	1	–	1	–
>60	5.557 (3.633–8.499)	0.000	2.307 (1.427–3.728)	0.001
Lymphocyte (×10/L)				
>1.0	1	–	1	–
≤1.0	3.814 (2.440–5.961)	0.000	1.234 (0.741–2.054)	0.419
LDH (U/L)				
≤250	1	–	1	–
250–500	3.944 (2.526–6.158)	0.000	1.531 (0.918–2.553)	0.103
>500	4.215 (1.487–11.943)	0.007	2.572 (0.869–7.612)	0.088
ALB (g/L)				
≥40	1	–	1	–
<40	7.899 (4.374–14.267)	0.000	3.663 (1.912–7.018)	0.000
CRP (mg/L)				
<10	1	–	1	–
≥10	7.022 (4.076–12.098)	0.000	3.161 (1.677–5.961)	0.000

RESULTS

Clinical Characteristics of COVID-19 Patients in the Severe Group and the Stable Group

A total of 419 eligible COVID-19 patients included in the stable group and the severe group were recruited from five hospitals in Shanghai, Jiangsu, and Hubei Provinces, China. The flowchart of patient enrollment is shown in **Figure 1**. The clinical characteristics of these patients are summarized in **Table 1**. In these 419 patients, the average age was 47.1 ± 16.0 years, 207 (49.4%) were male, 88 (21.0%) were older than 60 years, 139 (33.2%) had at least one underlying comorbidity, the average hospitalization time was 16.5 ± 7.9 days, and 87 (20.8%) patients became severe and critically ill during the observation period. Three hundred fourteen (74.9%) patients were Generations III and IV.

When the clinical characteristics in the stable group and the severe group were compared, the results showed that age, comorbidity, lymphocyte count, albumin (ALB), D-dimer, C-reactive protein (CRP), and lactate dehydrogenase (LDH) levels were significantly different between the two groups (**Table 1** and **Figure 2**).

High-Risk Factors for Severe COVID-19

We performed Cox regression analysis, which demonstrated that comorbidity (HR 3.17, 95% CI 1.96–5.11), ALB level (HR 3.67, 95% CI 1.91–7.02), CRP level (HR 3.16, 95% CI 1.68–5.96), and age ≥60 years (HR 2.31, 95% CI 1.43–3.73) were independent risk factors for severe COVID-19 in these patients (**Table 2**).

We also used OPLS-DA to evaluate the influence of parameters on the severity of COVID-19. The severe group was unambiguously distinguished from the stable group (**Figures 3A,B**). The top five parameters that influenced the severity of COVID-19 were CRP, ALB, age ≥60 years, comorbidity, and LDH (**Figures 3C,D**).

Hence, comorbidity, ALB, CRP, and age ≥60 years were identified as the most influential risk factors for the severity of COVID-19 in these patients.

Development and Validation of a Predictive Nomogram for the Probability of Severe COVID-19

Based on the above independent risk factors associated with the severity of COVID-19, we developed a predictive nomogram and validated it using the bootstrap method (**Figure 4A**). Calibration tests were used to evaluate the predictive accuracy for progression of COVID-19 using the nomogram. The C-index for predicting the severity of COVID-19 with the nomogram was 0.86 (0.83–0.89), which indicated good accuracy. The calibration curve showed optimal agreement between the predictive nomogram and the actual observation with a slope of 0.95 ($R^2 = 0.89$) in the 7-day prediction and 0.96 ($R^2 = 0.92$) in the 14-day prediction after 1,000 bootstrap sampling (**Figures 4B,C**).

Development and Assessment of the Novel Scoring Model for COVID-19 Severity

Based on the above nomogram, we further developed a novel scoring model, which may facilitate the clinical assessment of COVID-19 severity. We named the model COVID-19-American Association for Clinical Chemistry (AACC) (age ≥60 years, ALB, comorbidity, and CRP), and the score ranged from 0 to 5 points (**Figure 5**). CRP (<10 mg/L) and ALB (<40 g/L) were chosen as the cut-off values, respectively, to score the ALB and CRP.

The AUROC of the COVID-19-AACC scoring model for predicting the probability of severe COVID-19 in these patients was 0.85 (95% CI 0.81–0.90).

With the cut-off value of 0 point, the positive predictive value and positive likelihood ratio of the scoring model were 27.2 (95% CI 22.3–32.5) and 1.42 (95% CI 1.3–1.5). The negative predictive value and negative likelihood ratio were 97.3 (95% CI 92.2–99.4) and 0.11 (95% CI 0.03–0.3), with a sensitivity of 96.6 (95% CI 90.3–99.3) and a specificity of 32.2 (95% CI 27.2–37.5).

Using a cut-off value of 4 points, the positive predictive value and positive likelihood ratio of the scoring model were 80.6 (95% CI 62.5–92.5) and 15.90 (95% CI 6.7–37.5). The negative predictive value and negative likelihood ratio were 84.0 (95% CI 80.0–87.5) and 0.73 (95% CI 0.6–0.8), with a sensitivity of 28.7 (95% CI 19.5–39.4) and a specificity of 98.2 (95% CI 96.1–99.3) (**Table 3**).

The following three risk groups according to their probability of severe COVID-19 were developed: low risk (Class A: 0–1

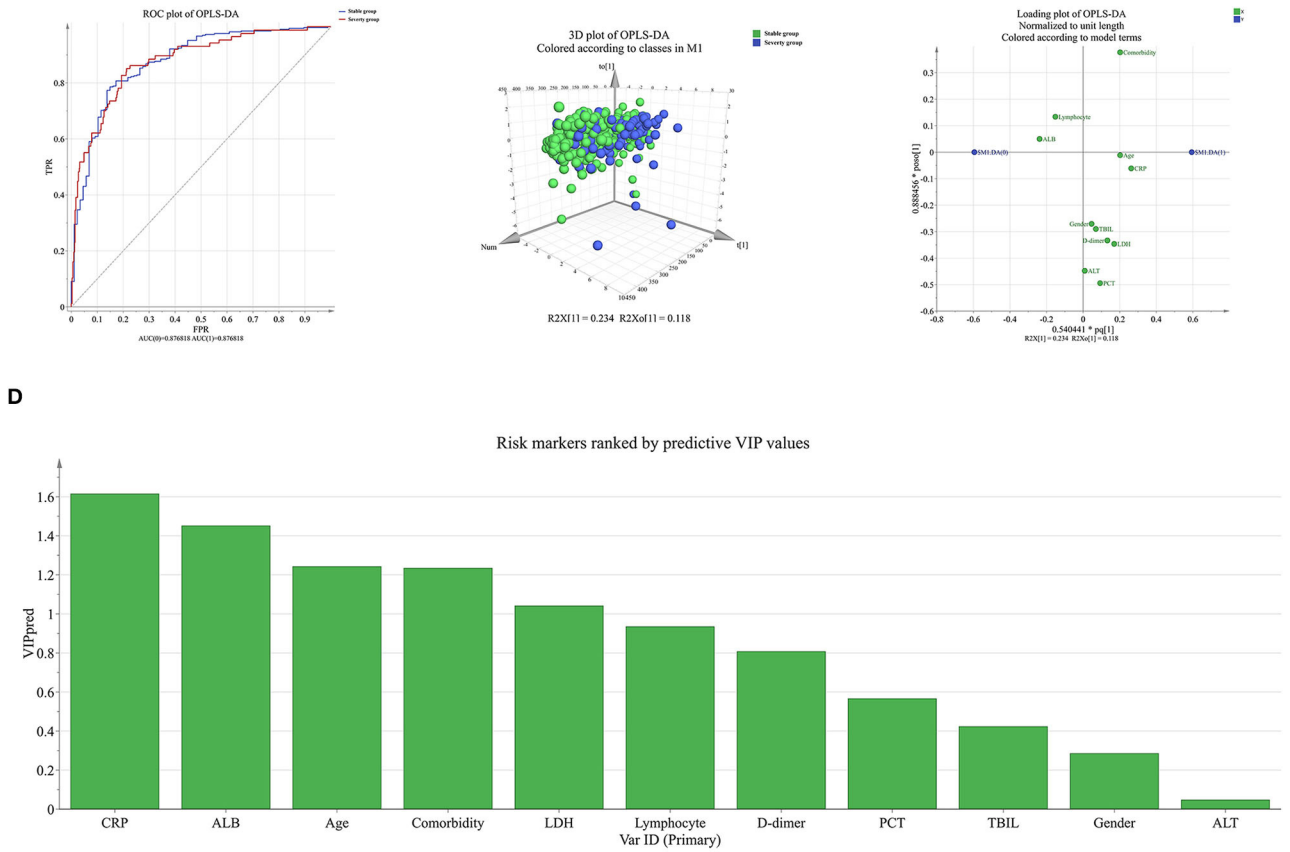

FIGURE 3 | OPLS-DA to evaluate the influence of parameters on the severity of COVID-19. **(A)** ROC of OPLS-DA. **(B)** In the three-dimensional scatter plot of all samples in the OPLS-DA model, the predictive component was used in the stable group and the severe group. **(C)** Loading plot showing the relationship of each parameter to the predictive component (x) and the first orthogonal component (y); parameters that deviated from zero on the x-axis were considered potentially predictive. **(D)** The higher predictive VIP (VIP pred) value.

point) group, with a risk of severe disease of <5%; intermediate risk (Class B: 2–3 points) group, with a risk of 10–30%; and high-risk (Class C: 4–5 points) group, with a risk of more than 50% (**Figure 5**).

DISCUSSION

Coronavirus is distributed throughout the world and has many subtypes. SARS in 2003 and Middle East respiratory syndrome (MERS) in 2013 were caused by coronavirus infection (15, 16). At present, the rapid spread of SARS-CoV-2 worldwide has resulted in a heavy burden to society. To date, the global control of COVID-19 was still not optimistic (17, 18). Although the overall mortality of COVID-19 is not high internationally, the mortality of patients with severe and critical disease is relatively high (19). According to the WHO, the death rate in critically ill patients was over 50% (20). Obviously, it is extremely important to manage these serious cases in a timely and appropriate manner. In fact, in the majority of regions and countries, rapid diagnosis of suspected cases has been possible (21). Thus, how to control the progression from mild to severe disease in these patients is the key to the treatment of COVID-19 by clinicians.

In view of this issue, several studies (22, 23) have shown the factors that may affect the severity of COVID-19. Ji et al. (24) showed that comorbidity, older age, lower lymphocyte count, and higher LDH level were associated with the progression of COVID-19. Yan et al. (25) described the clinical and laboratory characteristics of 193 patients with severe COVID-19. Of these patients, 48 with severe COVID-19 had diabetes. Diabetes was associated with an increased risk of death. Another study (26) showed that severe CO_2 retention and acidosis prior to extracorporeal membrane oxygenation were confirmed to be risk factors for severe COVID-19 and poor prognosis.

In this study, we retrospectively studied 419 patients from five hospitals in Shanghai, Hubei, and Jiangsu Provinces and determined several risk factors for the severity of COVID-19 in these patients, including age ≥60 years, ALB level, comorbidity, and CRP level. Of the 419 enrolled cases, both median age and the proportion of patients over 60 years in the severe COVID-19 group were significantly higher than those in the stable

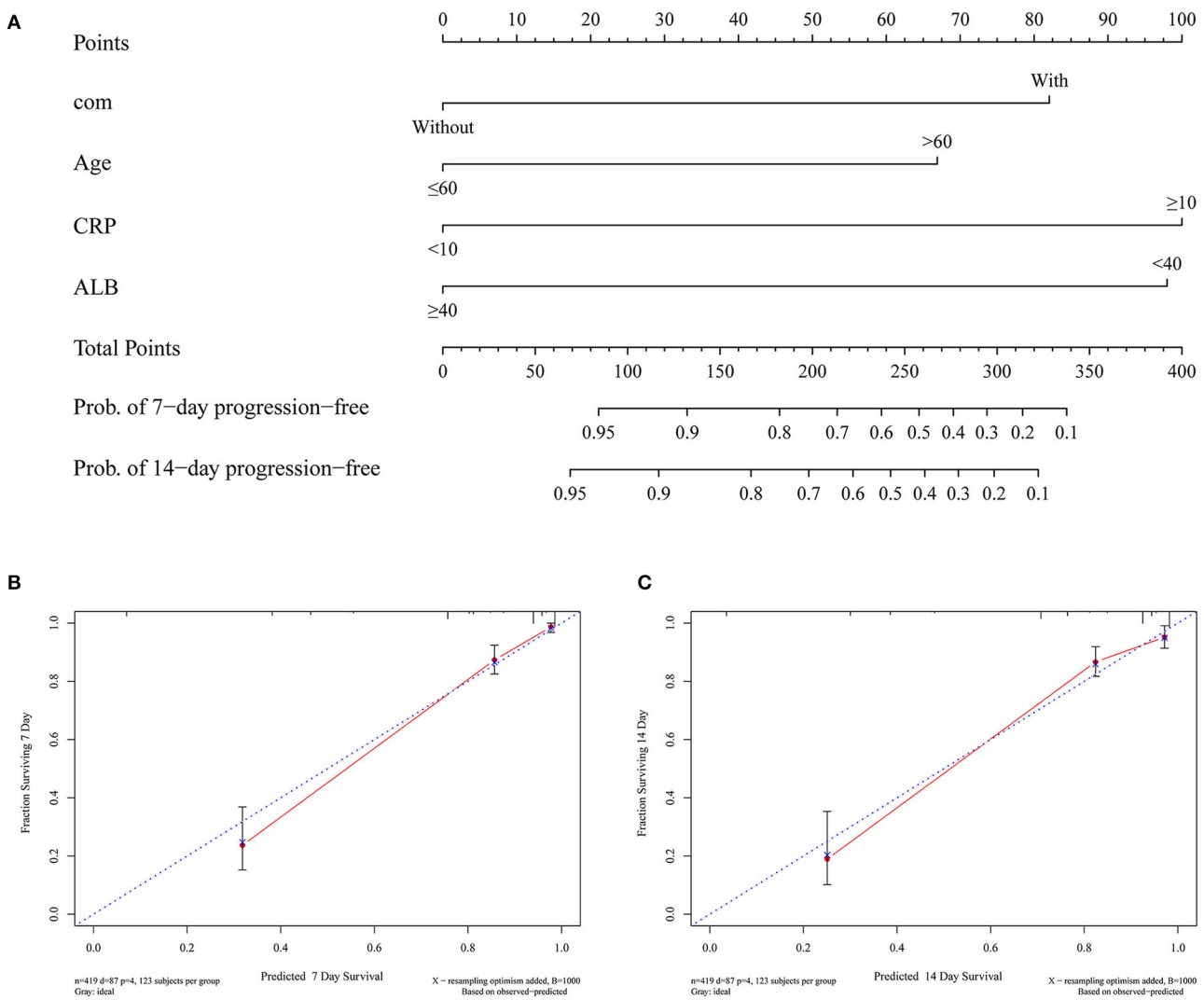

FIGURE 4 | Development and validation of a predictive nomogram for the probability of severe COVID-19. **(A)** A predictive nomogram was developed based on the independent risk factors associated with the severity of COVID-19. **(B)** The 7-day predictive performance of the nomogram with a slope of 0.95 ($R^2 = 0.89$). **(C)** The 7-day predictive performance of the nomogram with a slope of 0.96 ($R^2 = 0.92$).

group (**Table 1**). The above conclusions were consistent with most previous studies, such as those by Wang et al. (27). It is notable that patients with comorbidities, especially diabetes and cardiovascular diseases, were prone to severe COVID-19. Ji et al. (24) showed that comorbidity, older age, lower lymphocyte count, and higher LDH level at presentation were independent high-risk factors for COVID-19 progression. Zhang et al. (28) selected risk factors for severe and even fatal pneumonia and created a predictive scoring system, including age, white blood cell count, neutrophil count, glomerular filtration rate, and myoglobin level as candidates for the scoring system to predict the severity of COVID-19. We also considered the reasons for the decline in physical function and immune function in the elderly, which could increase the probability of severe COVID-19. The study by Cai et al. (29) indicated that CRP, procalcitonin (PCT), and D-dimer may predict the severity of COVID-19. The study by Zhou et al. (30) showed no significant differences in CRP between the non-aggravation group and the aggravation group. In our study, the levels of CRP in the severe group were significantly higher than those in the stable group, and the proportion of patients with CRP levels ≥10 mg/L was also significantly higher than that in the stable group. Mishra et al. (31) recommended serum ALB for the therapy of SARS-CoV-2. Bi et al. (32) showed that ALB was much lower in severe patients, but was not an independent risk factor for disease progression. Our study has confirmed that ALB is a risk factor for the severity of COVID-19. In the present study, we also assessed the critical factors for disease severity using logistic analysis and OPLS-DA, respectively. The results of both analyses showed that comorbidity, ALB, CRP, and age ≥60 years

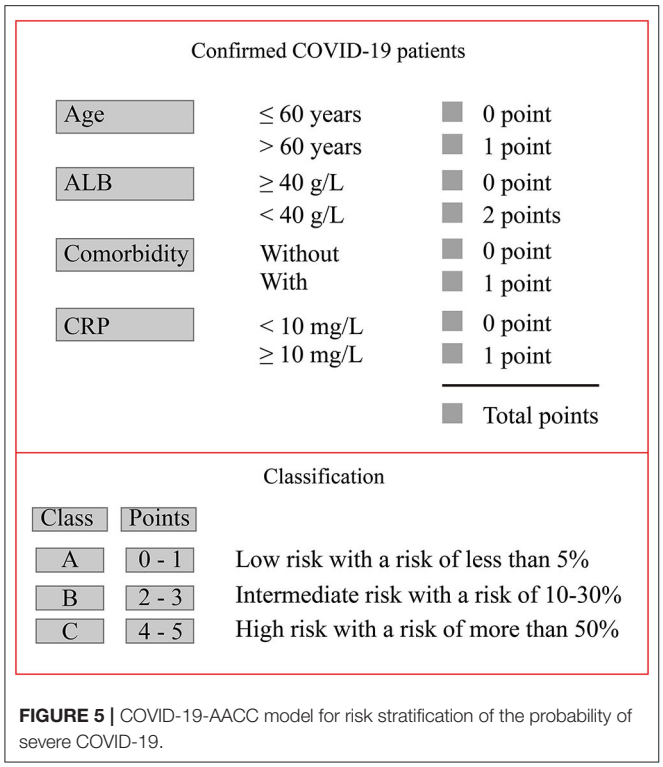

FIGURE 5 | COVID-19-AACC model for risk stratification of the probability of severe COVID-19.

TABLE 3 | The performances of COVID-19-AACC model for risk stratification of probabilities for severity of COVID-19 patients.

Variable	(*n* = 419)
AUROC	0.85 (0.81–0.90)
Cutoff value (95% CI)	0
Sensitivity, %	96.6 (90.3–99.3)
Specificity, %	32.2 (27.2–37.5)
Positive predictive value, %	27.2 (22.3–32.5)
Negative predictive value, %	97.3 (92.2–99.4)
Positive likelihood ratio	1.42 (1.3–1.5)
Negative likelihood ratio	0.11 (0.03–0.3)
Cutoff value (95% CI)	4
Sensitivity, %	28.7 (19.5–39.4)
Specificity, %	98.2 (96.1–99.3)
Positive predictive value, %	80.6 (62.5–92.5)
Negative predictive value, %	84.0 (80.0–87.5)
Positive likelihood ratio	15.90 (6.7–37.5)
Negative likelihood ratio	0.73 (0.6–0.8)

were the most influential risk factors for severe COVID-19 in these patients.

Based on the above risk factors, we developed a predictive nomogram for the probability of severe COVID-19. The nomogram had a good concordance index of 0.86 (95% CI 0.83–0.89) and well-fitted calibration curves in both the 7-day prediction and the 14-day prediction. We then constructed a scoring model (COVID-19-AACC) based on the above nomogram, which also had a good concordance index of 0.85 (95% CI 0.81–0.90). The COVID-19-AACC scoring model was used to identify COVID-19 patients at low risk (Class A), intermediate risk (Class B), and high risk (Class C) of severe disease. Of the 419 patients enrolled, 254 (60.6%) scored 0–1 point and were considered low risk, and 134 (32.0%) scored 2–3 points and were considered intermediate risk, whereas 31 (7.4) scored 4–5 points and were considered high risk. These high-risk patients should be transferred to tertiary centers as early as possible for appropriate treatment.

Of note, there were several limitations in the present study. Firstly, this study is a retrospective, multicenter study, and the possibility of recall bias cannot be completely excluded. The results from a limited sample size do not necessarily represent the overall results of patients in China or even in the world. Secondly, a validation group should be included to further validate the scoring model. Finally, more indicators, including genes and images, should be included to further optimize the model.

In summary, the COVID-19-AACC scoring model will be of significant help to clinicians in evaluating COVID-19 patients in the early stage, especially in non-tertiary hospitals. For high-risk groups, early intervention can effectively reduce the rate of severe disease and mortality.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the present retrospective study was performed in accordance with the Helsinki Declaration and was approved by the Ethics Committee of the Shanghai Public Health Clinical Center (YJ-2020-S089-02). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

ZD, DC, DW, and DZ contributed to the study concept and design, conducted the literature search, and wrote the manuscript. YF, JX, WG, and YY contributed to the data analysis and produced the tables and figures. YS, LZ, and XZ contributed to the collection of patient samples and medical information. JX and SS obtained funding. DL, YZ, MW, and AW contributed to the acquisition and analysis of data. HL and SS contributed to the study concept and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

CASE REPORT
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Case Report: Recurrence of Positive SARS-CoV-2 Results in Patients Recovered From COVID-19

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Background: Coronavirus disease 2019 (COVID-19) is spreading throughout the world. Limited data are available for recurrence of positive severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) results in patients with long duration of COVID-19.

Methods: We reported four cases recovered from COVID-19 with recurrence of positive SARS-CoV-2 results during the long-term follow-up.

Results: The four patients recovered from COVID-19 showed recurrence of positive SARS-CoV-2 results for more than 120 days with no symptoms and normal chest CT scan.

Conclusions: The dynamic surveillance of SARS-CoV-2 by nucleic acid detection and serological assays is important for asymptomatic patients who might be potentially infectious.

Keywords: coronavirus disease 2019, severe acute respiratory syndrome coronavirus 2, IgM, IgG, nucleic acid detection

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INTRODUCTION

With the increasing number of patients recovered from Coronavirus disease 2019 (COVID-19), more attention should be paid to the follow-up of these patients. Here we reported four cases with recurrence of positive SARS-CoV-2 results in patients recovered from COVID-19 for more than 120 days.

CASE PRESENTATION

Case 1

A 36-year-old woman had a fever of 38.3°C with cough and shortness of breath on January 29, 2020. She lived with four family members, all of whom were diagnosed as COVID-19 on January 26. Real-time reverse-transcription polymerase chain reaction (qRT-PCR) assay of nasopharyngeal swab-obtained materials showed a positive result of SARS-CoV-2. The chest computed tomography (CT) scan showed ground-glass opacities in basal segment of the lower lobe of right lung (**Figure 1A**). The patient was admitted to Chongqing Public Health Medical Center. She was treated with lopinavir/ritonavir (200/50 mg, 2 tablets, biw) from January 30 to February 20, hydroxychloroquine (400 mg, biw on the first day, and 200 mg, biw on the following day) from

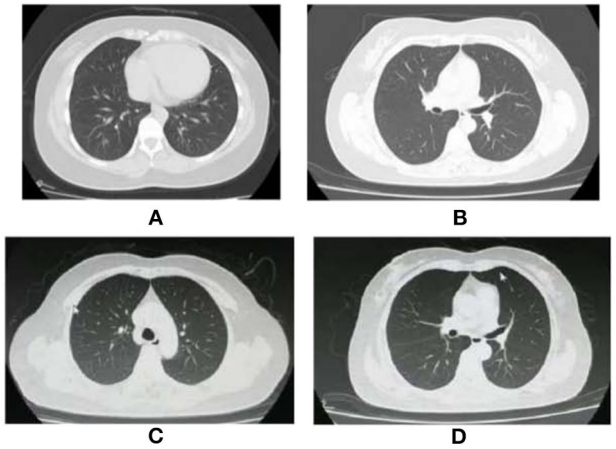

FIGURE 1 | Chest CT images of two cases with COVID-19. **(A)** The Chest CT images of the Case 1 showed ground-glass opacities in basal segment of the lower lobe of the right lung on January 2. **(B)** The Chest CT images of the Case 1 showed almost normal on March 23. **(C)** The Chest CT images of the Case 2 showed almost normal on April 7. **(D)** The Chest CT images of the Case 2 showed almost normal on June 21.

TABLE 1 | The IgG and IgM titers of four patients.

Case 1			Case 2		
Date	**IgG**	**IgM**	**Date**	**IgG**	**IgM**
5–21	12.515	1.573	5–21	22.614	2.682
5–24	10.805	1.512	5–24	20.311	2.451
5-–26	11.922	1.385	5–30	18.992	1.771
5–30	10.873	1.525	6–1	22.814	2.034
6–6	11.081	1.337	6–6	11.918	1.865
6-11	9.921	1.328	6–7	8.781	1.046
6-14	7.237	0.017	6–8	0.313	0.037
Case 3			**Case4**		
Date	**IgG**	**IgM**	**Date**	**IgG**	**IgM**
3–27	9.517	0.122	5–26	6.593	2.168
4–3	5.023	0.097	6–3	5.916	2.635
4–6	5.927	0.092	6–6	5.382	2.323
4–14	4.645	0.066	6–15	3.907	1.367
4–18	5.238	0.107	6–19	4.203	1.238
4–24	4.615	0.113	6–22	3.447	0.231
4–27	4.513	0.089			
5–1	5.367	0.409			
5–9	4.425	0.225			
5–15	3.988	0.279			

The normal range for IgG and IgM detection was 0–1.

February 20 to February 25, atomized inhalation of interferon α-2b (500,000 U, biw) from January 30 to March 1, and thymalfasin (1.6 mg, biw) from March 7 to March 9, supplemented with Chinese Medicinal therapy. The two nasopharyngeal swabs collected on March 8 and 9 were both negative for SARS-CoV-2 by RT-PCR tests. On March 9, the patient was discharged and went to a designated hospital for quarantine and observation. On March 23, the nasopharyngeal swab result of SARS-CoV-2 by RT-PCR test was positive again. She was readmitted to Chongqing Public Health Medical Center. The chest CT scan showed no abnormality (**Figure 1B**). The laboratory data contained no abnormalities. The patient was treated with thymalfasin (1.6 mg, biw) for April 17 to May 17 and hydroxychloroquine (400 mg, biw on the first day, and 200 mg, biw on the following day) for April 18 to April 27 in combination with Chinese herbs. Serum immunoglobulin M (IgM) and immunoglobulin G (IgG) specifically for SARS-CoV-2 antigens were detected on May 21 by using magnetic chemiluminescence enzyme immunoassay kits (Bioscience Biotechnology Co.) (**Table 1**). The treatment was switched to atomized inhalation of interferon α-2b (500,000 U, biw) from May 21 to July 2. On July 2, the patient was discharged and maintained home quarantine after 14 consecutively negative results of SARS-CoV-2 by nasopharyngeal swab tests.

Case 2

A 54-year-old woman had a fever, sore throat, and cough on February 14. She was diagnosed as COVID-19 by positive nucleic acid test for SARS-CoV-2 with a contact history of patients with fever in Wuhan, China. The chest CT showed multiple patchy shadows in the lower lobes of both lungs. The patient was treated with arbidol, lopinavir/ritonavir, and Chinese Medicine from February 16 to March 14 in Wuhan. Two consecutive results of nasopharyngeal swabs were negative. On March 14, the patient was discharged from hospital. During the 14-day isolation period, the patient had no symptoms and multiple nucleic acid tests were negative. She was required to be quarantined in a government-designated hospital for 14 days. She was transferred to Chongqing Public Health Medical Center due to the positive result of SARS-CoV-2 on April 7. The chest CT scan was almost normal (**Figure 1C**). The patient was treated with arbidol (200 g, biw) from April 7 to April 17, hydroxychloroquine (400 mg, biw on the first day, and 200 mg, biw on the following day) from April 16 to April 24, and thymalfasin (1.6 mg, biw) from April 16 to April 26, combined with Chinese herbs. However, the IgG and IgM against SARS-CoV-2 was positive on May 21 (**Table 1**). The treatment was changed to atomized inhalation of interferon α-2b (500,000 U, biw) from May 21 to June 21. On June 21, the chest CT scan was almost normal (**Figure 1D**). The patient was discharged and maintained home quarantine after 14 consecutively negative results of SARS-CoV-2 by nasopharyngeal swab tests.

Case 3

A 50-year-old woman had fever and cough with a contact history of residents from Wuhan presenting the similar symptoms. She was confirmed as COVID-19 by positive nasopharyngeal swab of SARS-CoV-2 and was admitted to a local hospital on February 19. The patient had a history of chronic viral hepatitis B for more than 10 years. The chest CT showed multiple patchy ground-glass opacities in bilateral subpleural areas. The laboratory data of hepatitis B virus load was low. Other laboratory parameters

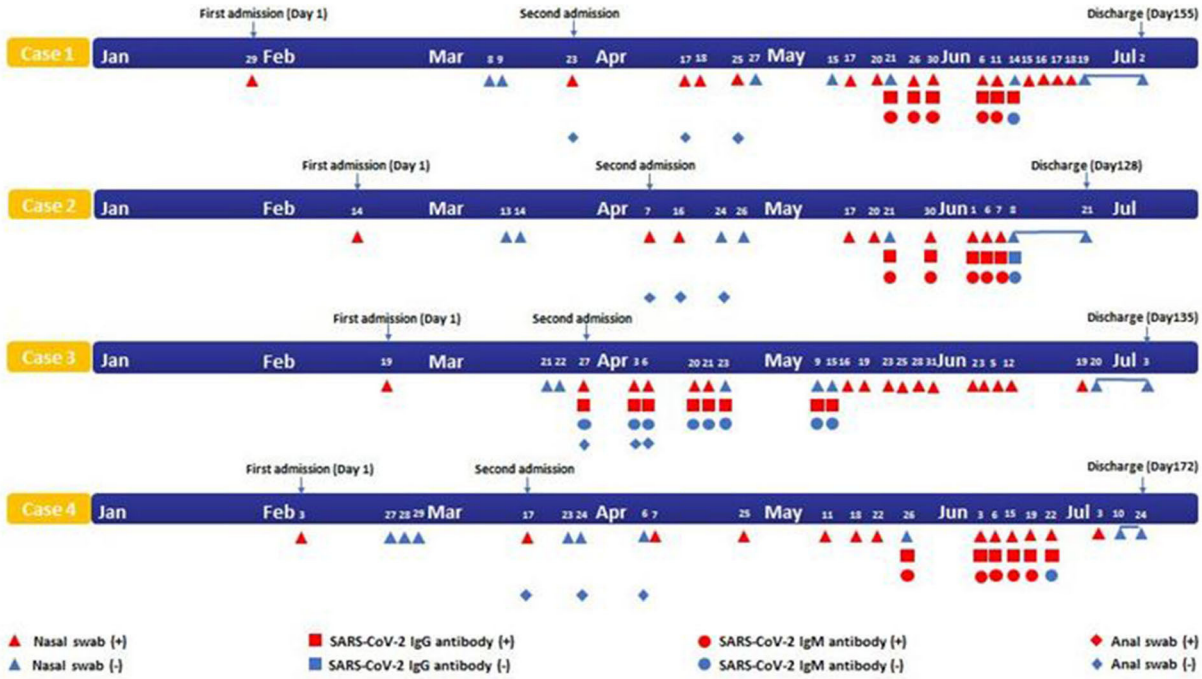

FIGURE 2 | Timeline of four cases with SARS-CoV-2 infection. SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2.

were normal. The patient was treated with arbidol (200 g, biw) from February 22 to March 2, hydroxychloroquine (400 mg, biw on the first day, and 200 mg, biw on the following day) from February 24 to March 3, and lopinavir/ritonavir (200/50 mg, 2 tablets, biw) from March 10 to March 20, combined with Chinese Medicinal therapy. On March 22, she was discharged and went to a designated hospital. On March 27, the patient was transferred to Chongqing Public Health Medical Center for further treatment due to the positive nucleic acid test for SARS-CoV-2.The CT scan was almost normal. The IgG for SARS-CoV-2 was positive (**Table 1**). The patient was treated with thymalfasin (1.6 mg, biw) from April 6 to May 9, atomized inhalation of interferon α-2b (500,000 U, biw) from April 20 to May 9 and from May 15 to July 3, and hydroxychloroquine (400 mg, biw on the first day, and 200 mg, biw on the following day) from April 21 to April 23, combined with Chinese herbs. On July 3, the patient was discharged and maintained home quarantine after 14 consecutively negative results of SARS-CoV-2 by nasopharyngeal swab tests.

Case 4

A 55-year-old man had fever of 37.5°C with headache and muscle soreness on February 3. He had a contact history of a confirmed COVID-19 case in the past 2 weeks. He was confirmed as COVID-19 by positive nucleic acid test. On admission, the chest CT images presented stripe shadows and focal ground-glass opacities in the upper and lower lobes of the left lung and middle and lower lobes of the right lung. The patient was treated with lopinavir/ritonavir, thymopentin, and Chinese Medicine. The three times of nasopharyngeal swabs collected on February 27, 28, and 29 were all negative for SARS-CoV-2, respectively. The anal swab test for SARS-CoV-2 by RT-PCR was negative on March 2. The patient was discharged on March 3. The patient was admitted to a government-designated hospital for centralized isolation after a 1-week home quarantine. On March 17, the nucleic acid test for SARS-CoV-2 was positive again. During the hospitalization, the nasopharyngeal swab tests for SARS-CoV-2 were two negative results. He went for medical observation for 2 weeks. On April 7, the nasopharyngeal swab test for SARS-CoV-2 was positive again. He was transferred to Chongqing Public Health Medical Center. The chest CT images presented stripe shadows and diffuse consolidation in both lungs. The patient was treated with thymalfasin (1.6 mg, biw) from April 7 to May 11 and hydroxychloroquine (400 mg, biw on the first day, and 200 mg, biw on the following day) from April 16 to April 24, combined with Chinese herbs. However, the IgG and IgM against SARS-CoV-2 were positive on May 26 (**Table 1**). The treatment was changed to atomized inhalation of interferon α-2b (500,000 U, biw) from May 26 to July 10. On July 24, the patient was discharged and maintained home quarantine after 14 consecutively negative results of SARS-CoV-2 by nasopharyngeal swab tests.

RESULTS

Four cases were confirmed as COVID-19 on the first admission. None had underlying diseases such as diabetes, hypertension, and cardiovascular disease. After treatment, they met the discharge

standard for two consecutively negative nucleic acid tests for SARS-CoV-2 and then were isolated in a government-designated hospital for at least 14 days. However, they had positive RT-PCR results of SARS-CoV-2 with no symptoms after discharge (**Figure 2**). On the second admission, the chest CT scan of three cases was normal. The serological assays of these three cases were positive for IgG and IgM. Only one case was positive for IgG (**Figure 2**). Multiple antiviral treatments were used in combination with Chinese Medical therapy. Four cases were treated with psychotherapy for anxiety disorders. The disease duration of four patients were 128, 135, 155, and 172 days, respectively, after discharge. All the four patients did not have additional recurrences during the follow-up visit with negative results of SARS-CoV-2 by nasopharyngeal swabs tests after discharge from July to September.

DISCUSSION

In this report, all four patients met the discharge standard with two consecutively negative nucleic acid tests on the first admission. However, the nasopharyngeal swab test of SARS-CoV-2 had recurrence of positive results during the follow-up period. There was little possibility for reinfection due to no contact with other patients with COVID-19 during the quarantine. The course of patients with COVID-19 for more than 90 days has been reported (1). Unexpectedly, the disease duration of the four patients lasted for more than 120 days, suggesting the longest duration reported outside Wuhan, China.

The nasopharyngeal swab test of SARS-CoV-2 by RT-PCR is currently the most common method for diagnosis of COVID-19. With the progression of SARS-CoV-2 infection, the virus could migrate from the upper respiratory tract to the lower respiratory tract and lungs (2, 3), resulting in insufficient viral load in the upper respiratory tract, which may explain the negative result of nasopharyngeal swab test (4). Currently, limited methods are available to determine the viral load and activity. Additionally, lack of experience with sample collection, transportation, and inspection could also lead to the false-negative results (5). Therefore, the detection rate and sensitivity could be improved by multisite specimen collection including alveolar lavage fluid, sputum, and serological assays (6). Furthermore, the nucleic acid test should be performed repeatedly for highly suspected patients to avoid misdiagnosis. The quarantine period should be prolonged for at least 50 days for recovered patients in order to avoid virus carrier transmission and identify patients that may pose a risk for the future outbreaks (7).

All four patients were discharged after meeting the discharge criteria on the first admission, indicating that the treatment was effective. The antiviral treatment temporarily cleared the virus, resulting in the negative result of SARS-CoV-2. However, it could still take days for the immune system to completely eliminate the remaining virus in the body (8), leading to intermittent release of the virus for recurrence of positive results. Once the virus had replication and intermittent shedding, the RT-PCR results reverted to positive in the discharged patients (7). Continuous antiviral therapy should be considered especially for the patients with repeatedly positive results of SARS-CoV-2 (9). The asymptomatic patients had a significantly longer duration of viral shedding than the symptomatic patients as previously reported (10). There still lacked the evidence for the viral clearance or the duration of viral shedding after initial infection. The "dead" virus or viral fragments without replication may also contribute to repeatedly positive results without potential infectiousness (4, 11).

All four patients were diagnosed as COVID-19 on the first admission. Case 3 had chronic hepatitis B virus for more than 10 years. Whether hepatitis B virus and SARS-CoV-2 affected each other still remains unclear. The nasopharyngeal swab tests of all four patients were recurrently positive on the second admission with negative results of anal swab tests, indicating the less possibility of oral-fecal transmission. The family members of Case 1 and Case 2 did not have recurrence of positive results of SARS-CoV-2 after discharge. Only four severe patients in total of 576 patients with COVID-19 were recurrently positive by nasopharyngeal swab tests in Chongqing, China. The four patients were isolated with the average duration of positive RT-PCR test for <40 days. The severe patients with COVID-19 may have a faster conversion from positive to negative by RT-PCR test.

The four patients were asymptomatic with normal chest CT scan and laboratory data on the second admission. The nasopharyngeal swab tests of SARS-COV-2 were recurrently positive. Case 1, Case 2, and Case 4 were also continuously positive for IgG and IgM. The weak production of the virus-specific IgG and IgM could lead to asymptomatic or mild patients which could be a long-term carrier transmission (12). Asymptomatic patients had lower IgG titers and shorter duration due to a reduced immune response (10), which suggested that likelihood of "herd immunity" was low. After recovery from COVID-19, prevention of reinfection by a protective antibody and the duration of antibody protection still need to be determined. Therefore, the use of "immunity passports" for COVID-19 could be risky. We speculate that the presence of IgG does not have a long-term protective effect in some situations.

Serological assays in combination with viral nucleic acid test should be performed to screen for asymptomatic or suspected patients in order to reducing the false-negativity by RT-PCR test alone (13). The dynamic surveillance of SARS-CoV-2 by RT-PCR test combined with serological assays should be of significant value for viral infectiousness. These tests will also be informatively important for the diagnosis and prognosis of COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RZ, WD, and DW had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis, designed the study, and wrote the paper. JH, CQ, and QY were responsible for the acquisition, analysis, or interpretation of the data. DW contributed to the revision of the manuscript for important intellectual content. All authors have read and approved the manuscript.

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ORIGINAL RESEARCH
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Epidemiological Investigation and Virus Tracing of a Measles Outbreak in Zhoushan Islands, China, 2019

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Background: Measles transmissions due to case importations challenge public health systems globally and herd immunities in all countries. In 2019, an imported measles case and its subsequently outbreak was found in the Zhoushan Islands. Here, the process of epidemiological investigation and virus tracing were summarized to provide references for the prevention and control of measles in the future.

Materials and methods: The data on the demographic, epidemiological, and clinical manifestation of measles cases in this outbreak were collected. The 450 bp fragments of the measles virus (MeV) N gene were amplified and sequenced. The genome of the first imported case was further isolated. Then, the maximum-likelihood and time-scaled phylogenetic analysis was conducted.

Results: A total of 28 measles cases were confirmed. Their onsets were between March 13 and May 18, 2019. The first patient was from the Ukraine. He was confirmed at the Fever Clinic in Zhoushan hospital on March 15, 2019 and at the same time, another patient had visited the hospital due to another illness and 10 days later, this second case had onset (March 25, 2019). The epidemic curve shows sustained community transmission. The majority of the following cases (19/26) were clustered on the Donggang street which was close to where the second case worked. The 22 measles virus strains successfully isolated from this outbreak all belonged to the D8.2a sub-cluster and clustered with the KY120864/MVs/GirSomnath.IND/42.16/[D8] which was the predominant genotype in the Ukraine during 2018-2019. The analysis of the complete D8 genotype genome pointed to the fact that this prevailing strain originated from India in 2015 and its substitution rate was estimated as 6.91×10^{-4} ($5.64–7.98 \times 10^{-4}$) nucleotide substitutions/site/year.

Conclusion: This outbreak was caused by an imported case from the Ukraine. There was a possible nosocomial infection between the first case and the second case. Then, the second case played an important role in the spread of virus due to her occupation. The molecular phylogenetic analysis could help to track the origin of the virus. Increasing and maintaining the high level of vaccination coverage (≥95%) and an efficient response to imported cases are essential to prevent and control the recurrence and outbreak of measles virus.

Keywords: measles virus, D8 genotype, outbreak, epidemiological investigation, phylogenetic analysis

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INTRODUCTION

Measles is a highly contagious disease characterized by a basic reproduction number (R0) ranging from 12 to 18 (1, 2). It is caused by measles virus (MeV) mainly spreading through respiratory routes with typical symptoms of fever and rash (3). At the present time, all member states/regions of the World Health Organization (WHO) have set a goal of eliminating measles (4). This, however, has had to be revised or suspended due to a global upsurge in measles cases in 2018–2019. This includes countries who had previously verified that they had eliminated measles, where the disease has been re-established. Although China has reported record low numbers (5).

Measles is vaccine-preventable but the global elimination of measles has constantly been impacted. This is mainly due to pockets of low vaccination coverage and repeated international importations of measles cases among unvaccinated persons contacting the affected communities (6). Therefore, case base surveillance and analysis of circulating MeV strains has become important in the documentation of the interruption of measles transmission and the sources of imported cases (7). MeV was classified into eight clades including 24 genotypes that show a distinct geographic distribution published in 2012 (8). The imported measles cases always led to the geographical dispersion of measles strains and outbreaks. For example, in 2008, an outbreak of >24,300 cases in Bulgaria was caused by the imported MV/D4-Hamburg virus (9). In 2017, the imported D3 and D8 genotypes caused one of the largest measles outbreaks in Italy with 5,404 notified cases and 4,347 confirmed cases (10). In the United States, 22% of measles cases were imported and caused 66 outbreaks during the 2009–2014 period (11).

The MeV genome is 15,894 nucleotides in length and encodes a total of eight proteins (N, P, M, F, H, L, C, and V). The carboxy-terminal 450 nucleotides of N were used for measles genotyping, established by WHO (12). In China, genotype H1 has been predominantly cocirculating since the early 1990s (13), among these, several reports stated that the outbreaks were related to new imported measles virus such as the D8, B3, B4, and D11 strains (14–17). Zhoushan consists of an archipelago of islands and is the only prefecture-level city in China. The Zhoushan Islands are located at the northeast of Zhejiang Province (121°30'E∼123°25'E, 29°32N∼31°04'N) with ∼22,200 km^2 in land area and 1.2 million in population. Genotype H1 of measles virus was dominantly circulating in Zhoushan before 2018 (18). As a result of the special geographic features of the area, there are many ports for foreign trade and transportation. Frequent movement of a population increases the risk of infectious disease importations. In 2008, the Zhoushan Islands experienced their largest measles outbreak (115 cases) caused by imported cases in the Putuo region. In this study, we report the epidemiological investigation and virus tracing of an imported measles case and the subsequent outbreak in the Zhoushan Islands to provide our recommendations for local measles elimination and eradication in the future.

MATERIALS AND METHODS

Case Definition, Epidemiology Investigation, and Specimen Collection

The case and outbreak definition are according to the China National Measles Surveillance Programme (19). In total, 28 patients with measles were reported by a local hospital from March to May, 2019. When the measles cases were clinically diagnosed and reported from the local hospital, the local/district (Zhoushan and Putuo) Center for Disease Control and Prevention (CDC) arrived at the scene within 24 h and then conducted an epidemiology investigation using a standardized questionnaire to collect the demographic, epidemiologic, and clinical data of these patients. The possible sources of infections, transmission routes, and close contacts were also recorded. Subsequently, staff at all levels of the medical organizations or clinics in the Zhoushan Islands were requested to react and continually seek possible measles cases based on the records of clinical surveillance. At the same time, throat swabs and serum specimens of patients were collected by the local hospital or CDC members and then transported to the Zhoushan CDC measles network lab for testing. The laboratory-confirmed patients were then isolated and treated in a specialized hospital for infectious diseases.

Measles Virus Detection, Amplification, and Sequencing

According to the standard operational procedures (SOP) developed by China's CDC, the throat swab specimens were tested for MeV nucleic acids by RT-qPCR (Bioperfectus, China) and the serum specimens were tested for the anti-measles IgM antibody (Serion, Germany) (19). The specimens which were identified as MeV positive by RT-qPCR were kept for virus isolation and gene sequencing. The QIAamp® Viral RNA Mini kit was used to extracted the measles virus RNA according to the manufacturer's instructions (QIAGEN, Hilden, Germany). Then, the RNA was subjected to RT-PCR with specific primers to amplify the 450 bp fragment of the N gene which was used for MeV genotyping (20). The PCR products were purified and sequenced by Sangon Biotech (Shanghai, China). The complete genome of the first strain in this outbreak was further amplified and sequenced by Sangon Biotech (Shanghai, China) using Sanger dideoxy sequencing.

Genotyping and Phylogenetic Analysis

The blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to genotype the measles virus. The online tool MAFFT (https://maft.cbrc.jp/alignment/server) was used to align the sequences. The IQTREE v.2.0 software (21) was used to construct the maximum-likelihood (ML) phylogenetic tree of both the N gene sequences and the complete genome of the D8 genotype strains with K3P and TIM+F+R3 nucleotide substitutions models which were best-ft models, respectively in the ModerFinder software according to the Bayesian information criterion (BIC). A temporal signal in the dataset was evaluated by a root-to-tip regression based on ML phylogeny inferred using the IQTREE v.2.0 software via the program TempEst v.1.5 (22).

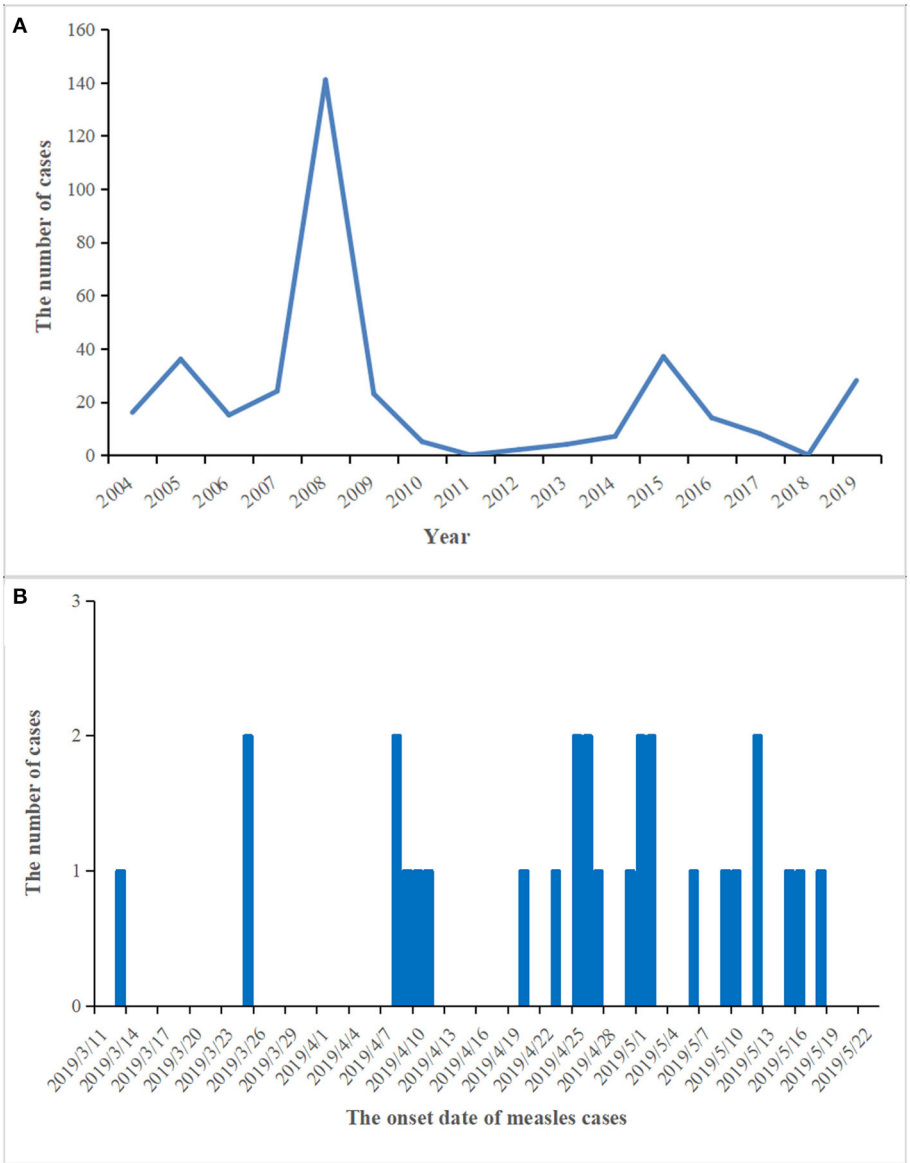

FIGURE 1 | The prevalence and epidemic curve of measles cases in the Zhoushan Islands. **(A)** The reported of measles cases in the Zhoushan Islands during 2004–2019. **(B)** The epidemic curve of measles outbreak in the Zhoushan Islands, 2019.

The BEAST v.1.8.2 software (23) was used to construct the time-scaled phylogenetic tree in order to estimate the time of the most recent common ancestor (TMRCA). The appropriate molecular clock and tree models were then determined by path-sampling/stepping sampling (PS/SS) and then the exponential growth-relaxed molecular clock, GTR+G+I+F4 substitution, and constant size coalescent model were chosen, with a single run of a Markov Chain Monte Carlo (MCMC) sample chain for 100 million steps and sampling every 10,000 steps. The MCMC analysis was run for more than twice as much, and then two of them were selected to check for convergence (24). After discarding the first 10% of the chain, the effective sample size (ESS $\geq$200) was used to evaluate the convergence of the continuous parameters using Tracer v.1.6 (http://tree.bio.ed.ac.uk/software/tracer/).

RESULTS

The Prevalence of Measles Cases in the Zhoushan Islands, China

From 2004 to 2018, the number of reported measles cases ranged from 0 (2011 and 2018) to 141 (2008) in the Zhoushan Islands (**Figure 1A**). We identified 28 measles cases in the Zhoushan Islands in 2019 (**Table 1**). The dates of rash onset in the measles cases were between March 13 and May 18, 2019. The incidence rate was 2.4/100,000. Out of the 28 patients, 15 (53.57%) were

TABLE 1 | The patient characteristics and measles virus strains in the Zhoushan Islands, China, 2019.

Case No.	Date of onset	Age (Year)	Gender	Region	Occupation	Vaccination history	Serological test (IgM)	Accession number/strain name
1	13/03/2019	39	Male	Putuo	Fisherman	Unknown	+	MT738528/MVs/Zhoushan.CHN/09.19[D8]
2	25/03/2019	40	Female	Putuo	Self-employed laborer	Unknown	+	MT738529/MVs/Zhoushan.CHN/12.19/1[D8]
3	25/03/2019	51	Female	Putuo	Housework	Unknown	+	MT738530/MVs/Zhoushan.CHN/12.19/2[D8]
4	08/04/2019	29	Male	Dinghai	Self-employed laborer	Unknown	+	MT738531/MVs/Zhoushan.CHN/14.19/1[D8]
5	08/04/2019	44	Male	Putuo	Worker	Unknown	−	MT738532/MVs/Zhoushan.CHN/14.19/2[D8]
6	09/04/2019	36	Female	Dinghai	Public servant	Unknown	+	MT738533/MVs/Zhoushan.CHN/14.19/3[D8]
7	10/04/2019	48	Female	Putuo	Catering industry	Unknown	+	MT738534/MVs/Zhoushan.CHN/14.19/4[D8]
8	11/04/2019	37	Male	Putuo	Business	Unknown	+	MT738535/MVs/Zhoushan.CHN/14.19/5[D8]
9	20/04/2019	29	Female	Dinghai	Teacher	Unknown	+	MT738536/MVs/Zhoushan.CHN/15.19[D8]
10	23/04/2019	8	Female	Dinghai	Student	Unknown	+	–
11	25/04/2019	39	Male	Putuo	Sailor	Unknown	+	–
12	25/04/2019	28	Female	Putuo	Business	Unknown	+	MT738537/MVs/Zhoushan.CHN/16.19/1[D8]
13	26/04/2019	1	Male	Putuo	N/A	None	+	MT738538/MVs/Zhoushan.CHN/16.19/2[D8]
14	26/04/2019	0.5	Male	Putuo	Scattered living	None	+	MT738539/MVs/Zhoushan.CHN/16.19/3[D8]
15	27/4/2019	50	Female	Putuo	Teacher	Unknown	+	–
16	30/04/2019	39	Male	Putuo	Self-employed laborer	Unknown	−	MT738540/MVs/Zhoushan.CHN/17.19/1[D8]
17	01/05/2019	35	Female	Putuo	Medical staff	Unknown	−	MT738541/MVs/Zhoushan.CHN/17.19/2[D8]
18	01/05/2019	58	Male	Putuo	Worker	Unknown	+	MT738542/MVs/Zhoushan.CHN/17.19/3[D8]
19	02/05/2019	49	Male	Putuo	Businessman	Unknown	−	–
20	02/05/2019	35	Male	Putuo	Catering industry	Unknown	+	MT738543/MVs/Zhoushan.CHN/17.19/4[D8]
21	06/05/2019	50	Male	Putuo	Self-employed laborer	Unknown	+	MT738544/MVs/Zhoushan.CHN/18.19/1[D8]
22	09/05/2019	51	Female	Putuo	Housework	Unknown	−	MT738545/MVs/Zhoushan.CHN/18.19/2[D8]
23	10/05/2019	53	Female	Putuo	Tourist	None	+	MT738546/MVs/Zhoushan.CHN/18.19/3[D8]
24	12/05/2019	0.5	Male	Putuo	Scattered living	None	−	MT738547/MVs/Zhoushan.CHN/18.19/4[D8]
25	12/05/2019	49	Male	Putuo	Driver	Unknown	+	MT738548/MVs/Zhoushan.CHN/18.19/5[D8]
26	15/05/2019	3	Male	Putuo	Child care worker	None	+	MT738549/MVs/Zhoushan.CHN/19.19/1[D8]
27	16/05/2019	47	Female	Putuo	Public servant	Unknown	+	–
28	18/05/2019	45	Female	Putuo	Worker	Unknown	+	–

+/−: + represents positive for IgM test while − represents negative.

male and the mean age was 35.5 years (range: 0.5–53 years). All cases had fever and rash, of which 65% had coughs, 35% had conjunctivitis, and 35% had oral mucosal plaques. Four cases were under 6 years of age and none had a history of vaccination. There were no deaths or severe complications among any of the patients. All of the 28 patients were laboratory confirmed as having measles using RT-qPCR; however, six of the 28 swab samples with low viral load (Ct > 36) were negative by RT-PCR, which resulted in no specific genome product available for genotyping. Serologically, only 22 of the 28 patients were measles IgM positive, though all serum samples were collected quickly within 24 h of visiting the hospital (**Table 1**).

Epidemiological Investigation of the Outbreak

Out of the 28 cases, 24 were reported in the Putuo region (Donggang: 21, Shenjiamen: 3), and four in the Dinghai region (Lincheng: 4). The first case was from the Ukraine, who entered China on March 8, 2019 by air directly from the Ukraine to Shanghai Pudong Airport. He arrived in the Zhoushan Islands (Donggang, Putuo region) for a job as a fisherman and then went on a fishing boat on March 9, 2019. He had no history of traveling in other countries 30 days before coming to China. The patient presented with fever and was sent to Zhoushan hospital on March 13, 2019. Two days later (March 15), he was diagnosed with measles infection by RT-qPCR and transferred to a designated hospital for infectious diseases in the Zhoushan Islands. The second case had rash onset on March 25, 2019, 10 days after she visited Zhoushan hospital on March 15, 2019 due to another illness (cough and fever) when both the first and second patient visited the Fever Clinic in Zhoushan hospital. After visiting Zhoushan hospital, the second patient as a self-employed worker went back to work in the Donggang Farm Product Market which is located on the Donggang street in the Putuo region (**Figure 2**).

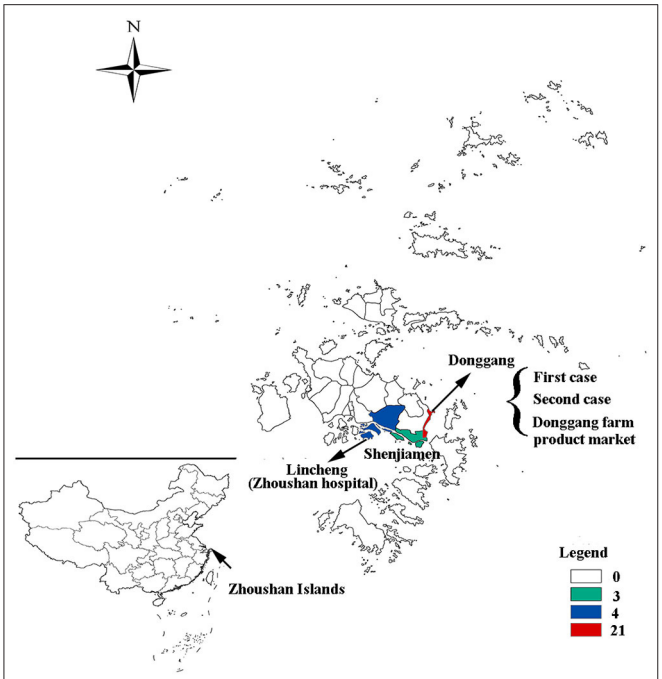

FIGURE 2 | The geographical distribution of measles cases in the Zhoushan Islands. The map showing the geographical distribution of measles cases in the Zhoushan Islands. The colors represent the number of cases in each street in which 21 were in the Donggang street, four were in Lincheng street, and three were in the Shenjiamen street. The first and second cases and Donggang Farm Product Market were located in the Donggang street and Zhoushan hospital was located in the Lincheng street.

She was confirmed as having measles by RT-qPCR on March 28 then isolated from March 29, 2019. Her main contacts were her family members, her husband, daughter [16 years old, previously vaccinated with the measles-contain vaccine (*MCV*)], staff at the Market, and customers. The third case, who also had rash onset on March 25, 2019, lived in the same residential area as case 2. Both had contact with the Donggang Market prior to their rash onset. Most of following cases (19/26) were clustered in the Donggang street which is close to the Donggang Farm Product Market (**Figure 2**). The epidemic curve shows sustained community transmission from March to May (**Figure 1B**).

Phylogenetic Analysis Based on the N Gene of Measles Viruses Detected

We obtained the 450 bp fragments of the N gene from 22 of the 28 measles cases and a complete genome from the first case. All sequences are available in GenBank under accession numbers MT738528–MT738550. The phylogenetic analysis was performed based on 103 MeV strains including the 22 strains from the Zhoushan Islands in 2019, a WHO D8 reference strain, 22 WHO-named strains, 49 D8 strains detected in China, and 10 strains which had the closest proximity to the Zhoushan Islands in the initial blast analysis. The measles virus D8 genotype was classified into two groups, D8.1 cluster and D8.2 cluster (25). The D8.1 cluster was further divided into three sub-clusters (D8.1a, D8.1b, and D8.1c) and the D8.2 cluster was divided into two further sub-clusters (D8.2a and D8.2b). The 22 measles virus strains of the Zhoushan Islands were identical and shared 100% identities with KY120864/MVs/GirSomnath.IND/42.16/[D8] based on the N450 region and belonged to genotype D8 (**Figure 3**).

Maximum-Likelihood and Time-Scaled Phylogenetic Analysis of the Complete Genome of Measles Virus D8 Strain

The maximum-likelihood phylogenetic analysis of the complete genome obtained from the first patient showed that the MeV strain was clustered with MVi/Bhavnagar.IND/17.16[D8] and MVi/GirSomnath.IND/00.17[D8] with 100 bootstrap values (**Figure 4**) and shared 99.83 and 99.75% identities, respectively. The dataset exhibited a positive correlation between genetic divergence and sampling time ($R^2 = 0.5028$) with which the presence of the temporal structure in the sequence data allowed us to proceed with Bayesian molecular dating analyses in BEAST (**Figure 5**). The complete genome of 80 D8 strains isolated from 2009 to 2019 were collected to construct a time-scaled phylogenetic tree which indicated that measles virus D8 evolved at a rate of 6.91×10^{-4} (95% HPD: $5.64–7.98 \times 10^{-4}$) nucleotide substitutions/site/year. The TMRCA for the D8 genotype was estimated to be 1992 (95% HPD interval: 1978–2003) and TMRCA for strains imported to the Zhoushan Islands was estimated to be 2015.61 (95% HPD interval: 2015.36–2015.97) (**Figure 6**).

DISCUSSION

In this study, we described a measles outbreak involving 28 cases in the Zhoushan Islands in 2019 where the measles D8 virus was first detected. Apart from this outbreak, no other measles cases were reported in the Zhoushan Islands in 2019. There were no cases reported in 2011 and 2018. The latest measles case was confirmed in 2017 and genotype H1 has only so far been detected in the islands. This status might be caused by the natural recession of the population immunity barrier of MeV. Once measles cases are imported, it is more likely to cause outbreaks. Based on the schedule of the first case, a fisherman from the Ukraine, this outbreak might have been subsequently caused by the virus being imported from the Ukraine. There was then a possible nosocomial infection between the first and second case. Due to the occupation of the second case, she had contact with a large number of people each day and the frequent mobility of the population in the farm product market might have resulted in the spread of the measles virus. The majority of the following cases were clustered in the Donggang street which was close to the Donggang Farm Product Market. After case 2 was identified (March 28), her close contacts were tested and traced by Putuo CDC staff and they requested that she self-quarantine. At the same time, all medical units in the Zhoushan Islands were requested to take continual action in seeking possible measles cases based on clinical surveillance through medical records. During April 8 to April 11, five measles cases were diagnosed

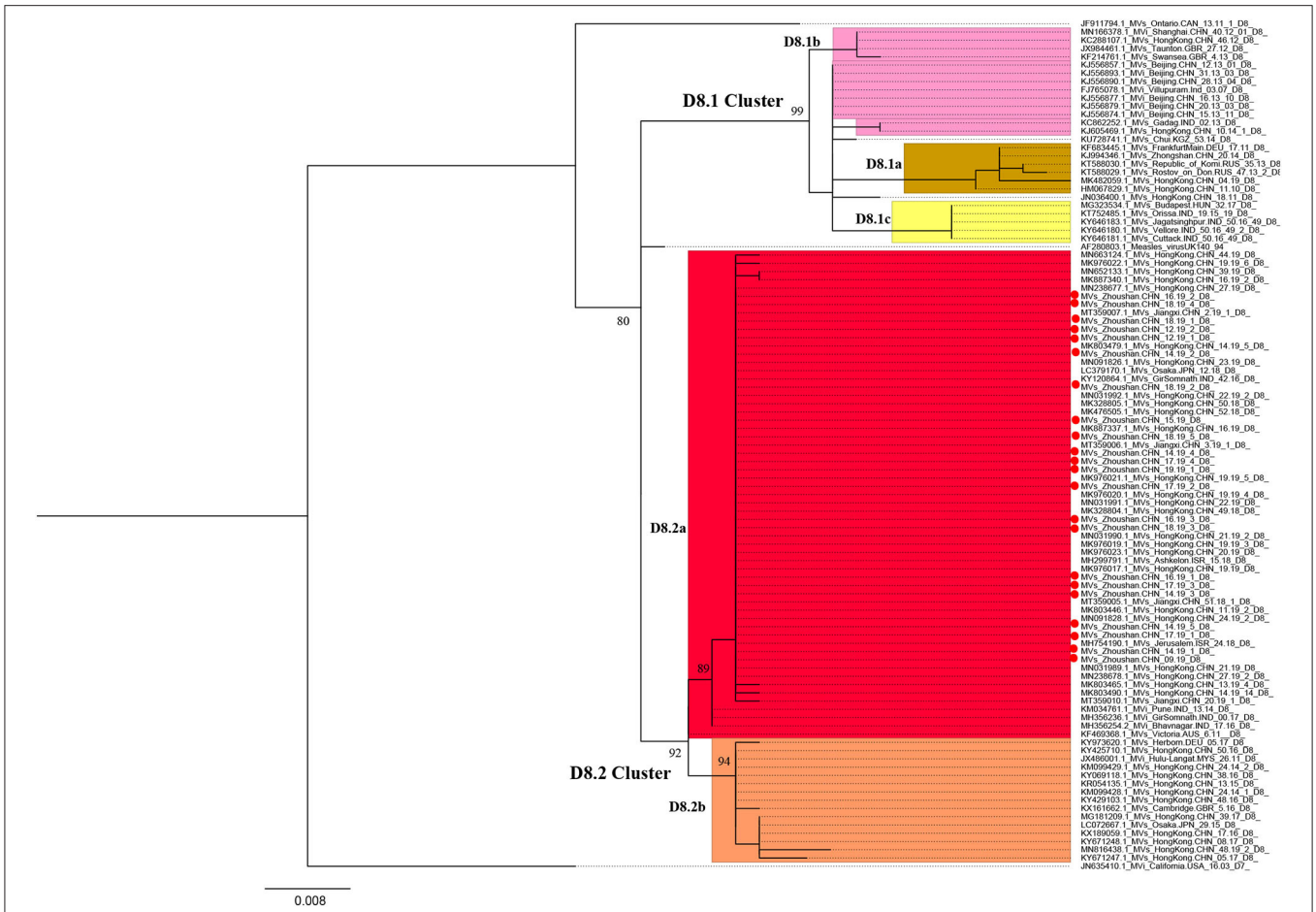

FIGURE 3 | Maximum-likelihood phylogenetic tree of D8 genotype strains based on the 450 bp fragment of the N gene sequences. ML phylogenetic tree showing the genetic relationship among MeV isolates. Strains isolated in this outbreak are represented in red dots. Bootstrap values (>70%) are shown at the corresponding branches and the scale bar is given in substitutions/site.

and all cases were located in the Donggang street. The Putuo CDC defined this event as an outbreak of measles and reported it to the Zhoushan CDC. Shortly after this, emergency vaccination was conducted among the close contacts as well as the people in the area surrounding where the measles cases worked and lived. Between March 15 and May 20, a total of 764 individuals with specific close contact with a measles case were identified and ∼2,000 people susceptible to exposure were vaccinated with the MCV.

In this incident, the vaccination history of 85.71% (24/28) of the patients was unknown, however, 22 of the 28 (78.6%) patients were measles IgM positive suggesting primary infection. The negative IgM in the other six patients could be due to either reinfection or a delayed antibody reaction, e.g., case 24 or the assay sensitivity (**Table 1**). The reasons for the four cases of young unvaccinated patients were their failure to reach vaccination age (case no. 14 and 24, 2/4) and contraindications associated with MCV (case no. 13 and 26, 2/4). WHO guidelines for the elimination of measles suggest that large-scale outbreaks of measles can be avoided if the MCV coverage is more than 90% in the whole population and that at least 95% coverage could eliminate measles entirely (26). The Chinese government launched the National Expanded Program on Immunization *(EPI)* in 2007 that vaccinated with the MCV at 8 and 18 months of age (27). With these efforts, the measles incidence in China decreased dramatically, from 99.4 per million in 2008 when 151 cases were reported in the Zhoushan Islands (**Figure 1A**) to 4.6 in 2012 when two cases were reported in the Zhoushan Islands (**Figure 1A**) (28). However, the actual vaccination coverage in China was estimated to be 80–90% (29). According to the surveillance of the antibody level of MeV in the Zhoushan Islands population in 2014. The overall positive rate was 88.56% and the positive rate of protective antibody was 46.59%. Among the 30–49 age group, the positive rate of antibody and protective antibody were both the lowest, with 84.64 and 40.20%, respectively (30). This indicates that the measles vaccine coverage is not enough in the Zhoushan Islands, especially among the 30–49 age group. When an imported case occurs, it easily causes measles virus infections and outbreaks. Recently, nationwide indigenous measles outbreaks and the resurgence

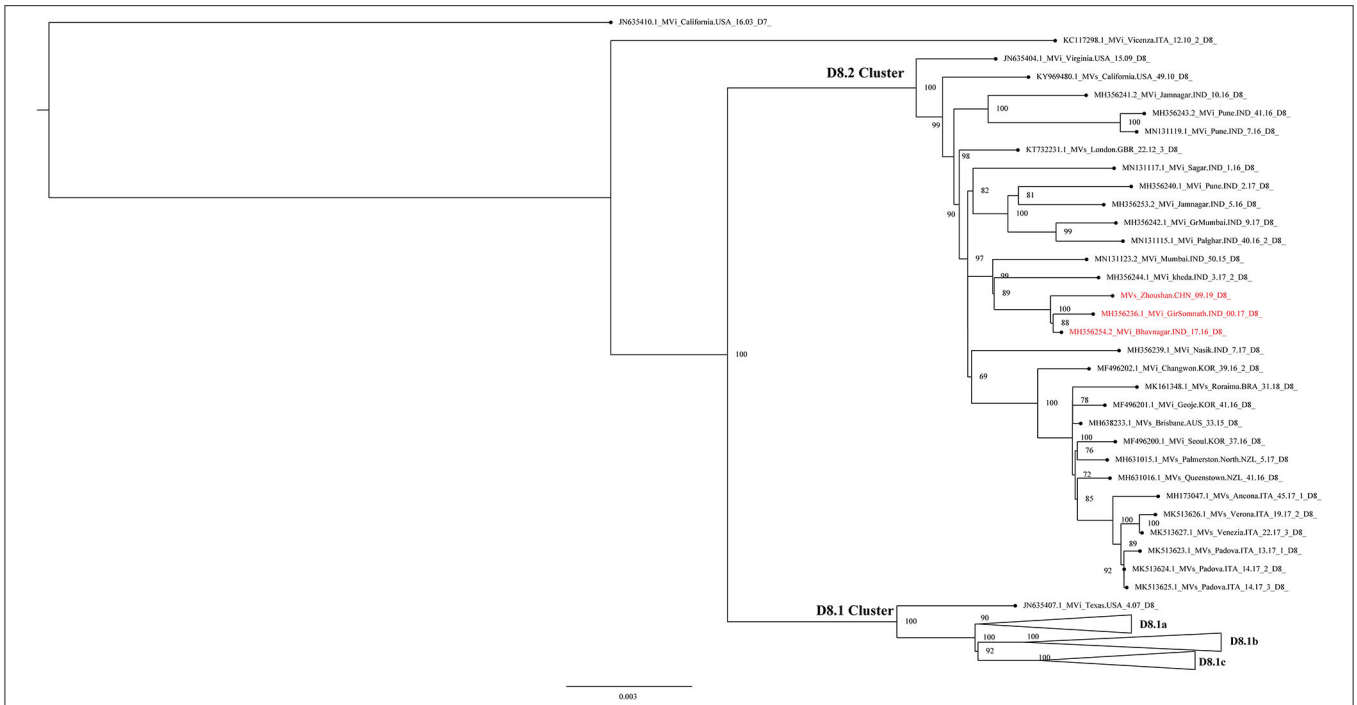

FIGURE 4 | Maximum-likelihood phylogenetic tree of D8 genotype strains based on the complete genome. ML phylogenetic tree showing the genetic relationship among MeV isolates. The triangle represents the D8.1 sub-cluster. The black dot represents each Mev sequence. The scale bar is given in substitutions/site.

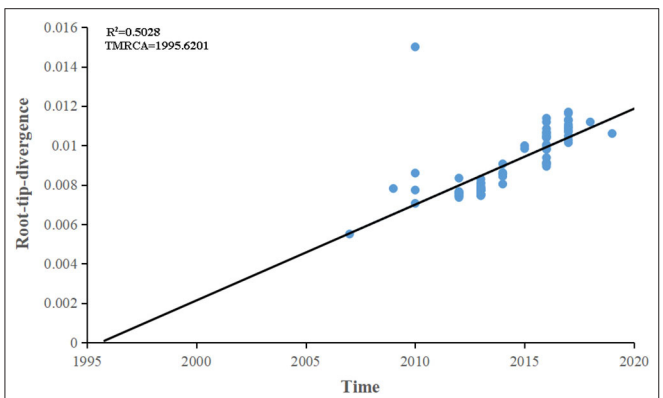

FIGURE 5 | Root-to-tip regression of a maximum-likelihood (ML) phylogenetic tree of complete genome of D8 genotype strains. The correlation between genetic divergence and sampling time was calculated by R^2. The time of the most recent common ancestor was designated by the X intercept.

of measles in unvaccinated children have been continuously reported (31, 32). Therefore, it is important to deliver two doses of MCV to children in a timely manner through routine immunization to increase and maintain high coverage (≥95%). Frequent monitoring and assessing coverage are also necessary to identify the pockets at risk that need to be strengthened.

The first measles D8 virus strains identified were AF280803.1/Measles/virusUK140/94 in 1994 in the UK (33). At that time, the D8 genotype had also been detected worldwide including in areas such as South Asia, the Middle East, Europe, and Africa (34, 35). Currently, the MeV genotype D8 is endemic in the Indian subcontinent. The genotype of the measles D8 virus has not been identified by WHO, but other research has classified the D8 virus into two genotypes including D8.1 (D8.1a, D8.1b, D8.1c) and D8.2 (D8.2a, D8.2b) (25). The D8 genotype strains are frequently imported from India into Europe, America, and Asia (36). Nowadays, the D8-Victoria lineage (D8.2a) is one of the predominant lineages worldwide (37). In 2018, measles cases tripled across the European region with nearly 83,000 reported. Among those, the Ukraine had more than 54,000 cases, where in 2016, only 31% of 6 year-old children received the second MCV (38). Because of the Ukraine's large pool of unvaccinated or under-vaccinated people, it is at a high risk of measles outbreaks and importation to other regions. In the maximum-likelihood phylogenetic analysis based on the 450 bp N gene of measles, the strains isolated from this outbreak were clustered with KY120864/MVs/GirSomnath.IND/42.16/[D8] and shared 100% identity with the predominant genotype in the Ukraine during 2018–2019 (39). In this study, MeV genotyping was not available for six patients with low viral loads, however, the results of epidemiological investigation and tracking could prove that they were linked as a part of the outbreak, suggesting that the combination of field investigation and laboratory confirmation is very important in countries or regions working toward measles elimination.

The maximum-likelihood and time-scaled phylogenetic analysis of the complete D8 genotype genome pointed out

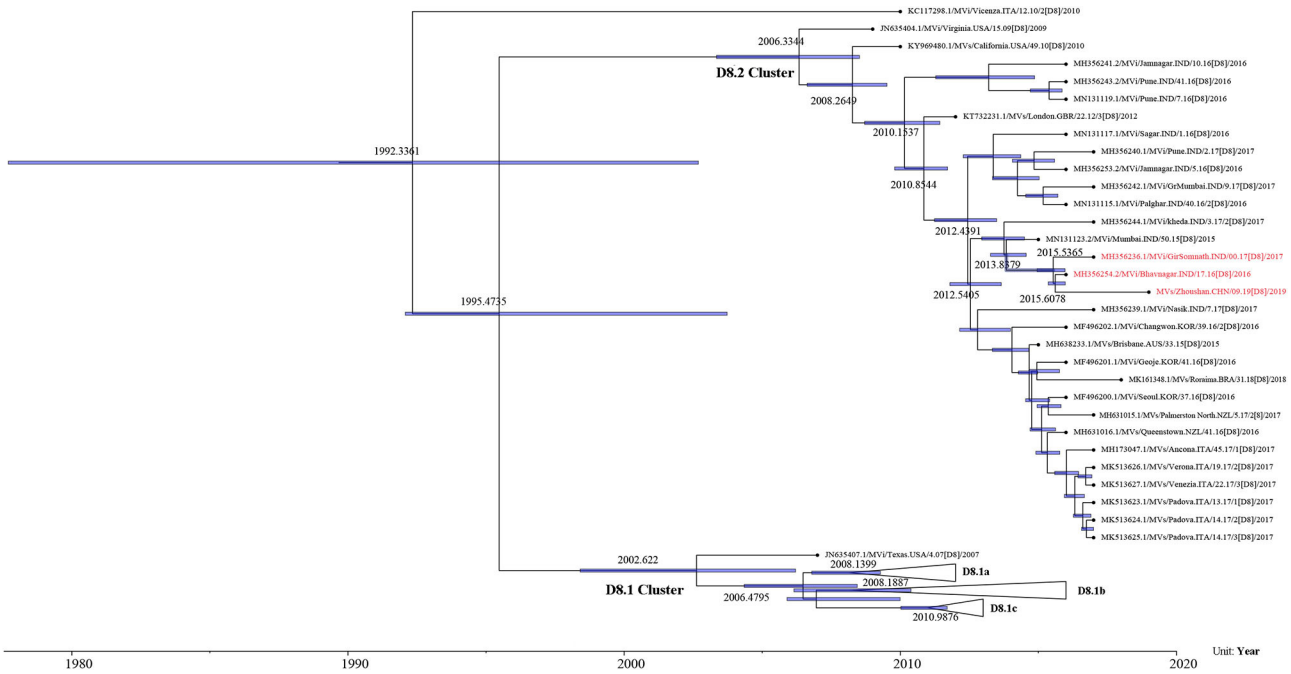

FIGURE 6 | Time-scaled phylogenetic tree based on the complete genome of D8 genotype strains detected from 2009 to 2019. Time-scaled phylogenetic tree showing the evolutionary relationships and timescale of MeV isolates. Branch lengths are scaled according to time. Purple node bars show the 95% credibility intervals of node-age estimates.

that this predominant strain originated from India in 2015. In a previous study conducted in India, the genotypes D4 and D8 were combined in the dataset instantaneously and the substitution rate of the D4 and D8 genotypes were estimated at (5.142×10^{-4} (95% HPD: $2.697–7.978 \times 10^{-4}$) nucleotide substitutions/site/year) (40). In our study, it was the first time the substitution rate of the D8 genotype genome was calculated independently and it was estimated at 6.91×10^{-4} (95% HPD: $5.64–7.98 \times 10^{-4}$) nucleotide substitutions/site/year based on 80 strains isolated from 2009 to 2019. This result would be helpful in evaluating the evolution status of the D8 measles genotype. The high substitution rate of the virus genome always indicates that new variants are more likely to develop which may cause large-scale outbreaks when it spreads to humans. This result was similar to previous studies in which the substitution rate of MeV was estimated to be $3.40–9.02 \times 10^{-4}$ substitution/site/year. The substitution rate of MeV is significantly lower and more static than many other RNA viruses which have the ability to undergo rapid genetic change such as norovirus, influenza A virus, HIV-1 virus, and foot-and-mouth disease which evolved at an approximate rate of 10^{-3} substitution/site/year (41, 42). The D8 strains isolated from Shanghai found that maintained glycosylation of the HA gene virus could still be neutralized by the Chinese measles vaccine strain S191 (H1 genotype) (43). In the first D8 genotype outbreak in Zhejiang province, it was also found that emergent vaccination could stop the transmission of the measles D8 virus, indicating that the current Chinese measles vaccine is effective in preventing measles D8 genotype virus infection (18). The molecular and phylogenetic analysis could provide evidence to confirm the pathways of virus transmission and find variants in a timely manner which could warn us to take immediate prevention and control actions.

Nowadays, the importations of measles cases are challenges to the public health system and herd immunity in all countries (44). Each imported case and related case should be proficiently confirmed and promptly isolated. Before and after an imported case is recognized, their close contacts should be rapidly followed-up (quarantining or exclusion). Vaccinating susceptible individuals and using immunoglobulin are effective in blocking measles transmission in exposed susceptible high-risk people (45).

CONCLUSION

In this study, we reported on the first measles D8 genotype outbreak in the Zhoushan Islands which was caused by an imported cases from the Ukraine. There was a possible nosocomial infection between the first case and the second case. Then, the second case played an important role in the spread of virus due to their occupation. The strains isolated in the Zhoushan Islands belonged to the D8.2a sub-cluster and were clustered with KY120864/MVs/GirSomnath.IND/42.16/[D8] which was the predominant genotype D8 strain in the Ukraine during 2018–2019. The analysis of the complete D8 genotype genome

pointed out that this predominant strain might have originated from India in 2015. The molecular and phylogenetic analysis could provide evidence to confirm the pathways of virus transmission and find variants in a timely manner. Increasing and maintaining the high level of vaccination coverage (≥95%) and an efficient response to imported cases are essential to prevent and control the recurrence and outbreak of measles virus.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HW designed the study. CC, HZ, AT, BW, LL, and MW collected the data. CC and HZ analyzed the data. AT, BW, LL, MW, and HW checked the data and results. CC and HZ interpreted the data and wrote the report. HW revised the report from the preliminary draft to submission. All authors have read and approved the manuscript.

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CASE REPORT
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Persistent SARS-CoV-2 RNA Positive in Feces but Negative in Breastmilk: A Case Report of COVID-19 in a Breastfeeding Patient

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COVID-19 is a pandemic infectious disease. Whether SARS-CoV-2 was transmitted through breast milk is unknown. Here, we report a breastfeeding woman with COVID-19 presenting with gastrointestinal symptoms and persistent SARS-CoV-2 RNA positivity in both her oropharyngeal swabs and feces, but negativity in her breastmilk. After appearance of serum SARS-CoV-2-IgG, she began to bottle feed her baby with breastmilk without transmission. This report facilitates the understanding of breastfeeding-related risks in COVID-19.

Keywords: breastfeeding transmission, breastfeeding, fecal-oral transmission, SARS-CoV-2 RNA, COVID-19

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INTRODUCTION

In December 2019, there was an outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the causative pathogen of Corona Virus Disease 2019 (COVID-19), in Wuhan, Hubei Province, China. COVID-19 spread rapidly from Wuhan to other areas in the world and there were 85,320 confirmed cases in China and 11,327,790 confirmed cases worldwide until July 6 (1, 2). COVID-19 has been considered as a public health emergency of international concern by the World Health Organization (WHO) as it seriously threatens human health and quality of life.

COVID-19 mainly affects the lower respiratory tract and manifests as pneumonia in humans, and is mainly transmitted among subjects by respiratory droplets and contact route (3). Besides, a small cluster of patients initially presented with gastrointestinal symptoms (4). SARS-CoV-2 RNA could be detected in fecal samples from 53.42% of patients (5). This indicates that SARS-CoV-2 probably can be transmitted by the fecal-oral route (6, 7). However, few studies report the transmission of SARS-CoV-2 during breastfeeding. Whether SARS-CoV-2 can be transmitted from mother to their breast-fed babies via breastmilk is still unknown. In this study, we report a breastfeeding woman who was infected with SARS-CoV-2 and diagnosed with COVID-19 presenting with gastrointestinal symptoms with persistent SARS-CoV-2 RNA positivity in her feces but negativity in her breastmilk. She bottle fed her baby with her breastmilk after treatment. The baby seems healthy and unaffected after a 1-month follow up.

TABLE 1 | Results of RT-PCR Testing for the SARS-CoV-2.

	Oropharyngeal swabs	Fecal samples	Breastmilk samples
Illness day 15	Positive	NT	NT
Illness day 16	NT	NT	NT
Illness day 17	NT	NT	NT
Illness day 18	NT	NT	NT
Illness day 19	NT	NT	NT
Illness day 20	Negative	NT	NT
Illness day 21	Negative	NT	NT
Illness day 22	NT	Positive	NT
Illness day 23	Negative	NT	NT
Illness day 24	NT	NT	Negative
Illness day 25	NT	NT	Negative
Illness day 26	NT	Positive	NT
Illness day 27	NT	NT	NT
Illness day 28	NT	NT	NT
Illness day 29	NT	Positive	NT

"NT" represents samples were not tested. RT-PCR, Real-Time Reverse Transcriptase Polymerase Chain Reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

CASE REPORT

On January 24, 2020, a 30-year-old woman started to have changes of bowel habits. She used to have a bowel movement once a day with Bristol 3 feces. Now, she had a bowel movement 1–2 times per day with Bristol 4 and 5 feces, with increased borborygmi and urgency of defecation. Two days later (on January 26), she began to have fever at noon with a maximum body temperature of 37.8°C. Some tests were performed for her on January 30, 2020 to work up the cause of her fever. The blood routine examination showed that the count of lymphocytes was 0.54×10^9/L with a relative ratio of 11.1%, which was below the normal range (**Supplementary Table 1**). The level of high sensitive C reaction protein (hsCRP) was 5.87 mg/L, which was a little higher than the normal range (**Supplementary Table 1**). Even without any respiratory symptoms, she underwent a chest computerized tomography (CT) scan because of a history of recent contact with a COVID-19 patient. The CT scan reported as showing no abnormalities. Oropharyngeal swabs were tested positive for 2019-nCoV using real-time reverse-transcriptase–polymerase-chain-reaction (RT-PCR) which was performed by using a SARS-CoV-2 nucleic acid detection kit (Shanghai bio-germ Medical Technology Co) to detect the 2019-nCoV ORF1ab and N gene with a cycle threshold (Ct) value of 38 or less was defined as a positive on February 7, 2020 (**Table 1**). The extraction and detection methods were performed according to the manufacturer's protocol. Her body temperature fluctuated from 36.3 to 37.5°C after she took umifenovir (arbidol hydrochloride) 200 mg orally twice a day, which is an antiviral to treat influenza in China. She was admitted to the Union Hospital on February 9, 2020 with the diagnosis of COVID-2019. The patient has not reported any respiratory symptoms since January 24.

The patient stated that she was living in Wuhan, and two of her family members living with her were confirmed to be infected with SARS-CoV-2 on January 27 and January 30, 2020, respectively. Apart from a history of cesarean section to deliver an infant on January 16, 2020, the patient was an otherwise healthy non-smoker. The physical examination revealed a body temperature of 36.8°C, blood pressure of 120/87 mmHg, pulse of 90 beats per minute, respiratory rate of 14 breaths per minute, and subcutaneous oxygen saturation (SpO2) of 98%.

Blood routine examination showed that the count of lymphocyte increased to $0.9–1.3 \times 10^9$/L during hospitalization (**Supplementary Table 1**). A rapid nucleic acid amplification test (NAAT) for influenza A and B was negative. The antibodies of mycoplasma, chlamydia, Respiratory Syncytial Virus (RSA), adenovirus, and coxsackie virus were tested negative in her serum. In serum, the levels of D-dimer, alanine aminotransfease (ALT), aspartate transaminase (AST), lactic dehydrogenase (LDH), creatinine (Cr), and blood urea nitrogen (BUN) were in normal range (**Supplementary Table 1**). She repeated chest CT scans on February 7 and February 22, respectively, which were basically normal, showing no signs of viral pneumonia. She was treated with aerosolized interferon α2β from February 9, 2020.

Oropharyngeal swabs tests for SARS-CoV-2 were performed at the fourth and 5th days of the interferon treatment, and both of the results were negative. Because of her gastrointestinal symptoms, she was requested to have her stool examined. The stool routine test was normal, and no parasite eggs or fungi were detected, while fecal samples were persistently positive for SARS-CoV-2 RNA (**Table 1**). After taking the probiotics, *Saccharomyces boulardii* Sachets, her urgency of defecation subsided. Thus, she was discharged from the hospital after treatment and 1 week later, she began to bottle feed her baby with her breastmilk as breastmilk samples were tested negative for SARS-CoV-2 RNA (**Table 1**), and the IgG antibody of SARS-CoV-2 was tested positive in her serum. Since her fecal samples were positive for SARS-CoV-2 RNA, the patient did not contact her baby directly. She pumped the breastmilk, and her unaffected family member helped to feed the baby. The baby boy seems healthy during the following 1-month follow up, whose body temperature (**Figure 1**), consciousness, and growth were normal without any symptoms. The baby is still actively monitored by his family.

DISCUSSION

On the basis of previous reports from China, the most common transmission route of SARS-CoV-2 among subjects are respiratory droplets and contact, and fecal-oral route maybe one of the transmission routes (3). We have described a COVID-19 patient who was bottle feeding her baby with breastmilk without transmission. SARS-CoV-2 was not detected in her breastmilk at the time when her stool samples tested positive for SARS-CoV-2. The baby seems unaffected during the 1-month follow up. This indicated that it may be safe for a COVID-19 patient to feed her baby using breastmilk.

Most COVID-19 patients presented with cough and low-grade intermittent fevers with ground-glass opacity on chest CT

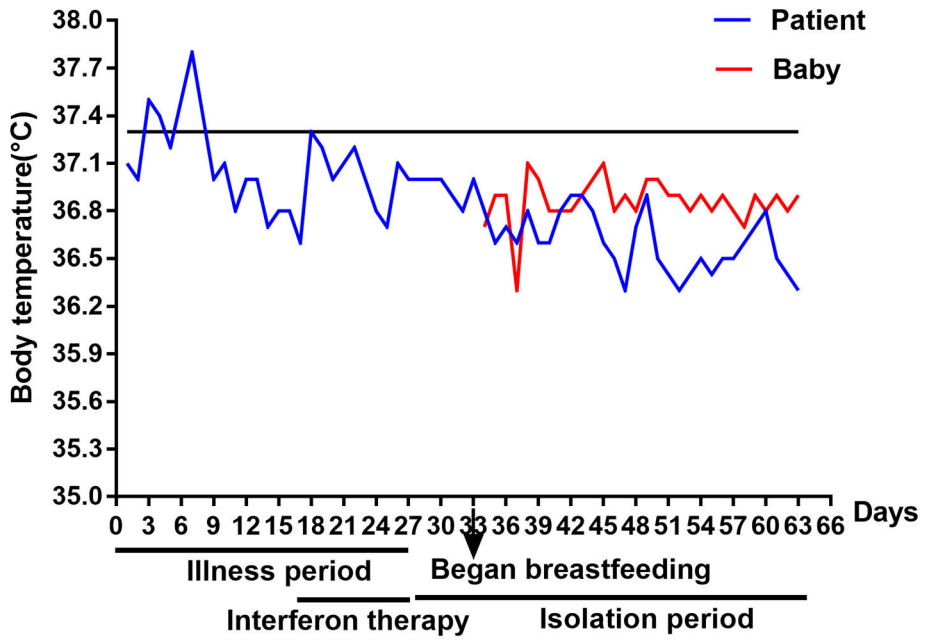

FIGURE 1 | Change of body temperature for the patient and her baby. The solid black line represents the lower limit of fever for subjects. The body temperature for the patient is higher from the 3rd day to the 8th day. The body temperature for the patient was in normal range during the following days including the breastfeeding period. And, the body temperature for the breast-fed baby was also in normal range during the 1-month follow up.

(8). While some patients initially presented with gastrointestinal symptoms, such as diarrhea and vomiting (4, 8). We have reported a COVID-19 patient initially presenting with change of bowel habits and increased borborygmi. These non-specific symptoms of mild illness in the clinical course of SARS-CoV-2 infection may be indistinguishable clinically from many other common gastrointestinal diseases. It is easy to be ignored by the patients. Key aspects of this case included the decision made by the patient to seek medical attention after recognition of the history of contacts with COVID-19 patients and prompt isolation and treatment of the patient. The mechanism that COVID-19 patients present with gastrointestinal symptoms is unclear. It was reported that SARS-CoV-2 uses angiotensin converting enzyme II (ACE2) for cell entry (9). Apart from the lung alveolar epithelial cells, enterocytes of the small intestine also express ACE2 (10). And, indeed, SARS-CoV-2 can enter enterocytes in the gastrointestinal tract and can possibly cause symptoms (5).

Detection of SARS-CoV-2 RNA in specimens from the upper respiratory tract suggests the potential transmissibility through droplets from the respiratory tract. Interestingly, we also detected SARS-CoV-2 RNA in stool samples from this patient. Several studies also reported that SARS-CoV-2 RNA could be detected from fecal samples of COVID-19 patients (4, 5). SARS-CoV-2 was also isolated from the mucus of intestine and esophagus (8). It was reported that the middle east respiratory syndrome (MERS) coronavirus could be detected in fecal samples from MERS patients (11). Further study confirmed that the middle east respiratory syndrome coronavirus could be transmitted through the fecal-oral route in the animal model (12). These indicated that the fecal-oral route may be the potential transmission route of SARS-CoV-2.

SARS-CoV-2 RNA was not detected in the breastmilk of our patient, which is consistent with another report (13). While other studies reported that SARS-CoV-2 RNA was detected in breastmilk for some cases with live viruses being undetected or not assessed (14–16). This maybe related with a low expression of ACE2 in breast (17). Moreover, there is no evidence showing that coronavirus can be transmitted via breastmilk in SARS or MERS cases (18, 19). In our study, the baby seems unaffected during breastfeeding. This indicates that SARS-CoV-2 probably is rarely transmitted through human milk. In this case, SARS-CoV-2 IgG antibody in serum of the patients may result in passive immunity during breastfeeding. Human milk testing of antibody from the patients and serum testing from the baby may provide a better understanding of the immune response to SARS-CoV-2 infection during feeding of human milk.

Limitations of this study was that we only observed clinical symptoms of the baby and did not test SARS-CoV-2 RNA or antibody from the baby. The baby may be asymptomatic despite being infected (20), which is probably related with the blunted immune response toward the SARS-CoV infection in children (21). Data on larger numbers of breastfeeding women infected with SARS-CoV-2 may help define infection risks and find prevention strategies.

In summary, this report, in conjunction with the reports from China, provides an initial view of the spectrum of illness and outcomes associated with breastfeeding-related SARS-CoV-2 infection. Multiple factors might contribute to this outcome, such as differences in host immune response, in the incubation period, and the presence of coexisting conditions.

Data on larger numbers of breastfeeding women infected with SARS-CoV-2 and long-term follow-up of babies are needed to fully understand the SARS-CoV-2 infection during breastfeeding. This will eventually provide a solid basis for clinical guidelines to manage future cases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The institutional board of Union Hospital, Huazhong University of Science and Technology (20200033). Affiliation: Union Hospital, Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HC was responsible for the acquisition, analysis, interpretation of data, and drafting of the manuscript. JL and JY provided assistance in data acquisition critical revision of the manuscript. TB provided assistance in data analysis. BS and LZ provided critical revision of the manuscript. LY and XH were responsible for the study concept and design, critical revision of the manuscript, and study supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.562700/full#supplementary-material

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OPINION
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Iterative Monitoring of Temperatures in Confinement for Early Screening of SARS-CoV-2 Infections

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Since the outbreak of the novel SARS-like coronavirus (SARS-CoV-2), more than 50,000,000 cases have been reported globally. The proportion of infected people with mild or no symptoms may be as high as 59% (1). However, they may still be contagious, as throat swabs detected high levels of the virus early in their illness, as was the case with symptomatic patients (2–8). It is worth noting that the peak viral load of SARS-CoV-2 was more than 1,000 times higher than SARS-CoV-1, and active SARS-CoV-2 replication in upper respiratory tract tissues has been found, whereas SARS-CoV-1 is not thought to replicate at this site (7). Some convenient and efficient large-scale-screening methods specific to mild/asymptomatic patients urgently need to be developed. Ferretti et al. (9) explored the possibility of protecting the population using questionnaires vs. algorithmic instantaneous coronavirus disease 2019 (COVID-19) contact tracing assisted by a mobile phone application. This tracing method may be very useful for identifying infected people (10), however some convenient and pre-diagnostic methods still need to be developed urgently, since people without symptoms do not always take the viral nucleic acid test, even if they have been in close contact with an infected individual.

As COVID-19 detection technology develops, national nucleic acid testing has become feasible in some countries. However, in other countries lacking diagnostic facilities, only symptomatic patients are subjected to the SARS-CoV-2 test. A high body temperature ($>37.3^{\circ}$C) is still the most distinguishable diagnostic criteria for SARS-CoV-2 infection.

A previous report using admission data of 41 patients in Wuhan, China, from Dec 16, 2019, to Jan 2, 2020 indicated that the most common symptom at onset of illness was fever, with a ratio of 98% (11). However, a later study with 99 patients in Wuhan from Jan 1 to Jan 20, 2020 demonstrated that 83% of the patients had a clinical manifestation of fever (12). Then a nation-wide clinical study of 1,099 cases from Dec 11, 2019 to Jan 29, 2020 in China found that fever was present in only 43.8% of the patients on admission but developed in 88.7% during hospitalization (13). Similarly, a study with a cohort of 366 patients with laboratory-confirmed COVID-19 in Sichuan, China, from January 2020 to February 2020 indicated that 69.3% of the mild patients and 79.1% of the severe patients showed body temperatures $<37.3^{\circ}$C on admission (14). These reports suggested that the virus has converted its infection strategy to adapt to the human body or that more asymptomatic patients (without fever) had been screened out as time went by. Considering that over 70% of COVID patients did not have fever on admission, the current definition of a viral fever of $\geq 37.3^{\circ}$C cannot discriminate all SARS-CoV-2 cases.

However, normal human body temperature has lowered (from 37°C to 36.5–36.7°C) over the past two centuries worldwide (15–17). The body temperature of older individuals is even lower,

with a basal body temperature of 0.23–0.56 degrees less than young individuals (18, 19). A rise of 1.0°C in some older infected individuals would not be defined as a fever case, if their basal body temperature was <36.3°C, which would result in missed diagnosis. Nevertheless, the older the infected individuals are, the more likely they are to develop severe acute respiratory distress syndrome (ARDS) and die (14, 20, 21). The presence of coexisting medical conditions was significantly higher in older patients compared with younger patients (55.15 vs. 21.93%), including hypertension, diabetes, heart disease, and chronic obstructive pulmonary disease. Thus, significantly higher rates of severe clinical type (16.18 vs. 5.98%), critical clinical type (8.82 vs. 0.77%), and shortness of breath (12.50 vs. 3.07%) were observed in older patients compared with younger patients (20). Accurate and early diagnosis of older people may be greatly helpful in reducing COVID-19 mortality.

LITERATURE SEARCH AND STUDY SELECTION

To further elucidate whether body temperature can be used as a pre-diagnostic indicator specific to mild/asymptomatic patients, we conducted a literature search of peer-reviewed publications in electronic databases from their inception to November 13, 2020. The databases used in the search procedure were PubMed, Embase, ISI Web of Science, and medRxiv. The following two key terms were employed for the literature search: "body temperature" and "SARS-CoV-2 or COVID-19." Through these searches, we obtained a total of 175 results in PubMed and 1,735 results in medRxiv, irrespective of the language, date of publication, and nationality, race, age, or gender of the participants. Two authors (SCJ and SY) independently screened the titles and abstracts to remove the ineligible studies. Disagreements were resolved by discussion. We retrieved the full text of the potentially eligible studies and examined full-text reports for further evaluation. In cases where there were multiple reports for the same study, we used the last published report. During the subsequent full-text screening, articles without the average (median) body temperature data from both mild and severe patients were excluded. Finally, only four reports met the criteria.

A RISE OF 0.5°C WOULD BE A DIAGNOSTIC CRITERIA FOR MOST SARS-CoV-2 INFECTIONS

Then the above four reports were analyzed further. A nationwide clinical study of 1,099 cases from Dec 11, 2019 to Jan 29, 2020 in China found that fever (≥37.5°C) was present in only 43.8% of the patients on admission but developed in 88.7% during hospitalization (13). The average body temperature of the patients on admission was 37.3°C, and the average maximum temperature during hospitalization was 38.3°C (13), which were 0.5°C and 1.5°C higher, respectively, than the normal body temperature of 36.8°C (19). Interestingly, there is no significant difference in the median temperature on admission (37.3 and 37.4°C for non-severe patients and severe patients respectively), although the proportion of the severe patients with body temperatures >38.0°C (21.6% of patients had body temperatures of 38.1–39.0°C and 4.7% of patients had body temperatures >39.0°C) was higher than that of the mild patients with temperatures >38.0°C (17.6% of patients had body temperatures of 38.1–39.0°C and 3.3% of patients had body temperatures >39.0°C) (13).

Clinical features of 5,279 patients with SARS-CoV-2 infections in New York City, USA, similarly showed that the average temperatures at presentation for non-hospitalized patients and hospitalized patients were 37.3 and 37.5°C, respectively (22). The proportions of temperatures ≥38°C at presentation were 5.0 and 33.5% for non-hospitalized patients and hospitalized patients respectively (22). Similar body temperatures on admission have been reported for adult inpatients from Korea (37.04 and 37.81°C for 198 cured patients and 13 transferred severe patients, respectively) (23). Slightly higher median temperatures have also been reported in 36 children patients on admission in Zhejiang province, China (37.6 and 38.0°C for mild cases and moderate cases, respectively) (24).

Thus, body temperature is a good indicator for viral infection (either symptomatic or asymptomatic). A rise of at least 0.5°C may be a diagnostic criteria. However, as mentioned above, many patients' basal body temperatures are below 36.8°C (especially in older patients), and a rise of 0.5°C would not be defined as a fever case, resulting in missed diagnosis.

THE METHOD OF ITERATIVE MONITORING OF BODY TEMPERATURES

How to accurately detect the 0.5°C rise in body temperature poses a big challenge to clinicians. An individual's body temperature can change significantly within a day (<1.0°C), influenced by diet, exercise state, mental factors, and so on (25). A rise of 0.5°C could not be discriminated accurately. In order to reflect the changing trend more accurately, we propose a method of iterative monitoring of body temperatures: recording axillary temperatures of close contacts every morning (immediately after getting up) and every night (about 2 h after supper). The average temperature of the first 3 days of the quarantine period (six measurements) should be taken as the basal value. Then if the average temperature of the next 3 days (the 4th, 5th, and 6th days) could be more than 0.5°C higher than the reference value, it would be suggested to do the SARS-CoV-2 infection test. If the temperature continued to rise in the following 2 days (both the average temperature of 5th, 6th, and 7th days ≥ the basic value + 0.5°C and the average temperature of 6th, 7th, and 8th days ≥ the basic value + 0.5°C), it would be strongly suggested to carry out the SARS-CoV-2 infection test. If it was not more than 0.5°C higher than the reference value, then the recording would continue until the end of the 14th day of the quarantine period (see the flow chart in **Figure 1**). However, the positive detection rate

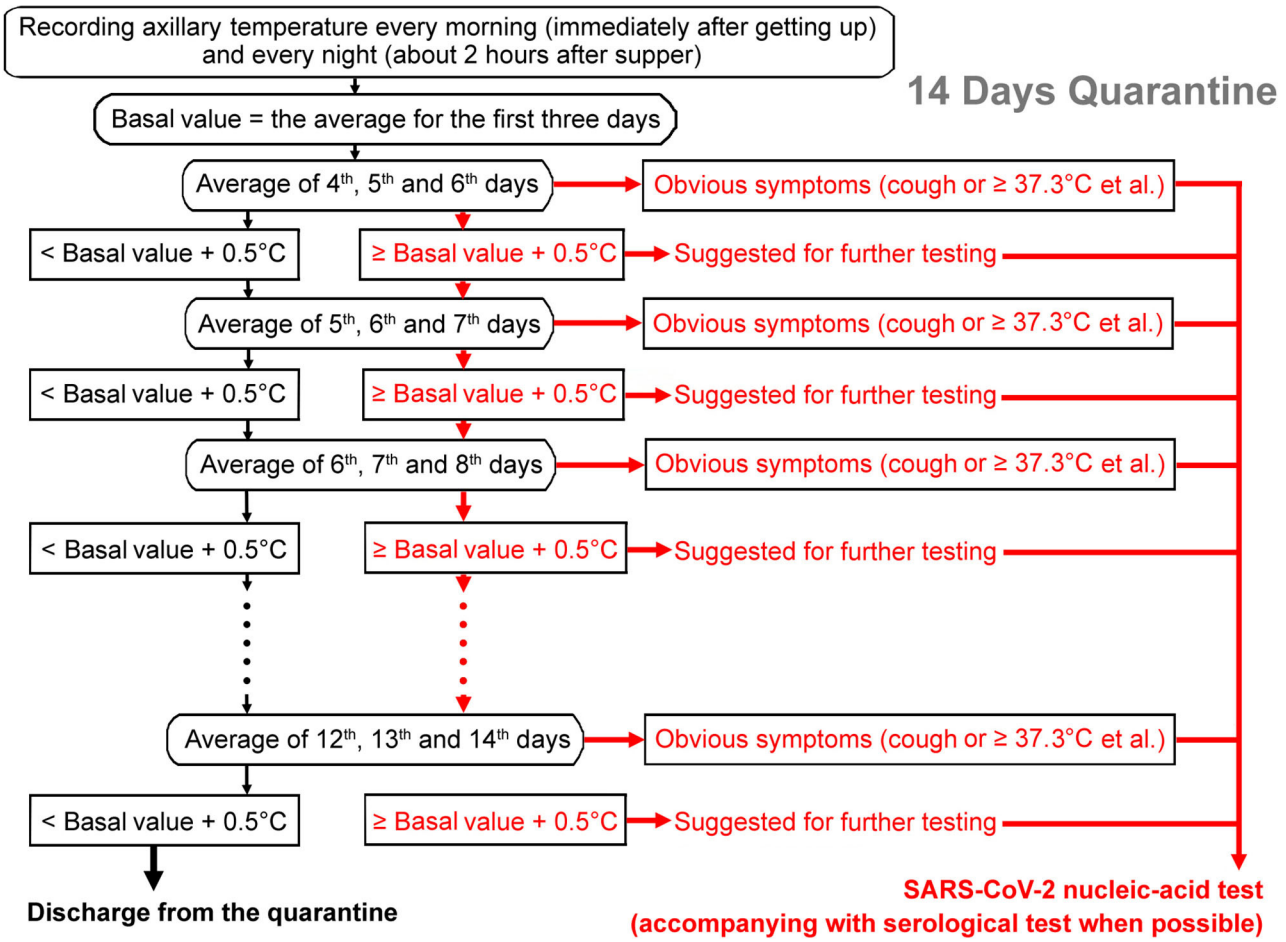

FIGURE 1 | Flow chart of the iterative temperature monitoring method to find possible infections of SARS-CoV-2. The average temperature of the first 3 days of the quarantine period would be taken as the basal value. Then if the average temperature of the next 3 days is more than 0.5°C higher than the reference value, it would be suggested to do a SARS-CoV-2 infection test (pink arrows). If the temperature continued to rise in the following 2 days or apparent symptoms (for example, fever or cough.) developed, it would be strongly suggested to carry out a SARS-CoV-2 infection test (red arrows). If the temperature is not higher than the reference value by more than 0.5°C, then the recording would continue until the end of the 14th day of the quarantine period (black arrows). In addition to a nucleic-acid test, adjunct of serological test is recommended when possible.

of a single nucleic-acid test is only 30–50% (26, 27). With the rapid advances in SARS-CoV-2 IgM-IgG antibody tests, adjuncts of serological tests would improve the accuracy in COVID-19 diagnosis. Thus, combined detection of SARS-CoV-2-specific antibodies and nucleic acid has been recommended when possible (26, 27).

This temperature monitoring method could be performed at home without any sophisticated equipment and therefore satisfies the need for large-scale screening, especially in countries lacking diagnostic facilities. The method would also build up an individual temperature reference and therefore satisfies the need for personalized diagnoses. Considering that not all patients could accurately calculate the average temperature by themselves, the everyday temperature recording may be uploaded to an online phone app, as Ferretti et al. (9) proposed, or the calculation could be performed by a computer program (28), and then the CDC would arrange the virus tests for the suspected patients.

CONCLUSIONS

Body temperature is a good indicator for SARS-CoV-2 infection. Evidence profiles of the two reports with 6378 patients showed that the average body temperature of the patients on admission was 37.3°C, which was 0.5°C higher than the normal body temperature of 36.8°C. However, many patients' basal body temperatures are below 36.8°C (especially in older patients with ages ≥60), and a rise of 0.5°C would not be defined as a fever symptom. The current definition of viral fever of ≥37.3°C cannot discriminate all SARS-CoV-2 infections. Here we propose that if the average temperature of 3 days is more than 0.5°C higher than the reference value, it would indicate an infection. If the temperature continued to rise in the following 2 days, it would be strongly suggested to carry out a SARS-CoV-2 infection test. It is now clear that many individuals who are infected (and even those with mild symptoms) do not exhibit fever. Thus, our approach could not discriminate all SARS-CoV-2

infections. However, this method may be helpful to quickly identify contagious asymptomatic patients and older infected individuals with higher risks of death.

AUTHOR CONTRIBUTIONS

SY conceptualized the analysis and wrote the original draft. S-CJ and Z-LL reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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Current and Promising Antivirals Against Chikungunya Virus

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Chikungunya virus (CHIKV) is the causative agent of chikungunya fever (CHIKF) and is categorized as a(n) (re)emerging arbovirus. CHIKV has repeatedly been responsible for outbreaks that caused serious economic and public health problems in the affected countries. To date, no vaccine or specific antiviral therapies are available. This review gives a summary on current antivirals that have been investigated as potential therapeutics against CHIKF. The mode of action as well as possible compound targets (viral and host targets) are being addressed. This review hopes to provide critical information on the *in vitro* efficacies of various compounds and might help researchers in their considerations for future experiments.

Keywords: antiviral design, CHIKV therapy, direct antiviral action, host-targeting antiviral, comparison of *in vitro* efficacies, favipiravir, ribavirin

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INTRODUCTION CHIKUNGUNYA VIRUS

Chikungunya virus (CHIKV) is a single-stranded RNA virus with a positive sense genome of about 11,800 nucleotides. CHIKV structure and genome organization follow those of all alphaviruses. The virion has a lipid-bilayer envelope that is tightly associated with an icosahedral nucleocapsid shell (240 capsid copies) which encapsidates genomic RNA (1). The genome contains two open reading frames (ORFs), which encode the non-structural (ns) or replicase polyprotein and the structural polyprotein.

CHIKV is primarily transmitted to humans by the bite of an infected mosquito, mainly of the *Aedes* species. CHIKV causes the so-called chikungunya fever (CHIKF) which is characterized by high fever, headache and the hallmarks of the disease, myalgia and polyarthralgia (1). The latter especially can last for months or even years after the acute phase of the illness has passed, causing a severely deteriorated quality of life for the patient. The resulting stooped bearing and rigid gait of infected individuals are described in the word origin of the disease "kungunyala," which is Makonde for "that which bends up." CHIKV was first described in 1955 by Robinson and Lumsden after an outbreak in present-day Tanzania in 1952 (2).

Until 2004, CHIKV was mainly distributed in tropical and subtropical regions of sub-Sahara Africa and Southeast Asia. It caused sporadic outbreaks mainly during the rainy season. In 2004, however, a massive outbreak in Kenya led to close to half a million infected people. This epidemic initiated the spread to more than 22 countries, including countries with a moderate climate such as France and Italy (**Figure 1**) (5).

Following the bite of a CHIKV infected mosquito, the virus is transported to the nearest lymph node and transferred to monocytes and macrophages which enter the bloodstream. At this point, viremia sets in by the active infection of human blood monocytes and other peripheral blood mononuclear cells. CHIKV then reaches the muscles and joints, where the infection causes the main symptoms of CHIKF—myalgia and arthralgia (6). Apart from muscles and joints, CHIKV may also target a range of secondary organs and thus cause severe complications in patients

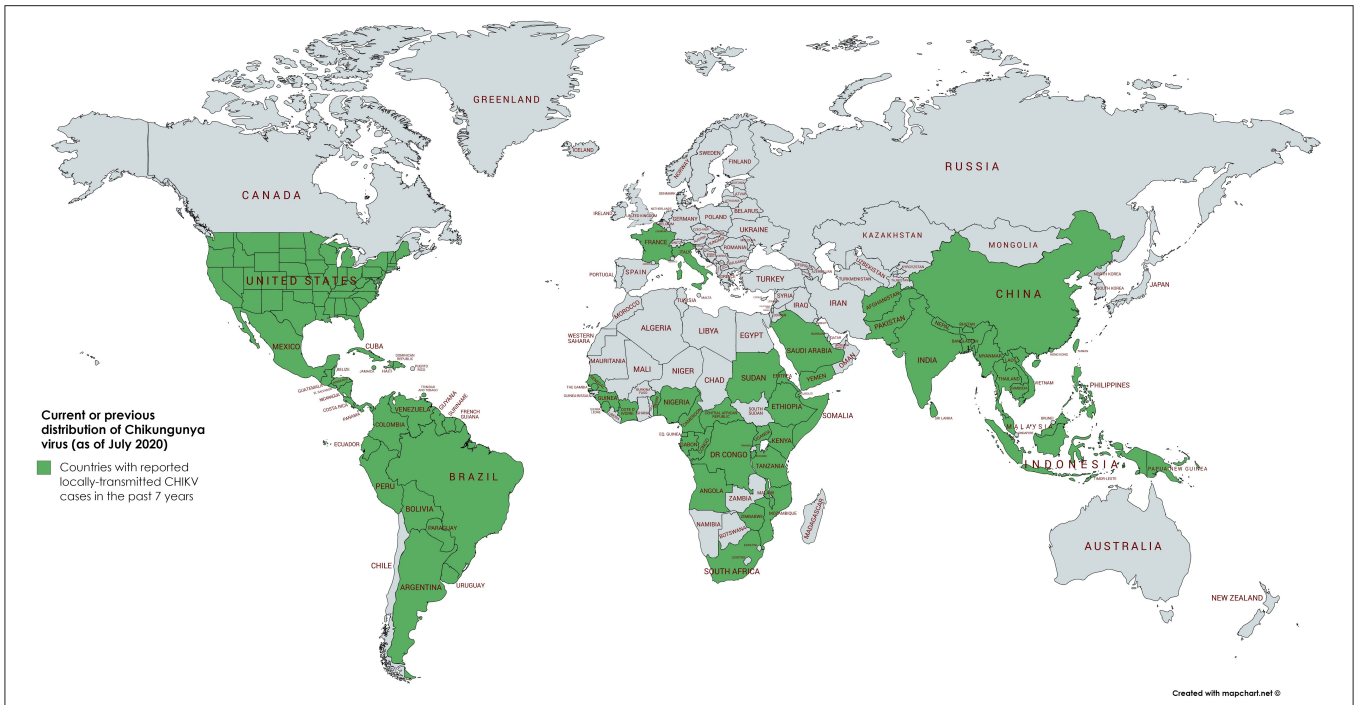

FIGURE 1 | Geographical distribution of CHIKV. Word map showing the distribution of autochthonous CHIKV transmissions in the past 7 years (2013–2020) highlighted in green. In the continental United States of America, only travel associated cases have been reported in the past 3 years. As of July 2020, the latest autochthonous cases in Europe have been recorded in France and Italy in 2017 (3, 4).

(i.e., renal, respiratory, hepatic, cardiac, and neural syndromes) (7). As neither specific antiviral drugs nor a licensed vaccine are available, the therapy of CHIKF is based on supportive measures and the treatment of symptoms [non-steroid anti-inflammatory drugs (NSAIDs) and fluid therapy (8)].

For detailed information on CHIKV epidemiology, replication, disease mechanism, and prophylaxis, we refer to the reviews of Silva and Dermody (1), Pietila et al. (9), and Hucke et al. (10).

ANTIVIRALS AGAINST CHIKUNGUNYA VIRUS

Direct-Acting Antivirals

The following chapter will deal with various compounds that are or have been in the focus of research and showed some promising results *in vitro* mainly against CHIKV and/or other relevant alphaviruses. Various compounds made it to *in vivo* studies but so far there is no licensed therapeutic drug acting directly against CHIKV or any other alphavirus. There are many compounds which are currently under investigation for their anti-CHIKV efficacy. However, as the scope of this review is limited, we will only discuss compounds that either showed efficacy in a variety of *in vitro* assays or were repeatedly investigated by different (independent) research groups. It must be noted that compound efficacy can vary considerably, depending on the cell line, virus strain, or assay method that is being used. **Table 1** illustrates this fact and gives an overview of EC_{50}/CC_{50} values of common substances used as experimental controls in *in vitro* trials. For the interested reader I refer to the reviews of Abdelnabi et al. (38), Subudhi et al. (39) and the review of da Silva-Junior et al. (40), focussing on the medicinal chemistry of synthetic and natural compounds against CHIKV. Furthermore, the review of Bugert et al. (41) inspects antivirals against alphaviruses and other viral agents relevant in medical biodefence.

CHIKV Entry Inhibitors

Chloroquine

Chloroquine is a licensed drug for the prophylaxis and treatment of malaria. Furthermore, it is prescribed for the treatment of systemic lupus erythematosus and rheumatoid arthritis (42). Chloroquine also shows *in vitro* antiviral activity against several viruses, such as human immunodeficiency virus (HIV), severe acute respiratory syndrome (SARS) coronavirus and alphaviruses (43). Khan demonstrated that chloroquine is able to inhibit CHIKV replication in VeroA cells in a dose-dependent manner. Apart from this mode of action, it is also assumed that the drug interferes with the endosome-mediated CHIKV internalization. Bernard et al. (44) showed that chloroquine raises the endosomal pH by interfering with the protonation of the endocytic vesicles and thereby prevents the E1 fusion step needed for the release of CHIKV RNA into the cell cytoplasm. Various research groups used chloroquine as a reference compound in their *in vitro* studies (**Table 2**) (11–15, 45).

Despite the promising results chloroquine displays in *in vitro* studies, clinical trials with the drug failed to prove any benefit

TABLE 1 | Comparison of compounds with anti-CHIKV property.

Compound	EC_{50} (μM)	CC_{50} (μM)	References
CHIKV entry inhibitors			
Chloroquine (reference)	5–11	>36–100	(11–17)
Suramin	8.8–62.1	350 to >700	(18)
Suramin conjugates	1.9–2.7	50 to >200	(19)
nsP1 inhibitors			
Lobaric acid	5.3–16.3	50–76	(20)
[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-ones (lead)	<1.0	>668	(21)
[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one (compound 8)	1.1–5.3	>300	(14)
nsP2 inhibitors			
Bassettos *in silico* lead (compound 1)	5	72	(12)
1,3-thiazolidin-4-one (compound 8)	1.5	>200	(22)
Compound ID1452-2	31	n.d.	(23)
nsP4 inhibitors and inhibitors of viral genome replication			
Ribavirin	2.05–756.8	49 to >500	(16, 24–29)
β-d-N4-hydroxycytidine (NHC)	0.2–1.8	2.5–30.6	(30)
Favipiravir (T-705)	16–245.13	>636	(25, 31)
Defluorinated Favipiravir (T-1105)	7–47	>571	(31)
Sofosbuvir	1–17	402	(24)
Mycophenolic acid (MPA)	0.5–1.6	370	(16, 24, 32)
Protein kinase C inhibitors			
Prostratin	0.2–8	50 to >100	(11, 33)
12-O-tetradecanoylphorbol 13-acetate (TPA)	0.0029	5.7	(11)
Phorbol-12,13-didecanoate	0.006	∼4.1	(13)
12-O-decanoylphorbol 13-acetate (DPA)	2.4	4.6	(34)
12-O-decanoyl-7-hydroperoxy-5-ene-13-acetate phorbol	4.0	7.8	(34)
Neoguillauminin A	17.7	∼35	(15)
12-deoxy phorbol Compound 1	0.13	12.7	(15)
12-deoxyphorbol Compound 2	0.02	4.85	(15)
12-deoxyphorbol Compound 4	0.02	30.0	(15)
Trigocherrin A	1.5	35	(17)
Multiple/unidentified targets			
Micafungin	17.2–20.63	>100	(35)
Abamectine	1.4 ± 0.9 (Huh-7.5) and 1.5 ± 0.6 (BHK-21)	15.2 ± 1.0 (Huh-7.5) and 28.2 ± 1.1 (BHK-21)	(36)
Ivermectine	1.9 ± 0.8 (Huh-7.5) and 0.6 ± 0.1 (BHK-21)	8.0 ± 0.2 (Huh-7.5) and 37.9 ± 7.6 (BHK-21)	(36)
Berberine	1.9 ± 0.9 (Huh-7.5) and 1.8 ± 0.5 (BHK-21)	>100 (Huh-7.5 and BHK-21)	(36)
coumarin derivatives conjugated with guanosine	9.9–13.9	96.5–212	(37)

The above mentioned compounds have been in the focus of studies during the past 7 years (with the exception of the reference compounds chloroquine and ribavirin). The compounds have been arranged according to their (known) point of interaction. EC_{50} and CC_{50} may display a broad range due differences in cell line, virus strain, and assay method within the study. Unless stated otherwise, EC_{50} and CC_{50} were generated with Vero cell lines.

BHK, baby hamster kidney cells; CC50, cytotoxicity concentration 50%; CHIKV, Chikungunya virus; EC50, half maximal effective concentration; Huh, Human hepatocarcinoma cells; n.d., not determined; nsP, non-structural protein.

for the patient. Trials for prophylaxis or treatment of CHIKV infection either in macaque models or human patients could not demonstrate advantage of chloroquine over meloxicam (an NSAID) administration (46, 47). The discrepancy between *in vitro* and *in vivo* effectiveness of chloroquine has been described before.

TABLE 2 | Efficacy of selected compounds against CHIKV according to different studies.

Compound	Cell line	CHIKV strain; MOI	EC_{50} (μM)	CC_{50} (μM) (SI)	Assay method	References
Ribavirin (RBV)	Vero	Ross C347 strain; MOI = 0.001	341.53		plaque/microscope/trypan blue	(27)
	Vero	vaccine strain 181/clone 25; MOI = 0.0001	408.2	266.5 (SI = 0.65)	Tox: Viral ToxGlo (Promega), Inf: Virus quantification via plaque assay	(25)
	Huh-7	vaccine strain 181/clone 25; MOI = 0.1	10.56	49 (SI = 4.64)	Tox: Viral ToxGlo; Inf: plaque assay	(25)
	A549	vaccine strain 181/clone 25; MOI = 0.1	480.11	205.86 (SI = 0.43)	Tox: Viral ToxGlo; Inf: plaque assay	(25)
	Vero	ECSA clinical isolate; MOI = 2	10.95	n.d.	Tox: MTT; Inf: plaque formation assay, ELISA-like cell-based assay and IFT	(26)
	Vero	vaccine strain 181/clone 25 (NR-13222); MOI = 0.0001	419.43	n.d.	Inf: plaque assay	(28)
	BHK21	CHIKV-0708 Singapore not mutated; MOI = 1	2.05	n.d.	IFT	(29)
	Huh-7	CHIKV (Asian strain); MOI = 0.1	2.5 ± 0.3	298 ± 22 (SI = 120)	RNA level (RT-PCR)	(24)
	Huh-7	CHIKV (Asian strain); MOI = 0.1	5.5 ± 1.5	298 ± 22 (SI = 54)	Virus titer (yield) by plaque	(24)
	Vero E6	ITA07-RA1; MOI = 0.005	423.6 ± 27.5	>500 (SI > 1.18)	MTS (Promega)	(16)
	Vero E6	LS3; MOI = 0.005	756.8 ± 22.4	>500 (SI > 0.66)	MTS (Promega)	(16)
	Vero E6	LS3-GFP; MOI = 0.005	466.7 ± 38.0	>500 (SI > 1.07)	MTS (Promega)	(16)
	BHK21	ITA07-RA1; MOI = 0.005	20.8 ± 1.1	>500 (SI > 24.04)	MTS (Promega)	(16)
	BHK21	LS3; MOI = 0.005	15.6 ± 1.5	>500 (SI > 32.05)	MTS (Promega)	(16)
	BHK21	LS3-GFP; MOI = 0.005	17.5 ± 1.7	>500 (SI > 28.57)	MTS (Promega)	(16)
Favipiravir (T-705)	Vero	vaccine strain 181/clone 25; MOI = 0.0001	184.53	>6365.4 (SI > 34.5)	Tox: Viral ToxGlo (Promega), Inf: plaque assay	(25)
	Huh-7	vaccine strain 181/clone 25; MOI = 0.1	127.3	>6365.4 (SI > 50)	Tox: Viral ToxGlo; Inf: plaque assay	(25)
	A549	vaccine strain 181/clone 25; MOI = 0.1	245.13	>6365.4 (SI > 25)	Tox: Viral ToxGlo; Inf: plaque assay	(25)
	Vero A	Indian Ocean 899; MOI n.s.	60 ± 10	>636 (SI > 10.6)	MTS (Promega)	(31)
	Vero A	LR2006-OPY1; MOI = 0.1	25 ± 1	>636 (SI > 25.44)	MTS (Promega)	(31)
	Vero A	Italy 2008 (clin.); MOI = 0.1	16 ± 6	>636 (SI > 39.75)	MTS (Promega)	(31)
Sofosbuvir	Huh-7	CHIKV Asian strain; MOI = 0.1	1.0 ± 0.1	402 ± 32 (SI = 402)	Inf: RNA level (RT-PCR); Tox: XTT and PMS	(24)
	Huh-7	CHIKV Asian strain; MOI = 0.1	2.7 ± 0.5	402 ± 32 (SI = 149)	Inf: Virus titer (yield) by plaque; Tox: XTT and PMS	(24)
	Stem cells derived astrocytes (iPSCs)	CHIKV Asian strain; MOI = 1	17 ± 5	n.d.	Virus titer (yield) by plaque	(24)

(Continued)

TABLE 2 | Continued

Compound	Cell line	CHIKV strain; MOI	EC_{50} (μM)	CC_{50} (μM) (SI)	Assay method	References
Mycophenolic acid (MPA)	Huh-7	CHIKV Asian strain; MOI = 0.1	0.8 ± 0.05	370 ± 55 (SI = 463)	Inf: RNA level (RT-PCR); Tox: XTT and PMS assay	(24)
	Huh-7	CHIKV Asian strain; MOI = 0.1	1.1 ± 0.2	370 ± 55 (SI = 336)	Inf: Virus titer (yield) by plaque; Tox: XTT and PMS	(24)
	Huh-7	recombinant CHIKV-118- GFP; MOI = 0.5	1.6	> 100 (SI >62)	Resazurin reduction assay	(32)
	Vero E6	ITA07-RA1; MOI = 0.005	0.6 ± 0.03	>50 (SI > 83.3)	MTS (Promega)	(16)
	Vero E6	LS3; MOI = 0.005	0.6 ± 0.01	>50 (SI > 83.3)	MTS (Promega)	(16)
	Vero E6	recombinant LS3-GFP; MOI = 0.005	0.5 ± 0.07	>50 (SI > 100)	MTS (Promega)	(16)
Prostratin	Vero	CHIKV Indian Ocean strain 899; MOI n.s.	2.7 ± 1.2	~60 (SI~22.8)	MTS (Promega)	(11)
	BGM	CHIKV Indian Ocean strain 899; MOI = 0.001	8 ± 1.2	>100 (SI >12.5)	MTS/PMS (Promega)	(33)
	BGM	CHIKV Indian Ocean strain 899; MOI = 0.001	7.6 ± 1.3	>100 (SI > 13.16)	qRT-PCR	(33)
	BGM	CHIKV Indian Ocean strain 899; MOI = 0.001	7.1 ± 0.6	>100 (SI > 14.08)	titration assay	(33)
	human skin fibroblasts CRL-2522	Singapore (SGP011), Caribbean strain (CNR20235) + Reunion Island strain (LR2006 OPY1); MOI = 1	0.2-0.5	50 (SI = 100–250)	luciferase assay, qRT-PCR + titration assay	(33)
Chloroquine	Vero	CHIKV Indian Ocean strain 899	10–11	89–100 (SI = 8-9)	CPE reduction, RT-qPCR, MTS (Promega)	(11, 12, 15, 17)
	Vero E6	ITA07-RA1 MOI = 0.005	7.4 ± 1.1	>36 (SI >4.86)	MTS (Promega)	(16)
	Vero E6	LS3 MOI = 0.005	10.6 ± 1.6	>36 (SI >3.4)	MTS (Promega)	(16)
	Vero E6	LS3-GFP MOI = 0.005	5.0 ± 1.7	>36 (SI >7.2)	MTS (Promega)	(16)

The above mentioned compounds have been repeatedly used in in vitro studies as references. Compound efficacy may considerably between the different studies, depending on the cell line, virus strain, and assay method that is being used. The table aims to give an orientation at what range a control compound might be effective against CHIKV in different cell lines and assay methods.

A549, human lung carcinoma cells; BGM, buffalo green monkey kidney cells; BHK, baby hamster kidney cells; CC50, cytotoxicity concentration 50%; CHIKV, Chikungunya virus; CPE, cytopathic effect; EC50, half maximal effective concentration; ECSA, East/Central/South African strain; GFP, green fluorescent protein; Huh, Human hepatocarcinoma cells; IFT, immunofluorescence test/staining; Inf, infection assay; MOI, multiplicity of infection; MTT/MTS, 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide (salt) assay; n.d., not determined; n.s., not stated; RT-PCR, reverse transcriptase polymerase chain reaction; SI, selectivity index (= CC50/EC50); Tox, toxicity assay; Vero, African green monkey kidney cells.

Epigallocatechin Gallate (Green Tea Component)

Epigallocatechin gallate (EGCG) is an active polyphenolic catechin and the essential element of green tea (*Camellia sinensis*) extract. Various independent research groups discovered the antiviral properties of EGCG against a number of viruses and recent studies revealed that EGCG also inhibits CHIKV replication *in vitro*. Weber et al. (48) demonstrated that EGCG inhibits CHIKV replication in HEK 293T cells by blocking the entry of CHIKV pseudo-particles that carried the CHIKV envelope proteins. Thus, EGCG prevented the attachment of CHIKV to the target cells.

More recently, Lu et al. (49) showed the benefits of synergism in the combination treatment of CHIKV infected U2OS cells (human bone osteosarcoma cells) with EGCG and suramin. Lu tested EGCG combined with suramin against the CHIKV strain S27 and two clinical isolates. Besides the synergistic effect of the two compounds, Lu could confirm that the EGCG inhibits virus entry, replication, progeny yield as well as CPE of CHIKV *in vitro*.

Suramin

Suramin, also known as germanin or Bayer-205, is a symmetrical hexasulfonated naphthylurea compound that has been market-authorized by the U.S. Food and Drug Administration (FDA) for the treatment of trypanosomiasis (trypanosome-caused river blindness, onchocerciasis). The drug acts as a competitive inhibitor of sulphated glycosaminoglycans (GAGs) and heparin. As a number of viruses attach to cells via GAGs, suramin may consequently have anti-viral activity by inhibiting virus entry. The drug proved effective against a number of viruses, including DENV and Venezuelan equine encephalitis virus (VEEV) (50, 51). Against CHIKV, suramin proved effective in various *in vitro* studies (18, 52, 53). Suramin diminished CPE, virus replication and yield in a dose-dependent manner. Ho et al. (18) demonstrated that suramin was broadly effective *in vitro* against various CHIKV strains (**Table 1**). Ho used BHK-21, U2OS and MRC-5 cells. His group was the first to prove that the compound inhibits entry and transmission of CHIKV through binding onto E1/E2 glycoproteins. Furthermore, they showed that CHIKV infection was hampered in early stages. Virus binding and fusion was disrupted by the binding of suramin with viral glycoproteins. The compound also interfered with virus release. According to their research the EC_{50} of suramin for the inhibition of CHIKV *in vitro* (EC_{50} of 8.8–62.1 μM) is well within the range of non-toxic serum concentrations in humans (70 μM) when treated for river blindness (54).

Henß et al. (53) were also able to verify that suramin blocks CHIKV at early stages of the infection. Furthermore, her group tested the compound successfully against Ebola virus. All her tests were done *in vitro* (HEK 293T, MCF7, and Huh-7 cells). According to Henß however, the drug's side effects on the patient (nausea, vomiting, reversible urticarial rash, kidney damage, and exfoliative dermatitis; furthermore, suramin is connected to hepatic and bone marrow toxicity) might make suramin inappropriate for the treatment of CHIKV infections, a rather mild disease compared to Ebola. To avoid these side effects, Hwu et al. (19) chemically modified suramin and used 20 new conjugated compounds in a CPE screening assay against CHIKV. He identified six compounds with promising activity against CHIKV.

Inhibitors of Viral Genome Replication and Translation

RNA Interference (RNAi) Targeting CHIKV Genes

Small interfering RNA (siRNA) is able to regulate gene expression by the cleavage of the corresponding messenger RNA (mRNA) (55). The most commonly understood effect of this mechanism is the inhibition of the protein synthesis of certain genes because the mRNA is no longer available. This is referred to as "gene silencing." The discovery that siRNA is able to inhibit specific genes has led to a vast interest in this particular field. SiRNA was hoped to be used as a potential therapy for the treatment of genetic disorders, cancer, viruses, and other diseases. Bitko and Barik (56) showed that RNA interference (RNAi) was able to inhibit a negative-strand RNA virus.

Since RNAi is an endogenous biological process, potentially every gene can be supressed. In addition to that, siRNAs are easier to identify, synthesize and produce on a large scale than traditional drugs (57). Multiple studies have been conducted to test the possible efficacy of siRNA against viruses *in vitro* and *in vivo* (mice, guinea pigs, macaques and humans) (58). There are two approaches for recruiting RNA interference as antivirals: (1) targeting specific viral sequences; (2) targeting the host cell.

(1) Targeting specific viral sequences with synthetic siRNA:

SiRNA can be created in the laboratory and preferably targets conserved regions. Theoretically any specific viral gene can be disabled. This is an advantage over classical small drug molecules that have to be fitted to a target protein which usually is only present at certain sites in the cell (59).

Dash et al. (60) designed and evaluated siRNA sequences targeting CHIKV nsP3 and E1 genes in Vero cells. They could demonstrate that these siRNAs curbed CHIKV titres by 99.6% in siRNA transfected cells 24 h after infection. However, this reduction could not be sustained at 72 h, possibly because of the intracellular degradation of the siRNA. In 2013, Parashar et al. conducted *in vitro* studies in Vero-E6 cells, where he used siRNAs targeting nsP1 and/or E2 mRNA. He succeeded in downregulation of CHIKV replication for more than 90%. *In vivo* studies in CHIKV-infected Swiss albino and C57 BL/6 mice showed a complete inhibition of CHIKV replication when these siRNAs were administered 72 h post-infection (61). Lam et al. (62) could also demonstrate that CHIKV infection could effectively be supressed in the mouse model when pre-treating the animals with (small hairpin) shRNA (a precursor form of siRNA) against CHIKV E1 and nsP1 (62).

More recently, due to its advantages over siRNA and shRNA as far as stability, effectiveness, and toxicity are concerned, the artificial miRNA (amiRNA) based approach is in the focus of research. Bhomia et al. (63) showed the effectiveness of amiRNA for inhibition of Venezuelan equine encephalitis virus (VEEV). Saha et al. (64) successfully tested vector-delivered amiRNA against CHIKV infected Vero cells and efficiently inhibited CHIKV replication. One problem arising from this approach is the development of resistant mutants. A possible solution might be a combination therapy with a cocktail of various siRNAs.

(2) Targeting the host cell with siRNA:

It is also possible to target mRNAs for cellular accessory or entry proteins so that they can no longer be used by the virus during infection. Researchers tried to use the mutationally more stable host proteins as targets instead of the rapidly mutating viral proteins (58).

Rathore et al. were able to show in 2014 that by silencing the heat shock protein 90 (Hsp90) transcripts with siRNA, CHIKV replication is interrupted in cultured cells. Heat shock protein 90 (Hsp90) is known to play a key role in the replication of CHIKV and other viruses and is a highly abundant molecular chaperone (65). Rathore found out that Hsp90 interacts with the nsP3 and nsP4 proteins of CHIKV to promote virus replication (66). For further "Host-targeting Antivirals" (see section Antivirals Against Chikungunya Virus).

Both siRNA approaches (viral or host target approach) share the same issues in bioavailability, delivery, and specificity. siRNA is not very stable. It is rapidly degraded in the cell/organism. Furthermore, when systemically applied, siRNA has to reach the target cells. Effective pharmacological use of siRNA requires

"carriers" that deliver the siRNA to its intended site of action. siRNA displays poor cellular uptake and is not able to pass through the blood-brain-barrier (67). Small hairpin RNAs (shRNAs) present a solution to some of these flaws. shRNAs are ∼70 nt long precursor siRNAs that are introduced into the cell by viral or bacterial vectors (e.g., plasmids). After expression in the nucleus, the shRNA is being transported to the cytoplasm where it is further processed by Dicer proteins. It is subsequently loaded into the RISC for specific gene silencing activity in the same manner as synthetic siRNAs (68).

siRNA often turns out to be unspecific. The suppression of other genes (the so-called "off target effects") may lead to unknown consequences due to dangerous mutations and unwanted gene expression (69). SiRNA may also interfere with the host immune response (70). Consequently, the long-term safety of si/shRNA treatment is yet unclear as there are only few *in vivo* RNAi long-term studies (58).

Inhibitors of CHIKV nsP1

The non-structural protein 1 (nsP1) is a palmitoylated protein with methytransferase (MTase) and guanylyl transferase (GTase) activity. The protein consists of 535 amino acid residues and is responsible for the capping and the methylation of the newly synthesized viral and genomic RNAs (39). The added cap structure on the viral mRNA ensures the translation of the RNA and prevents its degradation from cellular 5′-endonucleases. On its N-terminal domain, the nsP1 has a α-helical amphipathic loop as well as a palmitoylation, which both act as anchors to attach the nsP1 and the nsP1-containing polyproteins/replication complex (RC) to the host's cellular membrane (71). Various studies could show that the palmitoylation of nsP1 is an important feature for the replication of some alphaviruses (72, 73). Depalmoylated Semliki Forest virus (SFV) mutants displayed a diminished pathogenesis in mice (72). Likewise, Zhang and colleagues (74) demonstrated *in vitro* that by inhibiting the enzyme responsible for the palmitoylation of proteins during CHIKV infection, CHIKV replication could be suppressed. There is evidence suggesting that nsP1 has additional functions during alphavirus infections like the development of cell filopodia and the rearrangement of actin filaments (73). Especially the MTase and GTase-like activities of nsP1 present a viable target for antiviral compounds since both enzymatic properties are essential for virus replication. The GT activity of nsP1 is dependent on successful MTase activity (75). Interestingly, unlike cellular MTase and GTase enzymes, the nsP1 does not contain canonical signature motifs and the mechanism of the enzymatic action differs from the cellular cap formation. Thus, there is the possibility of identifying molecules that selectively inhibit viral nsP1 without affecting the host cell capping enzymes' activity (76). Compared to the other nsPs, the research on antivirals that target nsP1 has been poor. Lampio et al. tested 50 guanosine/cap analogs for their activity of inhibiting SFV nsP1 20 years ago (77). Recently, Bullard-Feibelman developed an assay to screen and identify possible CHIKV nsP1 inhibitors (78). Two years later, the same research group presented their results on a high throughput screening (HTS) of 3,051 compounds and their successful identification of promising hit compounds like the naturally derived compound "lobaric acid" (**Table 1**) (20). Gigante et al. found a strong inhibitor of CHIKV replication among a new family of compounds named [1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-ones (**Table 1**) (21). It was not until 2016 when reverse genetics carried out by Delang et al. could identify the CHIKV nsP1 as the target for this potent compound (79). New derivatives of these compounds also inhibited the GTase activity of CHIKV and VEEV nsP1 (14). A report from Jones et al. (80) postulated that nsP1 was an antagonist of tetherin (an antiviral host factor that helps to retain the viruses at the surface of the infected cells). These findings gave rise to hope that nsP1 could be considered as a target for developing tetherin-mediated therapeutics against CHIKV (80). However, a more recent study on the subject could not confirm Jones' report since no evidence for tetherin-antagonists in alphaviruses was found (81).

Inhibitors of CHIKV nsP2

The CHIKV nsP2 has multiple enzymatic activities and thus plays a central role in CHIKV replication. nsP2 has auto-protease activity at its C-terminal end for cleaving the non-structural viral polyprotein (nsP1234) into the individual nsPs. There is a methyltransferase-like region of unknown function. The N-terminal half has terminal helicase, nucleoside triphosphatase (NTPase), and RNA triphosphatase activities (82). The triphosphatases are involved in RNA capping and also fuel the RNA helicase domain with energy. Additionally, CHIKV nsP2 is a virulence factor as it is able to stop the host cells mRNA transcription and translation, thus tampering with the hosts immune response. This is referred to as "transcriptional shut-off" (83). In fact, a recent study was able to show that nsP2 (as well as nsP3) exhibit RNA interference (RNAi) suppressor activity (84). Viral suppressors of the RNAi pathway (VSR) have been found encoded in various viruses (including flaviviruses) before. Yet, the report of Mathur et al. was the first to show VSR in alphaviruses. Moreover, Fros and colleagues found out that CHIKV nsP2 suppresses the type I/II interferon-stimulated JAK/STAT signaling pathway, which consequently inhibits the hosts antiviral response and defense mechanisms (82). It has previously been shown in other viruses that especially the protease function poses an interesting target for antiviral drugs (85).

Compounds designed in silico. Marcella Bassetto and colleagues applied a structure-based virtual screening strategy to find possible CHIKV nsP2 inhibitors. The molecules in question have been modeled to potentially fit and thus block the nsP2 protease binding site.

Based on this model, Bassetto performed a virtual screening of ∼5 million compounds and investigated the structure-activity relationship of the identified hits. After a final visual inspection, 15 derivates were selected to be potential CHIKV nsP2 inhibitors. As only 9 were commercially available, those were evaluated in a virus-cell-based CPE reduction assay. Compound 1 performed best and was predicted to fit the central portion of the nsP2 protease active site (**Table 1**). The compounds' ability to act as a selective CHIKV replication inhibitor was then further

investigated by performing a virus yield assay on Vero cells. The assay confirmed the findings of the CPE reduction assay.

Furthermore, Bassetto created structural analogs of Compound 1 and tried to chemically optimize the properties of the compounds. She designed and synthesized two new derivates with one showing a slightly better antiviral activity profile than compound 1. With her work Bassetto proved that a combination of molecular modeling with different *in silico* techniques and classical medical chemistry methods can lead to the discovery of novel and selective antiviral compounds.

Jadav et al. (86) tested a series of derivates of 1,3-thiazolidin-4-ones for their antiviral activity in a CPE reduction assay on Vero cells. Five compounds showed promising CHIKV inhibition properties. The authors assumed the mode of action may be that of protease inhibition, after they carried out molecular docking simulation with the available X-ray crystal structure of the CHIKV nsP2 protease (86). Here, the computer-aided binding model was used to explain possible mechanism of action, while Bassetto used the docking simulation to model compounds accordingly. Still, neither of these studies actually tested the ability of the predicted compounds to inhibit the protease activity of CHIKV nsP2.

It was the group of Das that actually designed and tested 12 compounds specifically on their ability to block the nsP2 (22). The researchers managed to create a test to validate whether the compounds actually inhibit nsP2. Das designed the compounds specifically to fit the nsP2 active site, using the same method as Bassetto and employing Compound 1 of Bassetto as a template for his products.

The group then systematically analyzed the ability of the compounds to inhibit the protease activity of the purified enzyme in cell-free assays. Two different cell free assays were employed, one being an end-point assay, the second one being continuous. In the end point assay, Das used full-length recombinant CHIKV nsP2 as the protease and a recombinant protein substrate containing the nsP2 cleavage site that was located between enhanced green fluorescent protein (EGFP) and thioredoxin. If the nsP2 was fully functional, the protein substrate was being processed, making it possible to detect the products by separating them by SDS-PAGE and visualizing the results with a Coomassie blue staining. The method on how to express and purify the recombinant proteins has been described earlier by the same group (87).

To verify his finding, Das additionally used a fluorescence resonance energy transfer (FRET)-based assay to compare the efficiencies of different inhibitors. This kind of assay had originally been described for the HIV protease by Matayoshi et al. (88). It is a continuous assay that makes it possible to collect information on the initial period of the reaction. In Das' assay, the nsP2 protease processed a peptide substrate with the nsP3/nsP4 cleavage site of CHIKV P1234 polyprotein (89). The substrate had a quencher at the N terminus and a fluorescent molecule at the C terminus. Cleavage of the substrate by nsP2 protease results in fluorescence that can be detected at an emission wavelength of 490 nm.

With these assays, Das managed to show that the majority of his compounds inhibited the nsP2s ability to process recombinant protein and synthetic peptide substrates. He also discovered that the original template molecule from Bassetto performed very poorly as a specific nsP2 inhibitor in these cell free assays, despite the fact that it had an EC_{50} of $\sim$5 μM in cell-based assays against CHIKV (12). Das then tested his compounds successfully in cell-based assays against CHIKV. The fact that some compounds did not inhibit the CHIKV nsP2 protease function in the cell free assays and yet managed to curb CHIKV infection in cell-based assays suggests that the antiviral activity of these compounds may be at least in part due to other mechanisms than the inhibition of protease activity of nsP2 (**Table 1**) (22).

Compounds inhibiting the nsP2 mediated "transcriptional shut-off". Lucas-Hourani et al. (23) developed a phenotypic cell-based functional assay to detect CHIKV nsP2 protease inhibitors. In particular, compounds that inhibited the nsP2 mediated "transcriptional shut-off" mechanism were to be detected. As mentioned before, the nsP2 protease is able to bind to cellular transcription factors and thus induce downregulation of the cell's immune response. In Lucas-Houranis' assay luciferase expression is induced when the cellular functions are working at a normal level. If nsP2 protease is blocked by antivirals, the cells mRNA transcription is properly restored and thus a replication of luciferase takes place, resulting in an increased signal.

The assay is thus based on a recombinant human cell line (HEK-293T) that expresses CHIKV nsP2 together with various reporter gene constructs (on three plasmids). Lucas-Hourani used this transfected cell line to establish an assay suitable for screening compounds for their nsP2 inhibition activity. From a pool of 3,040 molecules, he detected one with no toxicity that particularly blocked nsP2 activity *in vitro* (**Table 1**) (23).

Inhibitors of CHIKV nsP4 and Viral Genome Replication

The nsP4 is the sole protein with a polymerase function and is responsible for the RNA synthesis of the (replication complexes) RCs. The $\sim$100 residues at the N-terminal region are specific to alphaviruses. The nsP4 has $\sim$70 kDa and contains the core RNA-dependent RNA polymerase (RdRp) domain at its C-terminal end. The structure of the RdRp is typical and encompasses fingers, palm containing the GDD motif at the active site and thumb domains (90). The RdRp is able to copy the genome into a complementary minus-strand which is in turn copied into genomic and subgenomic RNAs by the polymerase with the help of the other viral nsPs in the RC. Mutation studies revealed a TATase (tyrosine aminotransferase) activity in the RdRp domain. Thus, the nsP4 may be generating the poly(A) tail at the 3'terminal of the genome (91). For more details on the nsP4s role during genome replication and its fundamental function I refer to the review of Pietila et al. (9).

Research has recently focussed on finding antiviral compounds against viruses of the *Flaviviridae* family [hepatitis C virus (HCV), Zika, Dengue, Yellow Fever virus (YFV), tick borne encephalitis virus (TBEV)], most of which are arboviruses. Especially Zika and Dengue can cause coinfections with CHIKV and the initial symptoms of the three diseases look very similar. Since the diagnosis is costly and time consuming, it is crucial to find a pan-antiviral that works against all of them. All three

viruses are +ssRNA viruses and there is a reasonable chance that they share conserved motifs in the orthologous RdRp enzyme (24, 91). The remarkable homology of the nsP4 among the alphaviruses makes it possible that antivirals blocking the nsP4 may exhibit their activity over a broad spectrum of viruses. With human cells lacking this specific polymerase the chances of adverse side effects of RdRp inhibitors are minimized (92).

Nucleoside analogs and proTides. Nucleoside analogs (NAs) are synthetic, chemically modified nucleosides consisting of a sugar and a nucleic acid analog. Nucleotide analogs additionally have one to three phosphate groups attached to the 5′-site. In the cell, they are processed the same way as the natural (endogenous) nucleosides. After their uptake into the cell and their metabolization, the NAs can act on cellular functions. They mimic their physiological counterparts and block cellular division or viral replication by impairing DNA/RNA synthesis (they usually cause termination of the nascent DNA/RNA chain) or by inhibition of cellular or viral enzymes involved in the nucleoside/tide metabolism (93, 94). The FDA has approved more than 25 nucleoside analog drugs used for the therapy of viral infections such as HIV/AIDS (tenofovir), hepatitis B (lamivudine/entecavir), and C (sofosbuvir) or herpes (acyclovir) (93, 95). Besides being antiviral agents, NA drugs are also applied in the therapy of cancer, rheumatologic diseases and even bacterial infections (96).

Before NAs can actually work as antivirals, they have to be phosphorylated in the host organism. Three consecutive phosphorylation reactions are necessary to activate the prodrug. The first reaction to the 5′-monophosphate is usually a rate-limiting step, which also means that if this first phosphorylation does not take place, the drugs remains inactive (97). This might happen either because the virus does not induce a specific kinase or has acquired a mutation in this particular enzyme resulting in resistance to the compound because the host cell is not able to phosphorylate the NA.

Monophosphate NAs have come into focus in order to avoid this problem and improve the therapeutic properties. However, these phosphate analogs (possessing a CO–P bond) proved to be prone to esterase and phosphatase hydrolysis. As an alternative, chemists investigated replacing the phosphate group by an isosteric and isoelectronic phosphonate moiety (CH2-P bond). This led to the discovery of nucleoside phosphonate analogs (NPs), which are chemically and enzymatically more stable than the phosphate analogs (98).

Toxicity and side effects of nucleoside/-tide analog drugs often result from their off-target use by host polymerases and their incorporation into RNA or DNA. The observed toxicities tend to be highly unpredictable and even closely related analogs may prove toxic for different organs (95). Various mechanisms for NAs toxicity have been discovered, the most characteristic is due to their affinity to host mitochondrial gamma polymerase (99). The NAs enter the mitochondria and are either incorporated into the mitochondrial DNA or block its synthesis.

Since NAs, nucleoside 5′-monophosphates or 5′-phosphonates are charged molecules and penetrate the cell membrane very poorly, they are not suited for oral administration. Research tried to improve the pharmacological properties and bioavailability of this class of compounds. This led to the discovery of the ProTides approach by McGuigan in 1998 (100, 101). The researchers designed a novel prodrug in which the phosphate was chemically protected or masked. This group of prodrugs became known as "ProTides" (pronucleotide) and as a result from the masked phosphate, this construct is able to pass the cell membrane via facilitated passive diffusion (94).

In the cell, the ProTide is enzymatically cleaved, thus releasing the masking groups from the nucleoside monophosphate/phosphonate which can be further transformed into the active 5′-triphosphate form of the NA. Various natural and unnatural amino acids can serve as the masking amino acid motif. All ProTide drugs that have reached the clinic, feature l-alanine (94). With the prodrug strategy, medical chemists were able to solve the main pharmacological problems associated with NAs, namely poor cellular uptake and poor metabolism into their phosphorylated forms.

Ribavirin. Ever since its discovery in 1972, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, also known as Virazole), a synthetic guanosine nucleoside analog, has been used as a compound against various viruses (102).

Ribavirin (RBV) is one of few FDA approved antiviral drugs in clinical use that is effective against respiratory syncytial virus in infants and chronic hepatitis C virus infections in combination with pegylated interferon (IFN)-α (103, 104). Apart from the FDA approved indications, RBV has shown efficacy against a variety of virus infections including haemorrhagic fever and measles (105, 106). Huggins and colleagues could also prove RBV's effectiveness against viruses of the alphavirus family *in vitro* (107). Multiple studies confirmed his findings by testing RBV *in vitro* against CHIKV either as a monotherapy (25) or in combination with doxycycline (26) or IFN-α (27, 28). Especially, Franco et al. (25) demonstrate that the effectiveness of antiviral agents against CHIKV differs considerably between host cell lines (**Table 2**).

Various different mechanisms of action have been attributed to RBV which might explain its broad-spectrum antiviral activity. The major mechanism, by which the replication of RNA viruses is being inhibited, is curbing the cellular guanosine triphosphate (GTP) pools by blocking the inosine monophosphate dehydrogenase (IMPDH) (108). Another indirect mechanism is the immunomodulation of the host's adaptive immune response: RBV triggers a suppression of the T-helper type 2 response and an induction of the T-helper type 1 response (109). The type 1 response is responsible for an increased clearance of infected cells. Additionally, RBV is believed to directly inhibit RNA capping. Other findings suggested that RBV interferes with the guanylyl transferase and/or methyltransferase activity of the nsP1, leading to a production of mRNAs that are not fit for translation (110). RBV is said to directly inhibit the viral polymerases, thus hampering the virus' genome replication (111). This has also been proposed by other studies that suggested RBV to directly inhibits nsP4 RdRp by interacting with its Cys483 residue, resulting in a decrease in replication fidelity (112). This would

confirm the theory that RBV leads to error catastrophe via increased mutation frequency (nucleotide transitions) because of the incorporation of ribavirin triphosphate (RTP) into the newly synthesized viral genomes (113). Others found indications that RBV promotes IFN signaling by modulating specific genes and thus potentiating IFN action (114).

RBV, albeit a success as a broad-spectrum antiviral *in vitro*, has rarely been reported to be the subject of *in vivo* trials against CHIKV in humans. Ravichandran and Manian (115) treated 10 patients with confirmed CHIKV infection. Before treatment the infection had not been resolved after 2 weeks and resulted in crippling lower limb pains and arthritis. The patients were treated with 200 mg RBV twice daily for 7 days. A control group of 10 similar patients was only given analgesics when required. According to Ravichandran and Manian the patients of the RBV group showed a significant improvement in the joint pains and 8 patients out of 10 had a reduction in tissue swelling. Ravichandran concluded that RBV may indeed have a direct antiviral property against CHIKV infection and might lead to a faster recovery of the patients. However, the study had some flaws: (1) only a small number of patients were considered; (2) the study was not a randomized controlled study (a so-called double-blind study) where the RBV group was compared with a group receiving placebo; (3) the drug was administered in the subacute phase of the disease, thus some of the improvement could be attributed to a normal course of healing. A recent *in vitro* study of Mishra et al. (116) suggested that RBV is only effective in the earlier stages of the CHIKV lifecycle; the benefit of giving the drug in a subacute or chronic phase might therefore be questioned.

The doses at which RBV would have to be administered in order to reach its full potential as an antiviral *in vivo* are associated with severe side effects such as haemolytic anemia, pulmonary, dermatologic, and teratogenic effects and can thus only be justified if the infection is life-threatening (117).

RBV's success as an antiviral is probably attributed to its ability to act simultaneously via multiple mechanisms. Usually, when an antiviral interacts at various cellular and viral processes, the chances for drug resistant mutants are diminished. But, in case of RBV, various resistant viruses have been reported, such as Sindbis virus, Hepatitis C Virus and CHIKV, showing yet again, how quickly viruses are able to adapt (52, 110, 118). Taking these developments into account, RBV might still be interesting as a component in an antiviral "cocktail" consisting of multiple drugs with various modes of action, where the dosages of the drugs themselves could be reduced due to synergism and the risk of adverse effects could thus be minimized.

β-*d-N4-hydroxycytidine (NHC).* A report from Ehteshami et al. (30) stated the outcome of experiments dealing with β-d-N4-hydroxycytidine (NHC), another modified NA. NHC was identified to successfully inhibit CHIKV replication in different replicon cell lines as well as in infectious models *in vitro* (**Table 1**). One year later, another group published that NHC was able to curb the release of genome RNA-containing VEE virions and their infectivity in *in vitro* test with Vero cells (119). This discovery supports the idea that the polymerase activity of the nsP4 is quite conserved and that drugs targeting this particular activity might show efficacy against various alphaviruses. The antiviral activities of NHC are probably due to the compound acting as a pyrimidine analog that may directly target the viral polymerase and cause chain-termination. Alternatively, the compound might induce accumulation of mutations in virus-specific RNAs which are either lethal or lead to viral genomes that are incapable of replication (30). Urakova suspects a dual effect of NHC on VEEV by causing a modest decrease *in virion* release and a strong decrease *in virion* infectivity. This idea supports the theory that mutations caused during the replication process lead to "error catastrophe" or "lethal defection" (119, 120).

Urakova reported that NHC only triggered the development of a low-level resistance in VEEV against NHC, which makes it a very promising compound that might substitute RBV. These findings are very encouraging. Nevertheless, further studies with more relevant human cell lines, animal models as well as other viruses are needed to confirm whether this compound has a future as a broad-spectrum antiviral.

Favipiravir (T-705) and its defluorinated analog (T-1105). Favipiravir (T-705, 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is an approved drug in Japan for the treatment of influenza virus infections (121, 122). The drug is a purine analog and functions as a broad-spectrum antiviral agent which has also been reported to inhibit (*in vitro* and *in vivo*) the replication of a number of RNA viruses such as arenaviruses, bunyaviruses (123) and alphaviruses (124–126). During the 2014/2015 Ebola epidemic in western Africa, T-705 proved beneficial for infected patients (127).

Favipiravir is a prodrug, which is phosphoribosylated in the cell into its active form, a ribofuranosyl 5′-triphosphate metabolite (favipiravir-RTP). It acts as a pseudo purine and inhibits the viral replication of influenza. Two modes of action have been suggested: There is evidence that favirpiravir-RTP specifically blocks the influenza virus RNA-dependent RNA polymerase (RdRp) by binding at certain domains of the enzyme (122). Others suggested that favipiravir-RTP is incorporated into the nascent viral RNA, thus leading to lethal mutagenesis or preventing further extension of the RNA strand entirely by chain termination (128, 129).

As favipiravir is relatively novel, the information on its *in vitro* efficacy is limited. Values vary depending on the assay, cell line and virus strain used (**Tables 1**, **2**). Apart from favipiravir itself, the defluorinated analog T-1105 has worked as an antiviral drug against CHIKV in *in vivo* experiments with mice (31). The drug prevented mice from developing severe neurological disease and reduced the mortality rate of the CHIKV infected animals. A dosage of 300 mg/kg T-705 daily and orally proved especially beneficial for CHIKV infected mice during the acute phase of the disease (125). Delang also identified T-705 resistant CHIKV variants *in vitro*. The mutant had acquired a mutation in the motif F1 of the RdRp, which seems to be important in the nucleoside triphosphate binding during and in the initiation of the viral RNA synthesis of +ssRNA viruses (130). Yet, Abdelnabi et al. (126) suggest that favipiravir has a high barrier of resistance. Abdelnabi made experiments in which he tried to create T-705-resistant coxsackievirus B3 (CVB3) (another +ssRNA virus), by

point-mutating the same F1 motif. These efforts resulted in either low-fidelity RdRp or unviable virus. Since NTP binding is a major fidelity checkpoint, point mutations in this F1 motif could destroy the activity of the polymerase or reduce catalysis (131).

The fact that resistant mutants develop, demonstrates how quickly RNA viruses can adapt to selective pressure via mutations. Understanding the role of conserved motifs like F1 is of great importance in order to understand the mode of action of certain drugs and possibly design more potent compounds.

Sofosbuvir. Sofosbuvir (β-D-2′-deoxy-2′-α-fluoro-2′-β-C-methyluridine, formerly known as PS-7977 or GS-7977) is a RdRp inhibitor approved by the FDA for the treatment of HCV infections (132). The drug is a nucleotide analog that is orally available and functions as a prodrug. In hepatocytes, sofosbuvir is metabolized to 2′-F-2′-C-methyluridine monophosphate (UMP) and further phosphorylated into its active triphosphate form (UTP). During the viral genome synthesis, UTP functions as a chain terminator, thus inhibiting HCV replication and production at the site of infection, in this case the liver (133). Sofosbuvir has recently been reported to inhibit YFV and ZIKV replication *in vitro* and *in vivo* (134–136).

Sofosbuvir has been tested against CHIKV *in vitro* (Huh-7 cells and astrocytes) and *in vivo* (mice) (24). The drug inhibited CHIKV replication and was three times more potent in inhibiting CHIKV in human hepatoma cells than RBV (**Table 2**). In human induced pluripotent stem cell-derived astrocytes, sofosbuvir did impair virus production and cell death in a MOI-dependent manner, yet not to such a degree as in the Huh-7 cells. This may be due to the fact that hepatocytes have the most effective system of turning the prodrug sofosbuvir into its active form (UTP), whereas astrocytes show less metabolic activity in this respect and thus have less of the active UTP form of the drug available (133). Furthermore, sofosbuvir prevented CHIKV-induced arthralgia-related paw oedema in adult mice as well as mortality in neonate mice (24). Since CHIKF can lead to chronic arthralgia, further studies are needed to evaluate if sofosbuvir in a combination therapy alongside anti-inflammatory drugs is beneficial to patients suffering from chronic CHIKV associated arthritis.

Interestingly, humans tolerate the drug better than mice. A 400 mg daily dose over a period of 12–24 weeks is the standard therapy for HCV patients (133), while doses of >33 mg/kg/day in a 7 day regime proved to be toxic to mice (136). The reason for this observation might be the decreased stability of sofosbuvir in rodent serum. This raises the question of how significant rodent models are for the evaluation of sofosbuvir or whether other (animal) models might be more representative.

Similar to favipiravir, sofosbuvir resistant HCV strains have been reported (137). Yet, the development of sofosbuvir resistant mutants seems to be slower compared to HCV inhibitors targeting other proteins. Researchers hold the high degree of amino acid conservation within the RdRp domain as well as the lack of fitness in mutated viruses responsible for this phenomenon (136).

Nevertheless, the fact that sofosbuvir blocks the viral replication of CHIKV as well as several flaviviruses is strong evidence for the presence of conserved motifs among RNA polymerases from +ssRNA viruses. The recent advances in elucidating the nsP4 structure and core domain function of CHIKV highlight these observations and may confirm that the RdRp is a feasible target for pan-antiviral molecules (91).

Other Viral Genome Replication Inhibitors

Mycophenolic acid (MPA). Mycophenolic acid (MPA) had already been discovered in 1893 and was isolated in 1896 as an antibacterial molecule produced by *Penicillium brevicompactum* (138). MPA is licensed by the FDA as a drug for transplantation rejection (139). The drug inhibits cellular inosine monophosphate dehydrogenase (IMPDH) and thus decreases the intracellular pools of guanosine triphosphate (GTP) and 2′-deoxyguanosine triphosphate (dGTP). This causes a disruption of viral and cellular RNA, DNA, and protein synthesis (140). Two derivatives of MPA are available for clinical use: mycophenolate mofetil (MMF, CellCept) and mycophenolate sodium (MPS, Myfortic). Mycophenolate mofetil is the orally bioavailable prodrug form of MPA. MPA has shown antiviral activity against DENV and Orthopoxvirus (141, 142).

Although MPA was reported to inhibit CHIKV *in vitro* in 2011, tests done in 2018 could not confirm these findings (143, 144). However, Ferreira tested MPA as a control alongside his compounds and indeed received good EC_{50} values, with MPA even performing slightly better than sofosbuvir and with a much better selectivity index ($SI = CC_{50}/IC_{50}$) than RBV (24). Likewise, other research groups used MPA as a reference against CHIKV and evaluated the efficacy against CHIKV (**Table 2**) (16, 32).

There are various studies confirming the antiviral, antibacterial, antifungal, immunosuppressive, and anticancer properties of MPA or its derivates (145). Yet it is important to deliberate whether the benefits of MPA as an antiviral outweigh its adverse effects as an immunosuppressant.

NsP3 and Possible Inhibitors

The nsP3 consists of three domains. The N-terminus has a macrodomain, while the C-terminus holds a hypervariable domain (HVD). The central part of the protein contains a zinc-binding domain which is sometimes referred to as the alphavirus-unique domain (AUD), a region that shares a strong sequence homology across the alphaviruses. The role of the AUD is so far undefined but the domain seems to be important in RNA replication and in the synthesis of negative sense and sub-genomic RNA (146).

There are hints indicating that the nsP3 is involved in inhibiting the assembly of the host cells stress granules (SG) which are essential for the degradation of viral mRNA (147). NsP3 is usually found in complex with other nsPs during infection. It also interacts with host factors. Saul et al. (148) discovered that the amount of nsP4 increased in a recombinant SFV with a duplicated nsP3-encoding sequence. Saul concluded that nsP3 is involved in the stabilization of nsP4. He could furthermore back other studies' findings that nsP3 is important for the (neuro-) virulence of old-world alphaviruses (148). In New-World alphaviruses, neurovirulence is mainly determined by structural proteins, particularly E2 (149).

So far, the complete function of the nsP3 macrodomain has not been fully unraveled although its crystal structure has been known since 2009 (PDB id: 3GPG and 3GPO) (150). The N-terminal macrodomain is highly conserved among alphaviruses but also occurs in other positive-strand RNA viruses such as coronaviruses and hepatitis E virus (151). There is evidence that the viral macrodomains bind ADP-ribose, dephosphorylate ADP-ribose-1"-phosphate and act as de-ADP-ribosylating enzymes thus counteracting antiviral ADP-ribosylation (152). Other studies indicated that the most likely biochemical function of viral macrodomains is de-ADP-ribosylation. By enzymatically removing mono- and poly-ADP-ribose from proteins, macrodomains might oppose the host cells' antiviral response (153). Furthermore, the mono(ADP-ribosyl)hydrolase activity of the nsP3 is critical for CHIKV replication in vertebrate hosts and insect vectors, and determines virulence in mice (154). These findings suggest that the macrodomain plays an important part in the host-pathogen conflict.

Nguyen et al. virtually screened a database of 1,541 compounds for possible hits that might block the nsP3 macrodomain of CHIKV (155). The group combined molecular docking, virtual screening, and molecular dynamics simulations to identify potential inhibitors. They ended up with three ligands that might have potential as nsP3 inhibitors. However, these findings were achieved *in silico* and still need to be verified by experimental studies *in vivo*.

Until Varjak et al. discovered a degradation signal at its C-terminus, nsP3 was thought to be a rather stable protein. Varjak could demonstrate that the nsP3 of SFV and Sinbis Virus (SINV) was degraded rapidly when the protein was expressed individually. On the other hand, nsP3 was significantly stabilized when it was expressed in the nsP123 polyprotein form (156). The role of this C-terminal degradation signal is still unknown but there are various hints that it may contribute to granting the optimal stoichiometry of the nsPs.

Especially the HVD at the C-terminal region of the nsP3 seems to be a center for interactions with host cell proteins, including stress granule (SG) components which might help the virus adapt to distinct cellular environments. Data suggests that the HVD interacts with several host factors through a conserved proline (P)-rich and duplicate FGDF motif. The letters of the motif correspond to the according amino acids, two phenylalanine residues which are separated from each other by a glycine and an aspartate residue (157). These interactions are needed for the assembly of virus genome replication complexes (158, 159). The FGDF motif seems particularly important for the successful replication of alphaviruses in mammalian cells. Experiments with CHIKV revealed that the virus' nsP3 has two FGDF motifs that bind to certain domains of the SG components in mammalian cells (160). SGs usually block host and viral translation. The interactions between the CHIKV nsP3 and the SG domains impede the organization of the SGs and thus may allow virus replication (147, 161, 162). When the alphavirus nsP3 HVD is mutated in a way that both FGDF motifs are disrupted, CHIKV is inactivated and SFV as well as SINV are attenuated in mammalian cells. If only one FGDF motif is present in CHIKV or SFV nsP3, the affinity for the SG domains is reduced and the virus is attenuated as well. This leads to the conclusion that alphaviruses need two FGDF motifs for a successful viral replication in mammalian cells (146, 160, 161).

The HVD seems also to be a determinant for virulence in some viruses. There is evidence that the conserved FGDF motifs in the HVD of chikungunya virus nsP3 are required for the effective transmission of the virus from *Aedes aegypti* mosquito saliva to a vertebrate host (163).

The nsP3 seems to be an important protein in determining vector specificity. ONNV, which is closely related to CHIKV, is the only alphavirus known to be transmitted by *Anopheline* mosquito species. CHIKV on the other hand, is mainly transmitted by *Aedes* mosquitoes. Experiments with chimeric CHIKV expressing ONNV nsP3 revealed that *Anopheles gambiae* mosquitoes become susceptible for CHIKV although being naturally immune to WT CHIKV (164). This observation is in line with previous findings suggesting that nsP3 might be involved in specific protein-protein interactions and thus carries out host cell-dependent functions (165). A recent study revealed that nsP3 suppresses RNAi alongside nsP2 in CHIKV infected insect cells (84). As RNAi is an antiviral defense mechanism in various organisms that leads to a degradation of viral RNA, the suppression of RNAi by viral proteins enhances infection.

The impact of these interactions on biological and biochemical processes of the host cell at early stages of the infection are still under heavy investigation. There is hope that the interacting regions might prove valuable targets for intervention and opens new possibilities for vaccine development and antiviral drug discovery.

Kaur et al. (29) reported the discovery of the anti-CHIKV properties of **harringtonine**, a cephalotoxin alkaloid from the *Cephalotaxus harrintonica* trees. It was suggested that the compound inhibits the early stages of CHIKV infection after cellular endocytosis (29). Harringtonine was proposed to interfere with the protein translation of CHIKV since it seemed to inhibit the production of nsP3, E2 proteins, and CHIKV RNA (29, 166). Harringtonine was approved in 2012 by the FDA as a drug for the treatment of chronic myeloid leukemia (167). **Homoharringtonine**, an analog of harringtonine with an additional methyl group, was reported to have anti-CHIKV properties as well. According to Kaur, both compounds display minimal cytotoxicity on BHK-21 cells and primary human skeletal myoblasts at the dosage needed for inhibiting CHIKV. However, the drug itself is labeled as a cytotoxic agent and according to the Globally Harmonized System (GHS) harringtonine is fatal if swallowed (H300), in contact with skin (H310) or if inhaled (330) (168). This may be the reason that although Kaur's original article has been cited repeatedly, no studies on the anti-alphavirus properties of harringtonine have been published in the past 7 years.

Host-Targeting Antivirals

Many viruses depend on host factors to ensure their replication or are inhibited by such. Host factors present a valuable target for drugs to interfere in the virus' life cycle either by inhibiting host factors on which the virus relies on or by promoting host

factors that curb virus infection. Since host factors also play vital roles in normal physiology, their inhibition or promotion can lead to abnormal physiological function and toxicity. The impact such interference may have on the host organism must thus be critically elucidated. Ideally therapeutics would target interactions between host and viral factors without disrupting essential cellular processes. For the interested reader we refer to the review of Wong and Chu (169) that summarizes the current knowledge on the interplay of viral and host factors in CHIKV infection as well as potential targets for antivirals.

Viperin, Hsp90 Inhibitors, and Interferons

Viperin

Viperin (virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible) is an interferon (IFN)-induced host cell protein that has come into focus because it is responsible for inhibiting viral replication via multiple pathways. It thus represents an interesting target for antiviral drugs (170). Viperin has been reported to inhibit a broad spectrum of DNA and RNA viruses, including members of the herpesvirus, flavivirus, alphavirus, orthomyxovirus, paramyxovirus, rhabdovirus, and retrovirus family (170). CHIKV infection is also curbed via IFN-induction of viperin and compounds leading to the up-regulation of viperin may present a strategy to manage CHIKV infections. Studies could demonstrate that CHIKV infection is controlled via type I IFNs that induce the interferon-stimulated gene (ISG) RSAD2 (radical SAM domain-containing 2) which encodes viperin (171). Teng et al. showed that mice lacking RSAD2/viperin had a higher rate of CHIKV replication and more severe inflammatory symptoms in the joints. A recent study tried to elucidate the role of viperin in shaping the pathogenic CHIKV-specific CD4 T-cell adaptive immune response during late acute disease phase (172). The group used viperin deficient mice in which CD4 T-cell had been depleted. They could demonstrate that increased late acute joint inflammation was exclusively mediated by CD4 T cells and that Th1-IFNγ-producing T cells played a pivotal role in the joint pathology. Further experiments showed that viperin expression contributes to reducing disease severity in both haematopoietic and non-haematopoietic cells (172).

Hsp90 Inhibitors

Chaperones help in the folding, assembly and maturation of host- and viral proteins. Almost all viruses depend on the chaperone Hsp90 (heat shock protein 90) especially during replication to ensure their life cycle (173). This causes viruses to be hypersensitive to Hsp90 inhibition and provides a way to curb virus replication. Compounds interfering in Hsp90 function have a potential as broad-spectrum antiviral drugs, especially since experiments with picornaviruses demonstrated that Hsp90 inhibitors are refractory to the development of drug resistance (174). As mentioned before, Hsp90 also plays an important role during CHIKV replication due to its interaction with the nsP3 and nsP4 of CHIKV. The chaperone furthermore stabilizes CHIKV nsP2 and thus promotes virus replication (65). Studies demonstrated that the Hsp90 inhibitor **geldanamycin** (GA) reduce CHIKV replication, particle formation and infection *in vitro* (65, 66). Yet, inhibiting Hsp90 very often results in toxicity, especially for the liver, presumably because Hsp90 is very abundant in liver cells and interacts with multiple proteins at crucial points in the cellular function. A lot of clinical trials with anti-Hsp90 drugs have been abandoned due to the *in vivo* toxicity (175). This also holds true for GA which is hepatotoxic as well as structurally instable, and thus has so far not been approved for clinical usage (176). Research is currently focussing on developing Hsp90 inhibitors with better pharmacological profile, such as **ganetespib**, which is relatively hydrophobic and less toxic (177). Ganetespib is currently under investigation in phase 1-3 clinical trials for the treatment of breast cancer, small cell lung cancer, acute myeloid leukemia, and myelodysplastic syndrome. However, its potential as an antiviral is not known but might be worth investigating once the drug is approved by the FDA.

Lillsunde et al. (178) investigated the antiviral activity of a number of **marine alkaloid-oroidin analogs** that are synthetic compounds and target the Hsp90. Lillsunde tested the compounds in replicon models against HCV and CHIKV. While 4 compounds selectively inhibited the HCV replicon, the compounds exhibited only moderate selectivity and efficacy against the CHIKV replicon in dose-response and cytotoxicity studies.

Interferons

Interferons (IFNs) play a vital role in the innate immune response to counter virus infections and thus have been the subjects of multiple studies. IFNs have been tested widely for their potential use as antivirals against a variety of viruses including HIV, Hepatitis C and B, and Influenza A (179). Type I IFNs [alpha/beta interferon (IFN-α/β)] are produced by the host cell upon sensing virus invasion. IFNs upregulate a variety of interferon-stimulated genes (ISGs). The protein products of the ISGs contribute to countering viral infections by suppressing viral spread and supporting the initiation of adaptive immunity [reviewed in (180)]. IFNs Type I are considered a "standard of care" in suppressing chronic HCV and HBV infections, while Type III IFNs have generated encouraging results as a treatment for HCV infection in phase III clinical trials (181). Various studies have confirmed that alphaviruses are also highly sensitive to the antiviral activity of Type-I IFNs (IFN-α/β) (182, 183).

Briolant et al. (27) compared the antiviral efficacy of IFN-α, glycyrrhizin, 6-azauridine, and RBV of inhibiting CHIKV and SFV infection *in vitro*. When combined with RBV, IFN-α2b had a sub-synergistic antiviral effect on both alphaviruses (27). A more recent study by Gallegos et al. (28) confirmed the highly synergistic effect of RBV and IFN α when administered as combination therapy *in vitro*.

In vivo studies with IFN-α/β receptor-deficient mice also demonstrated the importance of IFNs against CHIKV infection. The deficient mice lacked adequate IFN-α/β responses to the viral infection and CHIKV caused haemorrhagic fever, shock, and finally resulted in death (184).

Brehin et al. (185) investigated the role of IFN-induced 2′,5′-Oligoadenylate Synthetase (OAS) protein family in innate immunity to CHIKV. OAS proteins are critical components

of innate immunity and the group was able to show that the antiviral actions of IFN-α/β in HeLa cells are mediated due to the induction of these proteins. Various ISGs that affect alphavirus replication have been identified, including ISG15, ISG20, P56, ZAP, and Viperin (185).

Tetherin

Tetherin [also known as bone marrow stromal antigen 2 (BST-2)] is a host transmembrane protein with antiviral activity that is induced by IFN. Tetherin binds budded viral particles directly to the plasma membrane (PM) and thus restricts the release of enveloped viruses. The virus particles which are thus bound to the PM can then be endocytosed and degraded (186). Two isoforms of tetherin that differ in length are known. They are referred to as L-(long) and S-(short) tetherin and each has distinct biological properties (187). Tetherin showed antiviral activity against alphavirus release and studies demonstrated that tetherin does not affect viral entry or protein expression. L-tetherin is significantly more efficient in inhibiting the SFV release than the short isoform (186).

In response to this antiviral countermeasure, many viruses have evolved tetherin antagonists. Jones (80) postulated that CHIKV nsP1 is such a BST-2/tetherin antagonist. However, Wan et al. (81) could not confirm Jones' findings and suggested that the sole physical tethering of virus particles to the PM is not sufficient to restrict alphaviruses and that the subsequent virus endocytosis is a requirement for efficient inhibition of alphavirus release.

Silvestrol

The natural compound silvestrol (a cyclopenta[b]benzofuran flavagline) is an isolate from plants of the genus *Aglaia* and has been the focus of various antiviral studies over the past 5 years. Flavaglines have been the interest of anticancer research for more than two decades because they display antitumor activity (188). Silvestrol is a highly efficient, non-toxic and specific inhibitor of the host RNA helicase eIF4A (eukaryotic initiation factor-4A), which is part of the heterotrimeric translation initiation complex in eukaryotes (189). The host cell needs the RNA helicase eIF4A to unwind structured 5′-untranslated regions (UTRs) of mRNAs to allow translation. Since 5′-capped viral mRNAs often contain structured 5′-UTRs as well, it has been suggested that RNA viruses which have these structures might depend on eIF4A for their translation. Silvestrol proved to be a successful antiviral in multiple *in vitro* studies against a variety of RNA viruses, such as Ebola, Corona-, Picornaviruses and CHIKV (189–191).

Henß et al. (191) demonstrated that by delaying the protein synthesis of CHIKV nsPs and structural proteins, silvestrol also retarded the innate response to CHIKV infection. By curbing the amount of nsPs, silvestrol reduced CHIKV RNA replication. The compound also decreased the host protein shut-off which was induced by CHIKV infection, probably because of the lower total amount of nsP2. In accordance with this, silvestrol seemed not to impair the IFN-induced STAT1 phosphorylation and eIF2 did not become phosphorylated. All these *in vitro* findings suggest that inhibition of the host helicase eIF4A with silvestrol might be a therapeutic strategy to treat CHIKV infections. Further research is needed to find out how and if silvestrol can actually be of benefit against CHIKV infection *in vivo*.

Protein Kinase C Modulators and Plant Extracts

Plants have always been an important source of active substances and to date about 50% of the licensed drugs are natural products or were inspired by them (192). Natural compounds quite frequently have striking differences compared to chemical molecules, which often result in better pharmacological properties (193). The introduction of today's modern drug discovery process has led to a certain neglect of considering plants as a resource for bioactive compounds. But with the technological improvement in the field of natural product isolation, synthesis and screening, the interest in plants as a source for anti-infective natural compounds has been renewed (194).

After the massive CHIKV outbreak in the Indian Ocean region in 2005–2006, a large-scale quest for novel and selective antiviral compounds was initiated. A project called "Biodiversity and emerging viruses in the Indian Ocean: selection of drug candidates targeting the Chikungunya virus" was financially supported by the Center for Research and Monitoring of Emerging Diseases in the Indian Ocean (CRVOI) and carried out from March 2009 to December 2011 (195). Its goal was to find new selective antiviral compounds derived from plants from the Indian Ocean Region, an area with a vast botanical biodiversity. Soon after the program started, virologists, and natural product chemists discovered that the plant family with the most promising components was the *Euphorbiaceae*.

Especially **polycyclic** and **macrocyclic diterpenoids** as well as molecules derived from them came into focus of antiviral research. Within the family of *Eurphorbia* more than several hundred different macrocyclic diterpenoids of interest have been discovered. These molecules possess various types of carbon skeletons (e.g., jatrophane, lathyrane, myrsinane, ingenane, tigliane, daphnane, etc.). More than 20 skeletal types can only be found in this particular plant family (196). These molecules possess a broad structural diversity due to their different macrocyclic skeletons and the various aliphatic and aromatic ester groups.

Macrocyclic diterpenoids have the ability to modulate protein kinase C (PKC) activity (196). Particularly the **phorbol esters** or **phorboids** have a tendency to bind to phospholipid membrane receptors and activate the PKC (197). PKCs are a multigene family of related serine/threonine kinases that are involved in many signal transduction pathways and cellular responses. PKCs play a role in a multitude of cellular functions such as cell mitogenesis, differentiation and apoptosis, smooth muscle contraction, platelet aggregation, tumor-modulation, and anti-HIV activity (198). PKCs are classified into three sub-families with different isoforms depending on the way of their activation. The classical PKC (cPKC) isoforms (α, β, and γ) require calcium (Ca^{2+}) and the membrane-embedded ligand diacylglycerol (DAG) for activation, while the novel PKC (nPKC δ, ε, θ, η) are activated by DAG alone. The atypical PKC (aPKC Mζ-ι/λ) are not dependent on either ligand, but on proteins for activation (199).

All PKCs have an N-terminal regulatory moiety with a C1A domain and a C-terminal catalytic moiety for phosphorylation. Conventional and novel PKC isozymes have a second C1 domain (C1B) to which DAG binds (199). Phorbol esters have a two-order higher affinity to the C1B domain of conventional and novel PKC isoforms than DAG. This leads to the activation of the PKCs.

Recently a study reviewed the anti-CHIKV activity of about 80 naturally occurring macrocyclic diterpenes originating from the *Euphorbiaceae* plant family and about 30 commercially available natural diterpenoids (198) (**Table 1**). Some of these compounds have also been tested against other alphaviruses, like SFV or SINV. Other studies evaluated the antiviral properties of different plant compounds *in vitro* and found out that the phorbol esters **prostratin (12-deoxyphorbol 13-acetate)** and **12-O-tetradecanoylphorbol 13-acetate (TPA)** are potent inhibitors of CHIKV (11, 200). Allard et al. published on the anti-CHIKV properties of **trigocherrierin A**, an unusual chlorinated daphnane diterpenoid orthoester (DDO) from the plant *Trigonostemon cherrieri* (*Euphorbiaceae*), and analog compounds from the same plant (17, 45). Likewise, Nothias-Scaglia et al. found **Phorbol-12,13-didecanoate** to be the most potent inhibitor of CHIKV replication among 29 commercially available natural diterpenoids (201). Phorbol-12,13-didecanoate is structurally related to TPA. Corlay et al. tested **12-O-decanoylphorbol 13-acetate (DPA)**, a molecule that differs from TPA only by the length of the side chain that is attached at C-12 (10 carbons for DPA vs. 14 carbons for TPA) (34). DPA had anti-CHIKV properties but a small SI of 2.0 reflecting a narrow therapeutic window making this compound a poor choice as a future antiviral drug. A novel DDO called **neoguillauminin A** and four **12-deoxyphorbols** from *Euphorbiaceae* plants were recently found to have significant *in vitro* anti-CHIKV properties, three with an SI above 50 (**Table 1**) (15).

Yet despite the promising results of resent studies, the question of how said compounds manage to curb CHIKV replication has not been fully answered. Most studies assume that PKCs modulation is the key mechanism, but specifics are still outstanding. At the same time, the manner of how PKCs isoforms regulate intracellular signal transduction pathways and influence biological responses is still under heavy investigation and not completely understood. There are hints indicating that different translocation patters of the PKCs might lead to different intracellular signal transduction and cellular functions (202, 203). The cell type in which the PKCs are activated play a role as to how the response affects the organism. Additionally, the chemical properties (e.g., hydrophobicity) of different phorbol esters seem to play a critical role as well, since they induce different translocation patterns of PKCs in the cell. As conventional PKCs depend on plasma membrane bound Ca^{2+} and DAG as ligands, phorbol esters translocate them primarily to the PM, while the novel PKCs only depend on DAG and translocate to the more abundant and diacylglycerol-rich Golgi membrane (199). Studies showed that the stimulation of PKC δ by different phorbol esters induced distinct patterns of enzyme translocation. This indicates that lipophilicity of phorbol esters may contribute to differential PKC δ localization and thus to potentially different biological activities (203). Nothias-Scaglia et al. demonstrated that the HIV-1 and HIV-2 inhibitory effects of phorbol esters were strongly correlated with those of CHIKV (13). This observation is even more interesting since CHIKV and HIV belong to two different virus genera (alphavirus and lentivirus). Thus, the most probable explanation would be a common PKC-based mechanism of action. Yet a broad and potent PKC modulator with very good anti-HIV activity showed no anti-CHIKV activity, which might indicate that different PKC isoforms are involved in the two different virus life cycles. Abdelnabi et al. (33) tried to shed light on the role of PKCs in the cellular antiviral response to CHIKV infection by studying the mechanism of how **prostratin** works as an antiviral against CHIKV. The group found out that different cell lines express varied levels of diverse PKC isoform. Abdelnabi used four different cell lines [buffalo green monkey kidney (BGM) cells, African green monkey kidney cells (Vero cells), human embryonic lung fibroblasts (HEL), and human skin fibroblast cells] and four different CHIKV strains. Prostratin curbed CHIKV RNA synthesis and the production of infectious virus progeny at a post-entry step during virus replication. The antiviral action of the compound was dose- and cell- dependent. The most potent antiviral effect was observed in human skin fibroblast cells which also showed the highest gene expression levels of the classical PKC isoforms (**Table 1**). The antiviral activity of prostratin was significantly reduced when PKC inhibitors were present. These results suggest that the activation of mostly classical PKCs is the reason for the antiviral effect of prostratin (33).

Multiple or Unidentified Targets

Many other molecules have been tested against CHIKV and other alphaviruses in the past 5 years, with a special focus on plant extracts or drugs originally licensed for other diseases. Some seemed promising at first but then, upon closer investigation and with different assay methods, turned out to have a narrow SI or bad chemical properties. For some, the mode of action is still unknown. Here only the most recent or promising will be mentioned if they have been subject to repeated studies. For details on their efficacy (see **Table 1**).

Micafungin

Various researchers successfully tested the antifungal drug micafungin against viruses such as CHIKV, SFV, and SINV *in vitro* (35, 159). Micafungin has been licensed for the treatment of invasive candidiasis in 2005 (204, 205). According to Ho et al., micafungin significantly reduced CHIKV infection, cytopathic effects, and progeny yield (35). The question of how micafungin inhibits viral infection is still not answered. It was observed that the drug proved to be more effective in inhibiting CHIKV progeny yield than in reducing RNA replication (35, 159). The researchers thus deducted that micafungin might have a major influence on the later stages of CHIKV infection. On the other hand, the inhibitory effects of micafungin were stronger in the full-time treatment group than in the post-treatment group. This finding allows the speculation that micafungin might target different intracellular events during virus infection, such as viral replication, intracellular and extracellular transmission, and

virus stability. The antifungal action of micafungin comes from the non-reversible inhibition of the β-1,3-D-glucan synthase of fungi, thus blocking the cell wall synthesis (206). Since neither mammalian cells nor viruses contain 1,3-beta-D-glucan polymers, the mechanism of action of micafungin still has to be elucidated. On the other hand, the absence of these polymers in mammal cells indicates a lack of mechanism-based toxicity of the drug that might partially account for the good tolerability in patients.

Abamectin, Ivermectin, and Berberine

Varghese et al. (36) conducted HTS of about 3000 compounds for their ability to inhibit CHIKV infection. Some of the substances were already licensed drugs or under investigation in clinical trials. With the help of a *Renilla reniformis* luciferase (Rluc) reporter system in baby hamster kidney (BHK-21) cells, Varghese could evaluate the compounds' impact on viral replication. After a second validation with WT and reporter CHIKV infection essays of 25 initial hits, Varghese identified five compounds with the capacity to curb CHIKV replication (36). Among these secondary hit compounds, **abamectin**, **ivermectin,** and **berberine** performed best with an inhibition activity against CHIKV of over 85%. Toxicity evaluations of these three compounds were done in BHK-21 and human hepatocellular (Huh-7.5) cells (**Table 1**). All three compounds also exhibited antiviral action against other alphaviruses, including SFV and SINV (39).

Abamectin and ivermectin are macrocyclic lactones which originate from the fungus *Streptomyces avemitilis* and are the most commonly used compounds of the avermectin family. Both drugs are potent endo- and ectoparasitic agents with a broad spectrum of activity. Especially ivermectin has been used as an insecticide for vector control and it seems that apart from its insecticide properties against *Aedes* and *Anopheles* species, it also displays antiviral activity against some arboviruses (207). The fact that ivermectin has both mosquitocidal and antiviral action may come in handy for vector control and limiting virus spread as well as infection at the same time. The drug is currently under investigation in a phase 2 clinical trial as a therapeutic for Dengue haemorrhagic fever (ClinicalTrials.gov identifier: NCT03432442). In flaviviruses (DENV, YFV, TBEV) ivermectin inhibits the NS3 helicase activity and thus curbs viral replication (208). The mode of action of abamectin and ivermectin against CHIKV is not clear, but it is being speculated that the drugs inhibit the RNA synthesis and down-regulate the viral protein expression of the nsP1 and nsP3 (36).

Berberine is a plant-derived isoquinoline alkaloid that is also able to inhibit CHIKV replication in a dose-dependent manner. It is believed to curb RNA synthesis and interfere with the viral protein expression (39). However, berberine has a wide range of bioactivities and it is also possible that the alkaloid interferes with host factors which promote CHIKV replication (209). Berberine reduced the virus-induced activation of cellular mitogen-activated protein kinase signaling, a pathway which is relevant for maintaining the viral life cycle. Inhibiting this kinase cascade with specific drugs resulted in a decreased production of CHIKV progeny virions. Varghese tested berberine *in vivo* in a mouse model where it significantly reduced CHIKV-induced inflammatory disease (210). Berberine is currently under clinical investigation in a variety of trials; however, none of them test its use as an antiviral.

Coumarin Conjugates

Coumarins can be found in plants as well as certain microorganisms and animals. The (natural and/or synthetic) coumarins have a wide range of biological activities and they are in focus for the therapy of various conditions. A number of coumarins have been found to display antiviral, anticoagulant, anti-inflammatory, antimutagenic, antitumor, antitubercular, central nervous system stimulant, fungicidal or vasodilator activities (211).

Hwu designed and developed 22 compounds that were made up of uracil, arene, and coumarin derivatives (212). He tried to combine the antiviral properties previously described for uracil derivatives and coumarin compounds. Hwu tested the newly designed compounds against CHIKV *in vitro*. Five molecules displayed significant potency against CHIKV (212). In 2019, the same research group published a study after testing 21 new coumarin derivatives against CHIKV *in vitro*. This time coumarin derivatives had been conjugated with guanosine. Hwu had modified the design of the molecules and after HTS, three of these new conjugates were found to inhibit CHIKV in Vero cells with significant potency but with a better SI than the ones tested before (**Table 1**) (37). From the structure-activity relationship Hwu deduced that the coumarin moiety was essential and the presence of a –OMe group enhanced the antiviral activity. Still, Hwu did not try to elucidate the work mechanism of the antiviral activity of his compounds.

DISCUSSION

As CHIKV transmission depends on arthropod vectors in a complex interaction between virus host and the environment, a thorough understanding of these interactions is essential for the development of strategies to curb infections and the geographical spread of vectors. Especially climate change is one factor that may help arboviruses manifest in new areas that were formerly unsuitable for their vectors. International travel might further contribute to importing newly emerging arboviral diseases (like CHIK, Zika, or Dengue Fever virus). With autochthonous infections of CHIKV in France and Italy and established populations of *Aedes albopictus* in southern Germany, it is only a question of time until CHIKV manifests in moderate regions (3).

Thus, antiviral research remains of utmost importance to counter CHIKV infection. The different antiviral modes of action (MoAs), direct (by inhibiting the virus themselves), and indirect (by inhibiting host factors), have different merits, but both need to be considered and possibly combined for synergic effects of different MoAs.

A number of directly inhibiting antivirals against CHIKV that were tested *in vitro* were either discovered via *in silico* approach, high throughput screening of libraries or classical pharmacology. Especially plants have been rediscovered as a source for possible antivirals and yielded promising compounds like prostratin. Other drug candidates have been repurposed

and are already licensed for the treatment of different viral diseases, e.g., sofosbuvir, ribavirin, and favipiravir. As these molecules have already been intensely evaluated in patients, trials for them against CHIKV in humans may possibly be fast-tracked. Unfortunately, some failed to maintain their efficacy in *in vivo* experiments (e.g., chloroquine and ribavirin), while others (like favipiravir and sofosbuvir) look more promising in animal experiments but still have to be tested against CHIKV in humans.

Despite multiple efforts in antiviral research, there is no standardized protocol for determining efficacy and toxicity. This makes comparison of the different hits impossible. As demonstrated in **Tables 1**, **2**, efficacy and toxicity values vary considerably depending on the assay method, virus strain, and cell line. Some cell lines are refractory to the toxic effects of the molecules, possibly whitewashing the SI of the potential hit. The same applies for the assay methods, where each has its merits and its flaws. The lack of standardization as well as polypharmacology *in vivo* might be reasons why multiple drugs, although having achieved promising results *in vitro*, failed to be of benefit *in vivo*. Standardized efficacy and toxicity assays would help in calculating the SI which in turn is important for selecting molecules to test *in vivo*. So far, there is no defined cut-off for the SI, but a value of $\geq$10 is usually considered for animal models (39). A more thorough validation of potential hits in pre-clinical studies (e.g., multiple assay methods of selected hits *in vitro*) might help to avoid disappointment in *in vivo* assays.

Furthermore, as CHIKV infection often go hand in hand with other arboviral infections that are transmitted by the same *Aedes* species (e.g., DENV and ZIKV), a panantiviral which shows efficacy against these other viruses would be ideal. Apart from displaying anti-CHIKV activity, sofosbuvir, suramin, favipiravir, ribavirin, 6-azauridine, and ECGC also display antiviral activity against DENV or ZIKV or both *in vitro* (50, 136, 213–215).

Indirect antivirals targeting host factors yielded some promising results *in vitro*, but *in vivo* tests are still outstanding. This approach bears the risk of disrupting the physiological balance of the host factors which might lead to serious adverse effects.

Although research has brought forth a number of promising compounds, most of them still have to be validated *in vivo* and in clinical trials. The past epidemics caused by CHIKV demonstrated the impact a neglected or (re)emerging disease may have on a naïve population. Agents that have the potential to disable a population for a longer period with possible long-term sequelae, pose a vast threat to health and the economy. With no licensed vaccine and no specific antiviral treatment against CHIKF, research in the area of antiviral therapy is of utmost importance and the effort to find a specific treatment should be continued.

AUTHOR CONTRIBUTIONS

Both authors contributed to the article and approved the submitted version.

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Risk of Secondary Infection Waves of COVID-19 in an Insular Region: The Case of the Balearic Islands, Spain

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The Spanish government declared the lockdown on March 14th, 2020 to tackle the fast-spreading of COVID-19. As a consequence, the Balearic Islands remained almost fully isolated due to the closing of airports and ports, these isolation measures and the home-based confinement have led to a low prevalence of COVID-19 in this region. We propose a compartmental model for the spread of COVID-19 including five compartments (Susceptible, Exposed, Presymptomatic Infective, Diseased, and Recovered), and the mobility between municipalities. The model parameters are calibrated with the temporal series of confirmed cases provided by the Spanish Ministry of Health. After calibration, the proposed model captures the trend of the official confirmed cases before and after the lockdown. We show that the estimated number of cases depends strongly on the initial dates of the local outbreak onset and the number of imported cases before the lockdown. Our estimations indicate that the population has not reached the level of herd immunization necessary to prevent future outbreaks. While the low prevalence, in comparison to mainland Spain, has prevented the saturation of the health system, this low prevalence translates into low immunization rates, therefore facilitating the propagation of new outbreaks that could lead to secondary waves of COVID-19 in the region. These findings warn about scenarios regarding after-lockdown-policies and the risk of second outbreaks, emphasize the need for widespread testing, and could potentially be extrapolated to other insular and continental regions.

Keywords: COVID-19, epidemic projection, secondary outbreaks, computational modeling, herd immunization

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INTRODUCTION

The rapid propagation of the new COVID-19 pandemic requires timely responses, including the alignment of evidence generation by scientists and decision-making by policy stakeholders. As of the current date, several mathematical models have been developed to help policy-making in a wide arrange of interventions in various countries, encompassing from testing

strategies to lockdown measures (1–6). Although modeling pandemics is not without flaws, and its predicted scenarios cannot be uncritically adopted and therefore directly translated into policy (7), modeling can be a valuable support tool to guide policy when assessed in an integrated way.

Recent studies have dealt with the possibility of a second-wave of COVID-19 after the retirement of lockdown and confinement measures in China (1, 2). Recently, the value of restrictive social distancing measures has been largely proved since the first outbreak of the pandemic in European countries such as Italy (8). The analysis of data from closed confinements such as sea cruises allows us to address some key questions regarding the risk of second waves in an environment without external perturbations (9, 10). The study of the evolution of the pathogen in islands offers an opportunity to learn how the propagation occurs, and how the mobility restrictions are shaping the propagation in relatively isolated areas, either due to transport lockdowns implemented to contain COVID-19 dissemination or because of their geographical conditions.

The Balearic Islands archipelago is composed of four inhabited islands in the Mediterranean Sea, i.e., Majorca, Menorca, Ibiza, and Formentera, with a total population of 1,095,426, as per 2011 data (11). The main economic activity is tourism with principal connections to the UK and Germany. The first reported case in Spain was identified in the Canary Islands on January 31st, while in the Balearic Islands the first case (second in Spain) was confirmed on February 9th. He was a British citizen resident in Majorca who had been in contact with an infected person with SARS-CoV-2 during a stay in France from January 25–29. In Spain, the schools were closed on March 16th and the lockdown was implemented at the national scale from March 17th. As of April 11th, the number of infected cases in the Balearic Islands was 140 per 100,000 inhabitants (1,507 confirmed cases) to be compared with 345 in Spain (161,852 confirmed cases; data updated with values of April 11th, 2020) (12). The lockdown of the Balearic Islands includes the closing of airports and ports for passengers, rendering the archipelago a virtually closed system. In this regard, archipelagos are "living laboratories" suggesting insights about the ecology and evolution of infectious diseases and offering unique experimental testing protocols to reduce or eliminate the diseases not only in the islands but potentially across the world (13). Thus, the Balearic Islands present an opportunity to be used as a benchmark to explore how isolation and after-lockdown measures impact secondary COVID-19 waves.

COVID-19 has a particular structure in the timings of the disease that make it particularly dangerous in terms of a silent spreading potential. First, the incubation period, i.e., the time since infection to symptom onset, is relatively large around 5.2 days [95% confidence interval (CI), 4.1–7.0] (14). This itself is a driver of the predictability of the spatiotemporal patterns to expect from this disease (15). Furthermore, the latent period, i.e., the time from infection to the start of being infectious, does not align completely with the incubation period (4). Although latency periods of median 3.69 days (95% CI, 3.30–3.96) have been reported (16) infections can occur days 1 and 2 after exposure (4, 17). This leaves a period of presymptomatic infectivity, that increases R_0 through silent spreading, as not even the carrier might be aware of its own infectivity (18). The relative effectiveness of different non-pharmaceutical interventions will depend critically on the relation of those times (incubation and latent period) (19). Other related periods that shape the dynamics of the outbreaks are the generation interval (time between infection of infector-infectee pairs) (20) [example of mean values of generation intervals are 5.20 days for Singapore data, and 3.95 days for Tianjin, (21)] and the serial interval (the time between symptom onsets of an infector-infectee pair), which has also been used to estimate viral shedding dynamics for COVID-19 (example of serial interval values are characterized by a mean of 5.8 days [95% confidence interval (CI), 4.8–6.8 days] (4).

We aimed to study the dissemination of COVID-19 in an isolated system through a compartmental model that included, besides the susceptible (S), diseased (D) and recovered (R) compartments, an exposed (E) compartment, and a pre-symptomatic (I) infective compartment to account for the incubation period, as the times of transit between the latter two compartments are crucial for the modeling of COVID-19 (3, 4). Due to population size, we can implement an individual-based model where we consider each inhabitant as an individual in the model. In particular, first, we compare the results of an individual-based model tailored for the Balearic Islands and identify the parameter values that best fit the data. Second, we explore the likelihood of a second-wave scenario as a function of the initial date of the first imported case and the number of imported cases before the lockdown.

MATERIALS AND METHODS

Data

Population data for the 67 municipalities in the Balearic Islands were taken from the Instituto Nacional de Estadística (INE, Spain), which gathers all the census data (11). The census also provides the commuting flows for people that, according to the registry, are living in one municipality and work in another. This allows assigning a living location and working location to each individual. For small municipalities, these commuting fluxes are not included. We avoid the isolation of these municipalities (to be specific, Formentera with a population of 12,111 inhabitants, Escorca 280, Estellencs 389, Banyalbufar 605, and Deyá 755) considering commuting flows of 10 people toward each of the neighboring municipalities and Palma, the capital of Balearic Islands.

Data for the active infected and cumulative infected cases are obtained from the Ministry of Health (12). In particular, the official reports provide data on the cumulative number of infected, recovered, and deaths. The number of active infected cases is taken as the cumulative number of infected cases and subtracting deaths and recovered. Unfortunately, the values for recovered cases are only reported from March 22nd. Thus, for the fitting, we considered all the historical series for the cumulative number of cases while only values starting from March 22nd for the number of active cases.

Model

The relatively small population size of the Balearic Islands allows us to develop an individual-based model. Each individual is placed in one of 67 municipalities according to the census (**Supplementary Figure 1**). The mobility between municipalities is considered with commuting data from the 2011 census provided by the INE (11). For each simulation day, we consider two steps, one where each individual is located in its residence municipality, and a second step where each individual is placed in the working place. At each step, individuals can interact with any of the individuals placed at the same location (**Figure 1**). The number of individuals with residence in location i and commuting with location j is denoted by N_{ij}. Thus, the population at location i is given by $N_i = \Sigma_j N_{ij}$, and the population at location i during working time is given by $N_i^{'} = \Sigma_j N_{ji}$. The location assignment is done sequentially starting from $N_{1,1}$, then $N_{1,2}$, … until $N_{67,67}$. This assignment is done initially and such positions remain unchanged during the time evolution of the model.

The states of the individuals correspond to a SEIDR model: S, susceptible; E, exposed, corresponding to the latent period; I, infectious, corresponding to the presymptomatic infective period; D, diseased, corresponding to be infective with or without symptoms; and R, recovered. The transitions between these states are as follows, S becomes E in contact with an infected individual (I or D) with probability β. After $T_{\rm lat}$ (latent period) days, E becomes I; after $T_{\rm inf}$ (presymptomatic infective period) days I becomes D, and after $T_{\rm dis}$ (disease), D becomes R (**Figure 1A**).

The values of $T_{\rm lat}$, $T_{\rm inf}$, and $T_{\rm dis}$ were obtained from the time evolution of the number of active infected and cumulative infected cases in the Balearic Islands. The lockdown was imposed in Spain on March 16th and the effect of the mobility restrictions can be identified on March 22nd. The 6 days in this period are reflected in the incubation period, $T_{\rm lat} + T_{\rm inf}$ (**Supplementary Figures 2A,C**), and agrees with recent estimations (22). Finally, from the data on the cumulative number of infected cases, the change in slope is observed on April 2nd, that is, $T_{\rm dis} = 12$ days (**Supplementary Figures 2B,C**).

To implement the mobility restrictions, we observe from the data that the cumulative number of infected cases shows a bending every 7 days approximately, which is in accordance with the beginning of the lockdown, and the restriction imposed on March 15th, and later corrected on March 22nd and March 29th (**Supplementary Figure 2D**). Thus, the model has the freedom to adjust the infection probability every week after March 15th.

For a single day, the modeling proceeds as follows (**Figure 1**),

1. First, it considers the population in their residence location, for each municipality pairs of individuals in the same municipality are selected, say i and j. Then, i updates his/her state according to the dynamic rules. For each municipality p, N_p pairs are chosen randomly where N_p is the population size of the municipality p.
2. Second, we consider the individuals distributed in the municipalities of work. For each municipality p', we chose N_p' pairs of random individuals working in the same municipality p'.
3. Resume from 1.

Thus, on average, in a day, each individual is updated twice.

For calibration, the model is run exploring all the parameters: $T_{\rm lat} + T_{\rm inf} = 6$ and $T_{\rm dis} = 12$; and β is explored in the range [0, 1] in the following periods: β_1 from the origin of the infection on February 9th to March 15th, β_2 from March 16th to March 22nd, β_3 from March 23th to March 29th, β_4 from March 30th to April 5th, β_5 from April 7th to April 11th

The total number of infected cases depends on the date of the first infection. Models assume that the beginning of the outbreak is typically 30 days before the day when 10 infections are recorded (23). In the case of the Balearic Islands, on March 8th, 11 confirmed cases were reported. The first case reported in the Balearic Islands corresponds to an imported infection notified on February 9th. Consequently, the beginning of the outbreak was set on February 7th, 2 days before the first infected case was identified. Thus, we explore the date of the beginning of the infection between Jan 28th and Feb 7th.

Model Validation

The results of the model are validated with the official number of active infected and the cumulative number of infected cases between March 15th and April 11th. As the official values do not take into account the non-tested asymptomatic and the diseased not consulting to the healthcare systems, we assume that the reported values are a proportion of the values obtained from the model. Then, to validate the model parameters we minimize χ^2, $\chi^2 = \Sigma\ (\alpha\ Y_i - y_i)^2$, where α is a scale factor, that is, the ratio between estimated and confirmed cases, Y_i is the value obtained from the model in day i, and y_i is the official value in day i. Due to the initial exponential growth of the epidemics, we calculate χ^2 for the logarithm of the cases: $\chi^2 = \Sigma\ (\log(\alpha\ Y_i) - \log(y_i))^2$. The minimization of χ^2 leads to an optimal scale factor $\log(\alpha^*) = 1/n\ \Sigma\ (\log(y_i / Y_i))$, where n is the number of observation days. For this value of α^*, we finally calculate the optimal values. Our assumption implies that the scale factor should be similar to both the active and cumulative infected cases.

For each set of parameters, we report the χ^2 of the model values of the number of active infected cases with respect to the official values, the correction fraction $\alpha_{\rm active}$, and the $\chi^2_{\rm acc}$ of the model values of the number of cumulative infected cases with respect to the official vales and the correction fraction $\alpha_{\rm acc}$. For each set of parameters, the best fit is considered as the one leading to the minimum χ^2. Once the fitting values are determined, we calculate $\chi^2_{\rm acc}$ and $\alpha_{\rm acc}$. For each set of periods ($T_{\rm lat}$, $T_{\rm inf}$, $T_{\rm dis}$), we explore the infection probabilities that minimize χ^2 of the number of active infected cases. The value of χ^2 and scale factors α^* of the best fits are shown in **Supplementary Table 1** and the estimated prevalence in **Supplementary Table 2**.

Quarantining Mechanism

In order to explore the effect of quarantines on the impact of the second wave we implemented a stochastic quarantine mechanism. When an individual reaches the symptom onset, i.e.,

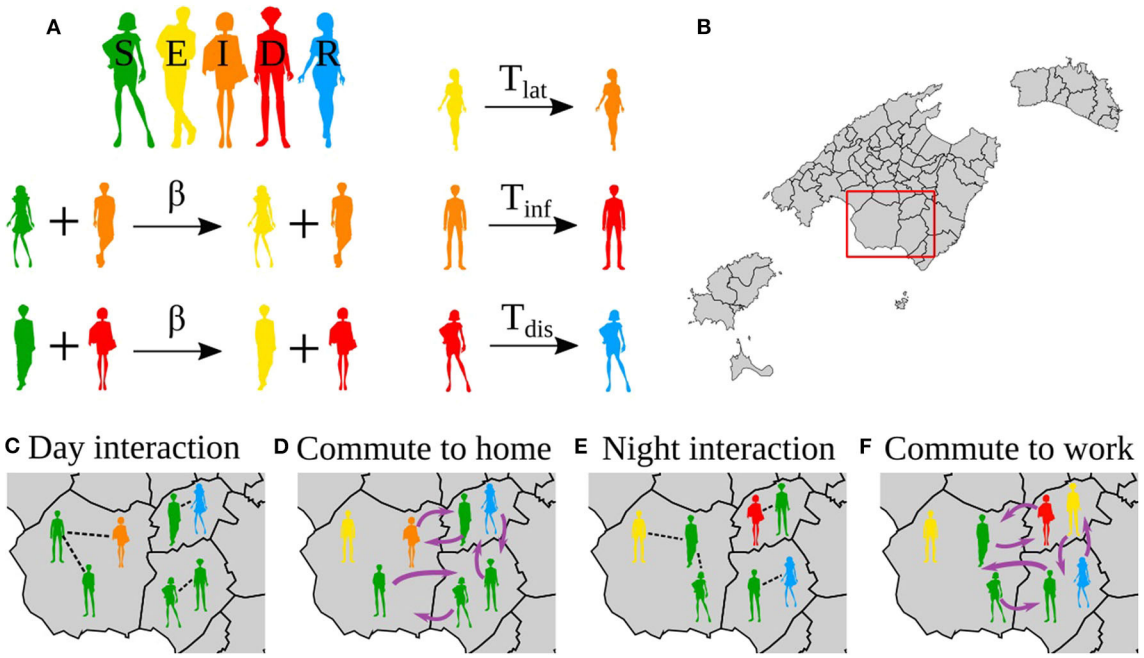

FIGURE 1 | Reactions in the model, interactions and movements. **(A)** Reactions in the epidemic model. **(B)** Map of the Balearic Islands showing all the municipalities in the islands. **(C–F)** Zoom to the region in the red box in **(B)**. **(C,E)** Show the interactions during the day and night, respectively. **(D,F)** Show the mobility to the night location (home) and day location (work), respectively. Note that the mobility patterns are reversed from **(D)** to **(E)**, i.e., the same agents always do the same commute.

when they reach the D state, with probability q the individual is isolated from further interaction with other individuals. The probability q can be understood as the fraction of cases that will be quarantined or isolated.

Herd Immunity Assumptions

An approximation to the herd immunity threshold is given by $1-1/R_0$ (24), which for COVID-19 is expected to be between 29 and 74%, taking R_0 between 1.4 and 3.9 (14, 25). To explore whether the number of cumulative infections reach the herd immunity threshold and therefore avoidance of potential second waves is to be expected, we run the model for the same parameters leading to the best fit (24). After the system has relaxed to zero infection, we select a random susceptible from the populations and infected her. As we are interested in whether the epidemics will spread again, we use the transmission rate obtained at the beginning of the epidemics in the Balearic Islands, that is, before any restriction on mobility had been applied. We can expect that once the mobility restrictions have been removed, the transmission will be reduced in comparison to the initial values, especially due to an improvement in the hygiene of the population. This will affect how fast the COVID-19 will spread and the intensity of the wave. If the estimated number of infected cases is lower than the threshold for herd immunization, we assume that SARS-CoV-2 will spread. In the **Supplementary Materials**, we show how the data can be collapsed using a proper combination of the initial number of infected cases and the time of the first infection.

RESULTS

Number of Active Infected Cases

The best fit of the model to the confirmed cases, allows us to extract the transmission probabilities and also the scaling factor that captures the ratio between the estimated and the confirmed cases. For the scenario where the initial date was on Feb 7th, and a latent period of 2 days ($T_{\rm lat} = 2$), an infective period of 4 days ($T_{\rm inf} = 4$), and disease period of 12 days ($T_{\rm dis} = 12$), the values of the infection probability leading to the best fit are $\beta_1 = 0.24$, $\beta_2 = 0.12$, $\beta_3 = 0.016$, and $\beta_4 = 0.036$ (**Figure 2**). This translates into an initial basic reproductive number $R_0 = 3.84$. The fitting of the data also informs us that the correction factor is 0.054, that is, that the confirmed cases are 5.4% of the model estimates. At the same time, we obtain that the percentage of added recovered cases and fatalities according to the official sources the model estimate are 5.3% of the confirmed cases. For the other values of the latent, infective, and disease periods, we obtain similar accuracy, given by χ^2 and similar scaling factors (**Supplementary Table 1**). The scaling factor, which gives the fraction of the model estimates, that corresponds to the confirmed case, increases to 10% in the case of $T_{\rm lat} = 5$, $T_{\rm inf} = 1$, which is the case with less infected individuals in the model.

The introduction of a single imported infected case after the first wave has expired produces a secondary wave that strongly depends on the first one (**Figure 3**). The intensity and duration of the second wave depend on specific values capturing the conditions applicable when newly infected cases appear, e.g.,

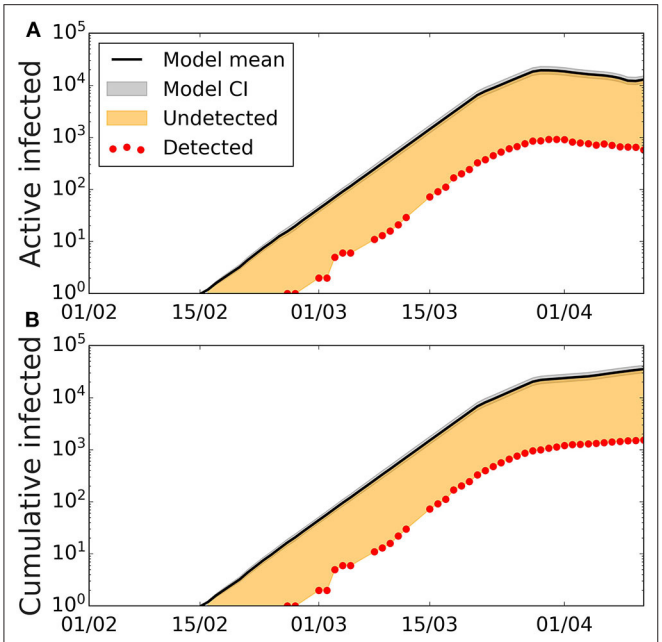

FIGURE 2 | Active and cumulative infected time series in the Balearic Islands. Time evolution of the average number of **(A)** active infected cases (black line) and **(B)** cumulative number of cases with the best-fitted parameters, and confidence interval CI 95% (gray area). Confidence intervals calculated using Cox's method (26). Red dots represent the data provided by the Ministry of Health of Spain, the solid lines depict the model with the fitted parameters $T_{lat} = 2$, $T_{inf} = 4$, $T_{dis} = 12$, $\beta_1 = 0.24$, $\beta_2 = 0.12$, $\beta_3 = 0.016$, $\beta_4 = 0.036$, $\beta_5 = 0$, the orange area represents the number of undetected cases when comparing model results with the data on detected cases.

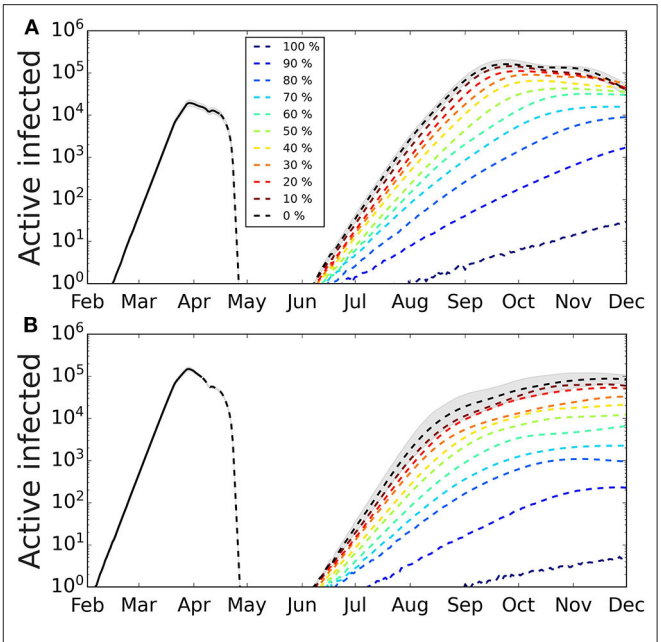

FIGURE 3 | Secondary outbreak appearing after the home-based confinement is removed. The lockdown is removed and the parameter values are as during the week of March 16th–22nd. We simulated different degrees of quarantines to explore its effect on the second wave. The greater the percentage of infected cases that are quarantined at symptom onset, the smaller the incidence of the second wave. The shaded area represents the 95% CI (for clarity, only shown for the case of no quarantine). Confidence intervals calculated using Cox's method (26). **(A)** First infected case is on February 7th, the number of infected in the first wave has not reached herd immunization and a second wave is triggered by a single infected case. The intensity of the second wave is one order of magnitude larger than the first. **(B)** First infected case is on January 28th, the number of infected cases in the first wave has reached a larger fraction of the population and the intensity of the second wave is, in the scenario, smaller than the first. Average over 100 realizations.

the transmission probability, which depends on the habits of the population, hygiene, and social distancing, and mobility restriction. Qualitatively similar results were obtained for the other set of values of the characteristic periods. The peak of the second wave is very sensitive to the date of the first exposure. If it happened on January 28th, the intensity of the second peak is less pronounced and similar to the one for the first peak, in contrast to the case of a more recent exposure, when the second peak can be more than one order of magnitude larger the first peak.

Effect of Quarantines on the Second Wave

We show the results of applying different levels of the stochastic quarantine mechanism in **Figure 3**. The greater the percentage of quarantined cases, the smaller is the peak of the second wave, and the longer is it in duration. We also observe that, depending on the fraction of cases that occurred during the first wave, the effect of quarantining varies. So for the case when the first infection occurred on Feb 7th (**Figure 3A**), the fraction of quarantined cases that we need to reduce the peak of the second wave by one order of magnitude is around 60%, while for a stronger first wave (**Figure 3B**) the reduction of one order of magnitude of the second wave is obtained with 40% quarantine.

Herd Immunization Estimates

Assuming recovered individuals get immunity, to estimate whether the Balearic Islands have reached herd immunization, we explored the estimated number of infected cases under two immunization scenarios based on the date of the first infection (**Figure 4A**) and the number of imported cases before air and maritime transport lockdown (**Figure 4B**). Firstly, we analyzed how the estimation of infected individuals is sensitive to the date of the first infection. We explored the time range of the first infection from January 28th (which corresponds to the stay in France before returning to Majorca) to Feb 7th (which corresponds to 30 days before the 10th confirmed case). Secondly, we explored the estimates under the assumption that more than one imported case could have gone unnoticed into the Balearic Islands before the closing of the airports. Depending on these two parameters, the range of immunization spans from 3% (for one initial infected on February 7th) to 64% (for 20 initial infected cases on January 28th). With the assumption of immunity after recovery, the achievement of herd immunization in the population is very sensitive to the date of the first infection and the number of imported cases before

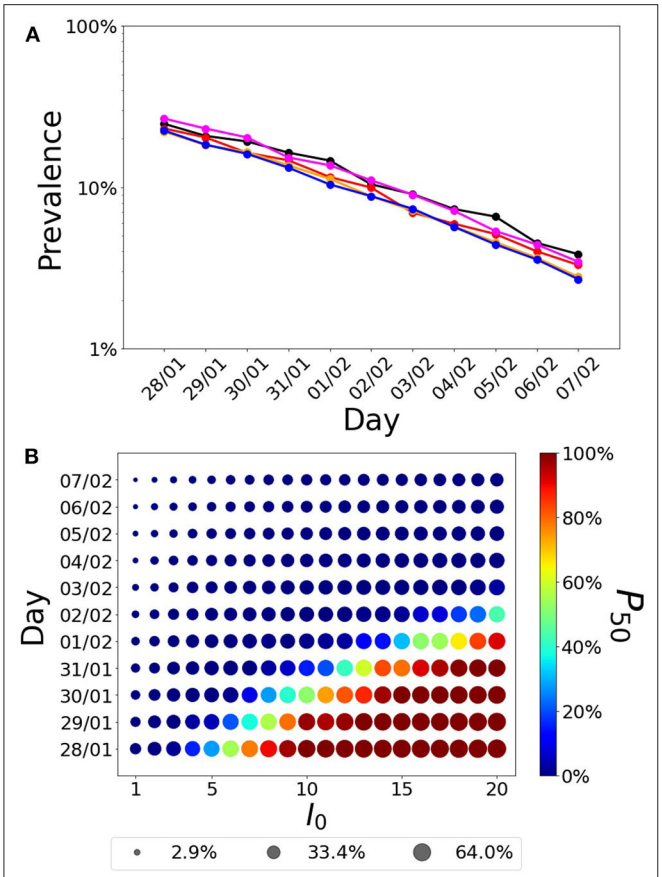

FIGURE 4 | Fraction of infected individuals (in logarithmic scale) as a function of the date of the first infection. **(A)** Each line corresponds to the following parameter values(T_{lat}, T_{inf})= (1,5) (black),(2,4) (red), (3,33) (orange), (4,2) (magenta), and (5,1) (blue). **(B)** Fraction of infected cases as a function of the first infection and the initial number of infected individuals. The radius of the symbol is proportional to the fraction of infected cases while the color indicates the probability that a realization of the model reaches at least 50% of infected cases in the population, P_{50}.

air and maritime transport lockdown. The interpretation of herd immunization indicates that if infected individuals become immune, then a percentage r of herd immunization prevents the spreading of reproductive numbers smaller than $1/(1\text{-}r)$. Assuming that the first case was exposed to the infection during his stay in France in the last days of January, the percentage of the population that was infected can be as high as 50%, which could prevent a high second peak, for values of the basic reproductive number below 2. Conversely, if the first case was infected 30 days before 11 confirmed cases were reported in the Balearic Islands, the percentage of infected individuals could lower to <10%, therefore falling far from potential herd immunity (only for values of the basic reproductive number below 1.1). The relation between the number of initial infected cases, the date of the first infection, the number of cases, and the number of confirmed cases is further explored in the **Supplementary Materials**.

CONCLUSIONS

Our study shows that a model including five compartments together with information on mobility between municipalities can be used to capture the spread of the epidemics in a closed community. The validation of the model with the official data allowed us to obtain the parameters that best fitted the data. Once the model was validated, we extracted an estimation of the number of the total infected in the Balearic Islands that indicates, assuming immunization after recovery, that these figures would reach the herd immunization threshold depending critically on the date of the first infection and the initial number of seeds, being herd immunization achievement more likely for an initial date before January 31st and number of initial infected above 10. Our exploration of the forecasted scenario of a newly infected individual entering the community after the lockdown confirmed that the number of potential cases widely varies according to the initial date of infection, which correlates with the percentages of immunity. Although we cannot determine with precision the start of the infection in the Balearic Islands, the model suggests that the Balearic Islands population is below the herd immunization threshold and thus, also susceptible to new outbreaks depending on how immunity is acquired and how the mobility restrictions are further implemented. In particular mobility and transmission probability, which depends on the general use of masks and hygiene protocols by the population, might alter the attack rate.

Focusing on second waves in insulated areas during the COVID-19 pandemic is of great value to analyze the spreading and containment of infectious diseases, where the lockdown of islands constitutes a paradigmatic scenario, with the potential to be applied to continental regions (13). For example, the risk of COVID-19 import to the Pacific Islands had been assessed and analyzed in the early stages (27–29). The use of modeling tools is a complement to field studies that can be used to anticipate the progress of a pandemic and thus help health authorities to make decisions. In the case of the Balearic Islands, there are two foremost advantages in terms of model precision. First, since the incidence during the first peak was relatively low and hospital capacity including ICU beds was not overpassed, the forecasted scenario of a second wave presenting with more intensity is more feasible than in other areas. Second, the relatively small size of the Balearic Islands and the organization of health and epidemiological surveillance systems make the official accounts of reported cases more reliable than in other areas were due to low rates of testing, overloaded hospitals, and lack of centralized data collection hampered the initial estimates.

The implications of the forecasted potential second wave yielded by our model for an insular territory can be useful also for other areas that are either naturally geographically isolated or closed to external perturbations due to strict lockdowns. According to our results, the date of the first infection and the import of cases while the airports and ports were open appear to be key to assess the likelihood and intensity of future waves and outbreaks. Knowing the approximate date of infection of the first reported case in an outbreak proved critical to estimate the current and foreseen number of cases. Whether a second

wave occurs and the intensity of the peak strongly depended on the date of the first infection, as the number of infected cases grows exponentially, but also on the number of imported cases, which contribute additively to the number of cases, and also on the real herd immunization. Our estimates rely on calculations assuming conditions far from the behavior of the population, and on the habits, for example, regarding hygiene, the use of masks, and social distancing, of the population after the lockdown is relaxed. We further show that the effect of quarantining measures strongly depends on the level of immunization reached by the population during the first wave.

Our model is an individual-based model for which, due to the population size, we identify each inhabitant with an individual in the simulation model. This approach is different from other models considering pan-mixing and ordinary differential equations (8), and forecasts based on iteration methods (24, 30). Other approaches implement recurrent mobility (3, 31, 32), which selects the individuals that perform the commuting randomly at each iteration step, thus increasing the mixing in the complete population. Our approach assigns a residence and a working municipality as initial condition and these locations remain unchanged during the time evolution for the model. Our implementation assumes that the same person commutes between two locations and thus it has to be fixed initially in the model. A random selection at each day will increase the number of effective connections, which could be compensated by a reduction in the transmission probability. We believe this approach is more comprehensive and better captures the reality of commuting under home confinement conditions, which essentially limit mobility from households to workplaces for those individuals that cannot work remotely or are not exempted from any work under the regulations of each country, while the rest of the population are not supposed to move from the vicinity of their households and even then only for justified reasons such as basic food supply. We use here a stochastic approach similar to other works (22, 33) which lets us compute confidence intervals even for single combinations of the parameters instead of deterministic ordinary differential equations (8, 34, 35) or discrete-time dynamic equations (3). We also use a fixed time for the transit through the E, I, and D compartments, T_{lat}, T_{inf}, and T_{dis}, respectively. We believe this approach is more realistic than an approach based on rates, where individuals transit the compartments at a given rate, giving rise to exponentially distributed times of transit through the compartments. In this case, infected cases will have the opportunity to be infectious immediately, or to transit the I compartment also immediately, bringing the start of secondary infections closer to the time they were infected for many individuals. A similar effect happens with the length of the disease (time in the D compartment), having then individuals that immediately recover.

The model also has several limitations. First, as it is constructed for fitting the global numbers of infected patients, it is missing finer structure, needed for the evaluation of risks of subpopulations that are differently exposed to the virus or have different outcomes, such as the population of elderly people or health workers, and the effect of city size (36, 37). Second, for COVID-19 there is evidence of three main transmission channels, namely direct contact with an infected individual with symptoms (14), contacts with an asymptomatic individual (38, 39), and environmental transmission (40). The present model takes into account the first two modes of transmission, but not the environmental one explicitly, although probably the fitting has assigned part of this transmission to the processes included in our model. Therefore, there is not a direct way of measuring the effect of interventions to reduce environmental transmission. Third, the model also considers asymptomatic and symptomatic individuals to be infectious in the same way, although the viral shedding in asymptomatic individuals is indeed lower (5). This can have an impact on the number of infected individuals and deserves future research. Fourth, the model assumes that the mobility restrictions are applied in the same way to all of the agents in the system and thus is lacking the fact that symptomatic infected individuals will modify their mobility drastically, either if they are quarantined at home or admitted to a hospital. We are therefore overestimating mobility, but this is probably passed to the infectivity in the fitting procedure. Fifth, the model also takes fixed times to transit through the E, I, and D compartments (T_{lat}, T_{inf}, and T_{dis}, respectively), which is artificial. More refined models would take these transit times from specified distributions matching the parameters of the disease (33, 35). While this will render the model more realistic, we believe that fixing the times is a good compromise between using rates for transiting the compartments and implementing distributions for those times, as it already captures the delays induced by these particular timings of the infection. Finally, the model also assumes that individuals are granted immunity to the virus, at least for the timescales explored here.

In conclusion, the risk of secondary infection waves should be comprehensively and cautiously addressed before removing confinement measures. Our study provides several relevant findings that could be useful to support policy design at avoiding second waves once measures to return to the societal usual activities start to be applied. First, the isolation of asymptomatic individuals that tested positive for SARS-CoV-2 and close contacts to infected individuals during the prior 2 weeks might reduce the number of new infections after the establishment of the usual activity by preventing dissemination from asymptomatic carriers during the incubation period. This requires proper testing strategies tailored according to the estimated prevalence of infection, population density, the openness of the community, and other relevant factors. Second, contact tracing measures are crucial, and digital tools might enhance the identification of high-risk individuals to be tested or preemptively isolated (41). Yet, data privacy and other relevant ethical considerations should be carefully balanced when designing contact tracing in the community. Third, progressive return to the normal activity instead of an abrupt change will facilitate the monitoring of new cases and may avoid a sharp growth of the number of infected individuals, which is expected when herd immunity has not been reached. Experience from other archipelagos illustrates the potential of pursuing an elimination strategy (including a full lockdown)

together with quarantine of travelers from abroad (42, 43). Further modeling studies on second-waves of COVID-19 are warranted to strengthen the knowledge on the best theoretical assumptions and data to be used to increase forecasting precision. In addition, these models should be validated through real-world data as these are collected during and after the pandemic.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. We obtained the data from official sources of the Spanish government, including the Ministry of Health and the Instituto de Salud Carlos III for the account of official cases (https://covid19.isciii.es/resources/serie_historica_acumulados.csv) and the Instituto Nacional de Estadística for the mobility data (https://www.ine.es). Computing codes are available at https://github.com/juanfernandezgracia/Balearic-epi.

AUTHOR CONTRIBUTIONS

VE and JF-G designed the work. VE performed the analysis. VE, JF-G, JR, JP, and CM prepared the figures, tables, wrote the first draft, and provided final approval to the manuscript. All authors contributed to the article and approved the submitted version.

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This manuscript has been released as a pre-print at medrxiv (44). The silhouettes in **Figure 1** were designed by brgfx/Freepik.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.563455/full#supplementary-material

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frontiers in Public Health

MINI REVIEW
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Africa's COVID-19 Situation in Focus and Recent Happenings: A Mini Review

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Given that COVID-19 (SARS-CoV-2) has crept into Africa, a major public health crisis or threat continues to linger on the continent. Many local governments and various stakeholders have stepped up efforts for early detection and management of COVID-19. This mini review highlights the current trend in Africa, history and general epidemiological information on the virus. Current ongoing efforts (e.g., improving testing capacity) and some effective ways (e.g., intensified surveillance, quick detection, contact tracing, isolation measures [e.g., quarantine], and social distancing) of preventing and managing COVID-19 in Africa are described. The review concludes by emphasizing the need for public health infrastructure development (e.g., laboratories, infectious disease centers, regional hospitals) and human capacity building for combating COVID-19 and potential future outbreaks. Additionally, regular public health educational campaigns are urgently required. Future epidemiological studies to ascertain case fatality and mortality trends across the continent for policy directions are necessary.

Keywords: COVID-19, intensified surveillance, detection, quarantine, contact tracing, Africa

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BACKGROUND

The recent novel coronavirus (COVID-19) pandemic has speedily escalated from China to other geographical boundaries, including Africa (1, 2). The initial ill-conceived thoughts that Africa is not conducive for the virus and that Africans have strong immune systems to combat the virus have been debunked with multiple confirmed cases (3). The swift rise of the pandemic across the continent is worrisome and has created a serious public health threat. This mini review provides the history and general epidemiological information on the virus as well as current trends in Africa. On-going concerted efforts and some effective ways of preventing and managing COVID-19 in Africa are also described.

HISTORY AND EPIDEMIOLOGICAL INFORMATION ON COVID-19

COVID-19 originated from a group of coronaviruses with RNA genome ranging from 60 nanometer [nm]to 140 nm in diameter with spike-like projections sprouting on a crown-like feature under the microscope; hence its coveted name coronavirus. Generally, four specific

coronaviruses, namely HKU1, NL63, 229E, and OC43 have been found in human beings to cause mild respiratory infection (4, 5). The pathological characteristics of the current COVID-19 is similar to the severe acute respiratory syndrome (SARS; 2002–2003) and Middle East respiratory syndrome (MERS; 2012) outbreaks. SARS originated through zoonotic transmission of a novel coronavirus from bats through palm civets in markets in Guangdong Province, China from 2002 to 2003. This virus was reported to have affected approximately 8,422 people mainly in China and Hong Kong, with 916 reported deaths (Case Fatality Rate [CFR], 17%) (6). MERS also emerged from zoonotic transmission of a group of viruses that had previously been detected in bats and cultured from respiratory secretions of a patient who had died from SARS in 2012 (7). The same causative organism was previously discovered in clinical trials from a severe occurrence of acute respiratory disease, which was first reported in humans in Jordan in 2012 (8, 9). By 2013, 55 laboratory-confirmed cases of MERS were reported in Jordan, Saudi Arabia, the UK, France, Italy, Germany, and Tunisia (10). According to Wu and McGoogan (7), all the 3 viral contagions commonly manifest with fever and cough, which regularly cause lower respiratory tract disease with poor clinical outcomes related to old age and primary health conditions. The detection of infection requires testing of respiratory tract samples (e.g., throat swabs) through clinical diagnosis which can also be done based on symptoms, exposures, and chest imaging.

Although early genetic assessment on COVID-19 in China revealed that the virus was similar to SARS-CoV and MERS-CoV, scientific evidence in the past months has revealed major differences between the other outbreaks and characteristics of COVID-19 (11). Reflecting on available epidemiological data on the current upsurge, since December 2019 when the first cases were recorded in Wuhan, China, the virus spread has solely been driven by human-to-human transmission and not only as a result of continuous global spillover (11). According to Heymann et al., COVID-19 reproduces in the upper respiratory tract and shows minimal symptoms comparable to the other human coronaviruses that manifest with common colds during winter. Affected persons create a large amount of virus in the upper respiratory tract at the onset or prodrome period, go about normal duties with ease and thus facilitate the spread of the disease. Comparatively, SARS-CoV transmission did not readily occur during the prodromal phase when infected persons were reported mildly ill, and most transmissions were believed to have happened when infected individuals showed severe illness. These symptoms made it easier to control SARS-CoV outbreaks compared to COVID-19 (12). Because the COVID-19 virus is transferred via droplets produced through coughing and sneezing by persons with notable symptoms and in some cases from asymptomatic individuals, virtually all ages are susceptible (13). Infection could be contracted either by inhaling these tiny microscopic droplets or touching surfaces infected by the virus and then touching the nose, mouth, eyes and/or face (13). According to Kampf et al. (14), the virus can stay active on surfaces for days in positive atmospheric conditions but can also be killed within a moment with common antiseptics or disinfectants.

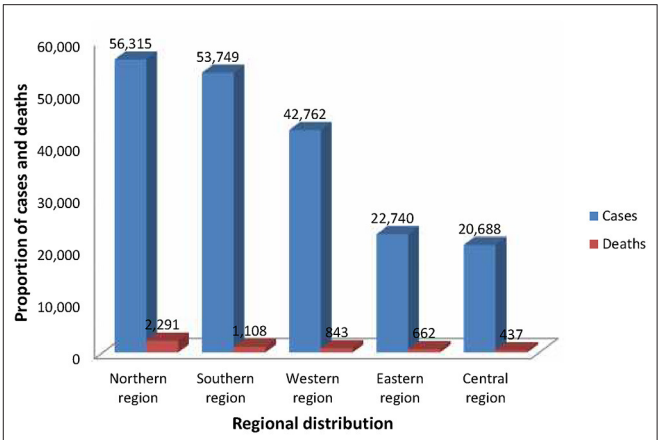

FIGURE 1 | Regional distribution of COVID-19 cases and deaths in Africa as of 9 am EAT 9 June 2020. This figure is original and based on data from Africa Center for Disease Control (4).

Reported clinical features of COVID-19 range from acute respiratory distress syndrome to organ dysfunctions. Common symptoms also include fever, headache, sore throat, cough, breathing difficulties, and exhaustion. The infection can progress within a week to pneumonia, respiratory failure, and death in different patients (15). According to Singhal (4), the duration from the onset of symptoms to dyspnea is 5 days, about 7 days to hospitalization, and development of acute respiratory distress syndrome (ARDS) in 8 days. Infected persons may require intensive care admission. Reported recovery may take 2 to 3 weeks. Serious consequences and deaths are more pronounced in the elderly and persons with co-morbidities (50–75% of fatal cases) and fatality rate of adult patients ranges from 4 to 11% whereas overall case fatality rate is estimated to range between 2 and 3% (16, 17).

RECENT COVID-19 TRENDS IN AFRICA

Available data shows that since the first case of COVID-19 was noted on the 14th February, 2020 in Egypt, a total of 196,254 COVID-19 confirmed cases and 5,341 deaths (Case Fatality Rate [CFR]: 2.7%) have been reported in 54 African countries as of 9 am EAT 9 June 2020 (18). This figure is an estimation of 2.8% of all cases reported globally. The proportion of confirmed COVID-19 cases and deaths reported by African sub-regions in the order of severity is as follows: Northern region (56,315 cases, 2,291 deaths), Southern region (53,749 cases; 1,108 deaths), Western region (42,762 cases, 843 deaths), Eastern region (22,740 cases, 662 deaths), and Central region (20,688 cases, 437 deaths, see **Figure 1**).

The top three countries worst affected in each region as at the beginning of 9th June 2020 9 am. EAT stood as follows: Northern region (Egypt, 35,444 cases, 1,271 deaths; Algeria,

Abbreviations: CFR, Case Fatality Rate; WHO, World Health Organization; IMF, International Monetary Fund; UNICEF, The United Nations Children's Fund.

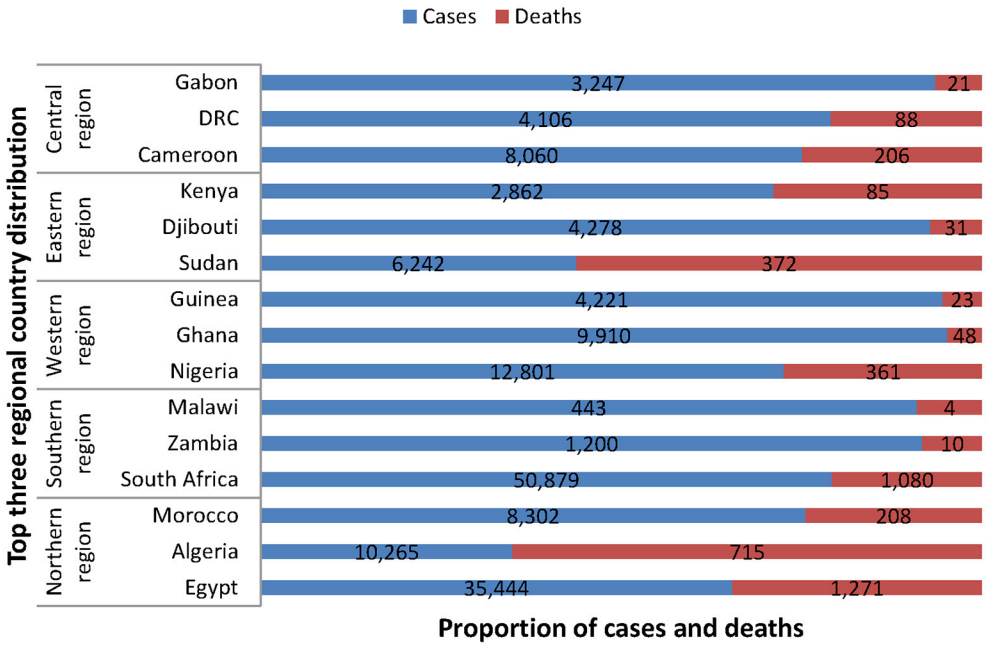

FIGURE 2 | Top three regional country distribution of COVID-19 cases and deaths in Africa as of 9 am EAT 9 June 2020. This figure is original and based on data from Africa Center for Disease Control (4).

10,265 confirmed cases, 715 deaths; Morocco, 8,302 cases, 208 deaths), Southern region (South Africa, 50,879 cases, 1,080 deaths; Zambia, 1,200 cases, 10 deaths; Malawi, 443 cases, 4 deaths), Western region (Nigeria, 12,801 cases, 361 deaths; Ghana, 9,910 cases, 48 deaths; Guinea, 4,221 cases, 23 deaths), Eastern region (Sudan, 6,242 cases, 372 deaths; Djibouti, 4,278 cases, 31 deaths; Kenya, 2,862, 85 deaths), and Central region (Cameroon, 8,060 cases, 206 deaths; Democratic Republic of Congo [DRC], 4,106 cases, 88 deaths; Gabon, 3,247 cases, 21 deaths, see **Figure 2**) (18).

By 4th August, 2020, a total of 968,020 COVID-19 cases and 20,612 deaths (CFR: 2%) had been reported in 55 African countries. The estimation of new COVID-19 cases reported by region still represented 5% of all cases globally. Below are the proportions of cases by sub-region: Southern region 65% (70,318), Northern region 12% (13,016), Western region 9% (9,638), Eastern region 11% (12,367), and Central region 3% (3,444). A total of 6 countries accounted for 80% of the new COVID-19 cases reported within the period in a descending order: South Africa (59%), Morocco (5%), Algeria (4%), Kenya (4%), Ghana (4%), and Ethiopia (4%). South Africa (890), Djibouti (524), Sao Tome and Principe (437), Cape Verde (431), and Gabon (364) reported the most cumulative COVID-19 cases per 100,000 in Africa. Reported case fatality rates comparable to or higher than the global case fatality rate of 4% were noted in 11 African countries; Chad (8%), Sudan (6%), Liberia (6%), Niger (6%), Egypt (5%), Mali (5%), Burkina Faso (5%), Angola (5%), Algeria (4%), Sierra Leone (4%), and Tanzania (4%) (19). The pictorial epidemiological information of COVID-19 per region as of 31st July, 2020 is captured in **Figures 3A–C** below (20).

Africa CDC brief report on public health and social measure implementation indicated that these new cases decreased by 23% between 28th July and 10th August while new deaths increased by 11% within the same time period. Total COVID-19 cases exceeded 1 million for the first time which was cited as the peak period. The decrease in new cases was attributed to South Africa, which recorded a 35% decline in newly reported cases, with data showing that the COVID-19 outbreak might have peaked in some provinces or districts that were earlier affected by the virus. Though South Africa still leads in new cases, Morocco, Kenya and Ethiopia reported rising case counts and new deaths. The number of tests performed per positive case has remained low (6 tests per case, see **Table 1**), suggesting that many cases are undetected. With the low tests per case ratios in many African countries (e.g., Algeria, DRC, Egypt, Madagascar, Somalia, South Sudan and Sudan), denoting poor testing capacity, indications are that many cases on the continent are less likely to be detected. Therefore, reported decreases in new cases ought to be interpreted with caution in many countries. The month of October saw a slight increase in the number of COVID-19 cases per 100,000 population. Again, 6 countries accounted for nearly 84% of the new COVID-19 cases; Morocco (31%), South Africa (18%), Libya (11%), Tunisia (9%), Kenya (7%), and Ethiopia (6%) by 27th October, 2020 (19).

Given that accurate mortality metrics are essential for assessing the risks and severity often associated with epidemic outbreaks like COVID-19, it is crucial to better understand these associated concepts unambiguously in the estimation of the current pandemic. The generally used metrics during infectious disease outbreaks are the case fatality rate (CFR), case fatality

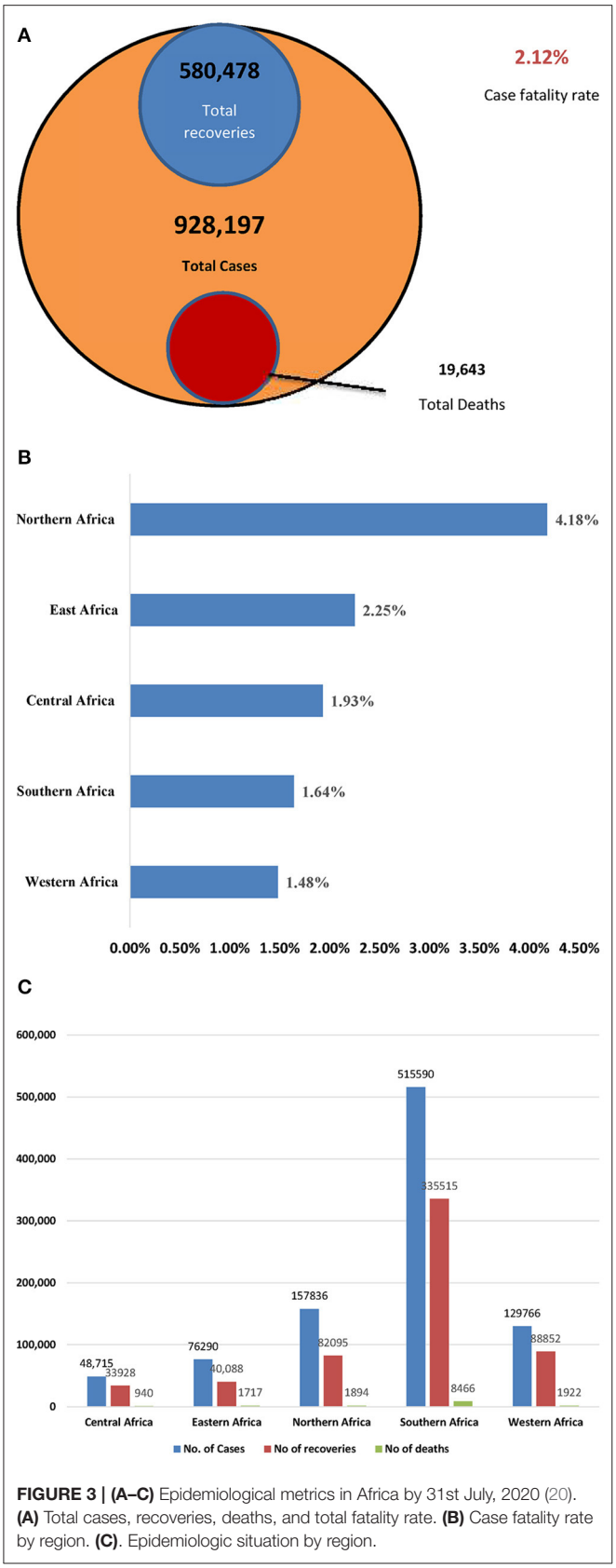

FIGURE 3 | (A–C) Epidemiological metrics in Africa by 31st July, 2020 (20). **(A)** Total cases, recoveries, deaths, and total fatality rate. **(B)** Case fatality rate by region. **(C)**. Epidemiologic situation by region.

ratio, and case fatality risk, often used interchangeably. Fatality *rate* means a change in deaths per unit time; *risk* denotes an individual probability; whereas *ratio* represents a fraction of two numbers, usually of populations. Therefore, CFR commonly represents the ratio of the total estimated number of deaths to date, $D(t)$, to the estimated number of all confirmed cases to date Nc (t) or an estimation of the number of deaths to date divided by the estimated total number of confirmed infected cases to date. Hence, these figures could be crucial for the estimation of the COVID-19 disease severity in Africa (21–23).

Conventionally, the reverse transcription polymerase chain reaction (RT-PCR) and antibody tests are used to confirm SARS-CoV-2-positive patients based on population data. To establish accuracy $D(t)$, the number of patients who actually die of COVID-19 should be appropriately be quantified. For instance, in Italy, deaths of patients with positive RT-PCR testing for SARSCoV-2 are reported as COVID-19 deaths and there could be similar cases across many countries. Therefore, the central criteria for COVID-19-related deaths as a function of CFR are currently not clearly defined and may vary from region to region (24). Other mortality metrics like case fatality risk, denoted as the probability of death of an individual confirmed case within a period of time and infection fatality ratios (IFR), representing the number of deaths to date divided by the number of all infected individuals add to the confusion. For example, IFR $= D(t)/N(t)$ requires an estimate of $N(t)$, the number of total (including unconfirmed) infected individuals [see (21) for detailed description].

According to Böttcher et al. (21), the further spread of SARS-CoV-2 in different countries, including those in Africa, makes it imperative that data on individual cases of death and recovery are easily stratified according to some specific COVID-19 mortality indices such as demographic and population heterogeneity, incubation period, health condition and a patient time of infection before confirmation is made on mortality estimates. Additionally, under-testing also confounds accurate estimation of actual causes of death. For instance, infected individuals who are untested in a given population consist of an unknown population which adds to deaths and recovery, and should be considered as "true" mortalities.

CURRENT COVID-19 EFFORTS IN AFRICA AND THE WAY FORWARD

The erratic COVID-19 increase and associated deaths in Africa underscore the essence for much attention, especially considering the weak public health systems of most African countries (19). Given the inherent challenges with general infrastructure network, poor transportation, neighborhood characteristics (e.g., over-crowding related issues) and inadequate trained health workers in many African countries, pragmatic measures have targeted preventive measures and effective management of confirmed cases. In a quick response to the current upsurge of the pandemic across Africa, the Africa Centers for Disease Control and Prevention in collaboration with WHO African

TABLE 1 | Africa COVID-19 Situation between late July and Early August 2020.

Africa Union Region	Total Death/Trend 28 July−10 August[a]	Total Deaths/Trend 28 July−10 August[a]	Countries where Test per cases[b] <10
Central	50353	961	Cameroon **(9)** Central African Republic **(6)** Chad **(9)** Congo **(6)** DRC **(5)** Equatorial Guinea **(9)** Sao Tome & Principe **(7)**
Eastern	91045	2007	Comoros **(6)** Madagascar **(4)** Somalia **(6)** S. Sudan **(6)** Sudan **(3)**
Northern	177118	7126	Algeria **(4)** Egypt (5)
Southern	589343	11093	Eswatini **(9)** Malawi **(7)** South Africa**(6)**
Western	140601	2087	Cote d'Ivoire **(6)** Gambia **(7)** Guinea **(8)** Guinea Bissau **(6)** Nigeria **(7)**

[a] *The total number of cases reported is the number of cases reported since the start of the epidemic. The trend compares new cases and new deaths during 28 July−10 August to 14–27 July to the previous 2-week period (14–27 July). The trend is illustrated in by an arrow icon: a green arrow indicates a decrease >5%, gray is a decrease/increase within 5%, and red means an increase >5%.*

[b] *The test per case is the number of tests performed per positive case. Countries with a low number of tests per case (<10) may not be testing widely enough to find all cases. Africa CDC recommends 10–30 tests per case, as a benchmark of adequate testing (19).*

Region instituted an Africa Task Force for Novel Coronavirus that recently launched the "Partnership to Accelerate COVID-19 Testing (PACT): Test, Trace, Treat in Africa" on 4th June, 2020 (18) to increase testing capacity in member states. PACT is to offer the needed support as joint continental strategy to assist member states limit COVID-19 transmission, through strategies such as staff training. As a result, a team of experts, community workers, supplies and other resources have been organized to help test, trace and treat COVID-19 cases in a timely manner to lessen the effect of the pandemic on the African continent. Key strategies such as an expansion of testing to sub-national, research, academic and private laboratories, increasing human resource capacity, reinforcing specimen collection, computerization of testing technologies, and use of pooled sample testing were endorsed (25).

Till date, over million test kits and several thousands of community health workers have been trained (i.e., with knowledge and key skills) and deployed. Again, 80 surveillance rapid responders for COVID-19 have been used in Africa. Additionally, 625,000 Polymerase Chain Reaction (PCR) tests have been advanced to 51 member countries and an extra support of 6,600 GeneXpert cartridges have also been advanced to three member states (i.e., Comoros, Guinea, Sao Tome) that have limited or no capacity for PCR testing. There has been extra supply of pathogen genomics equipment and reagents to member countries except Egypt (18). Laboratory testing capacity has increased from two to forty-four countries on the continent (26). Some countries (e.g., South Africa, Kenya, Morocco, Ghana, Uganda, Ethiopia) have made steady progress with testing capacity from a few hundreds to thousands. By August 2020, nearly 10 million tests have been done on the continent with 11% positivity rate. However, significant sub-regional variations exist in the testing capacity, with the Central Africa region contributing only 3.4% of the total conducted tests, showing that many countries on the continent are still struggling to increase testing capacity. Due to the limited testing capacity across many countries in the African region, pooled sample testing have been recommended to maximize the use of scarce test kits and other supplies to facilitate expansion in testing (27). Considerable efforts are currently ongoing to increase diagnostic capacity across the continent. For example, Morocco currently has 44 hospitals with 32 specialized centers that are fully equipped in response to the pandemic (28). South Africa, Ghana, Nigeria, Algeria, Senegal, and many African countries have laboratories for within country testing of COVID-19. Another testing strategy introduced by some countries (e.g., Ghana) is the Rapid Antibody

Tests (RAT). RAT uses a lateral flow technology, similar to home pregnancy tests using nasal swabs for point-of-care diagnosis of COVID-19 by measuring viral antigens or anti-viral antibodies, and results obtained within 10 min after sampling (29, 30). This lateral flow kits against COVID-19 have been developed and are either serology based (detecting host antibodies), or in some cases, antigen based (detecting specific viral proteins) (27). Many countries have created isolation and quarantine centers for the disease, with considerable efforts toward effective contact tracing of potential contacts with infected persons (31).

Besides all the instituted regional interventions, individual member states have adopted other mitigating strategies to reduce the spread of the virus at the national and community level. Since no specific drugs or vaccines are currently available, and health systems are overstretched, many affected countries adopted coercive and non-coercive community interventions with public engagement (32–35). Within local communities, continuation of some mitigation strategies to compel people to avoid crowded places or minimizing crowd sizes and exposure to body contacts (i.e., physical/social distancing) have been implemented. Such interventions included controlling essential social gatherings (e.g., funerals, church services, school attendance, remote working). Essential activities such as schooling and working were done with alternate arrangements such as remote or distance learning. New workplace interventions with work shifts and/ or rotational scheduling to reduce social density against the propagation of the virus have been employed. Conference calling and video conferencing helped working staff and tertiary students adhere to social distancing measures. Other activities such as attending night clubs, music festivals, cultural celebrations, and parties were temporarily suspended but have now been partially lifted with some restrictions (e.g., limited capacity, wearing of face/nose masks, physical distancing). Entertainment through virtual concerts with limited performers or artists has been introduced. Less-essential travels to places with ongoing transmission have been controlled. Since travel bans might trigger fear and affect economic life of the people, home delivery services of essential commodities have been adopted and still being encouraged, especially in the cities. Symptomatic individuals have been supported by telephone or online health consultation. Severe cases have been managed with the provision of essential life support such as oxygen supplies, and mechanical ventilators (32–36).

Adopting and compliance to low-cost evidence-based preventive measures in many African countries where socioeconomic status of the masses is low have been useful. For example, the use of locally made face or nose masks with non-medical cloth made from local fabric has been worn by people at public places and social gatherings. Senior public officials have been seen wearing these masks in public places to serve as an example for the local populace. Alcohol-based hand rub solutions have also been deployed at strategic locations (e.g., transport stations, market places, school/work environment, supermarkets) without restrictions (36). Continuous creation of awareness on the preventive and management measures related to COVD-19 through the media (e.g., radio, television in local languages and dialects) by public health officials and other properly trained analogous personnel across individual countries are still on-going on the African continent.

Protecting local jobs and employees, assets, technology, and infrastructure of critical sectors of economies have also been given a priority. African governments have tried protecting the livelihoods of citizens by preventing unemployment risks during this period. Private companies have been given financial assistance, tax and electricity waivers to maintain continuous production while observing preventive measures. Strategies to minimize workplace transmission have included daily pronouncements of being symptom free by all staff members, and where applicable, requested the screening of staff members. Therefore, screening everyone at a work place before resumption of work could be encouraged, especially in higher risk sectors like the hospitality industry (tourists and hotels), education, aviation or others with high risk of person-person interactions. Other areas such as occupational health service delivery should be crucial for the manufacturing and construction industries by offering close surveillance on workers and possibly test as well as facilitate the quarantine of any member with symptoms pending test results (37).

With the likelihood of reduction in importation during the current crisis, various local markets on the continent might significantly depend on domestic production, including agricultural products. Hence, support for local farmers through the provision of inputs such as fertilizers, weedicides, pesticides, outboard motors, fishing nets as well as mass spraying exercises on local farms could be part of policy interventions for food security. Local governments should provide social welfare interventions through the provision of food and other essential items for personal use, soap and shampoo, to persons living on the fringes of life during the crisis because of restrictions. These support mechanisms will help sustain families' income, preserve the productive capacity of the working population and human capital of enterprises as well as the overall economy of African countries (38). Amidst COVID-19, the entertainment industry should still be encouraged to use innovative virtual performances for the masses to help promote their psycho-social needs. Although country-specific data on health infrastructural expansion are not readily available, there are reported cases of on-going developmental projects (e.g., new disease centers, hospitals, ICUs) in the health sector across many countries (e.g., Morocco, Senegal, Kenya, Ghana, South Africa, Nigeria) through foreign aid to increase health facility capacity and recruitment/ training of healthcare professionals (39).

There are elements of fear, worry and panic about local transmissions and multiple infections because some individuals (e.g., internet bloggers, social commentators, opinion leaders, political officials) are disseminating diverse misinformation or unsubstantiated malicious information on the virus (37–39). This ill-advised COVID-19 related misinformation can rapidly spread the disease and can cause xenophobia on the continent (40–42). The distress, apprehension and other untruths about COVID-19 may have serious effects on disease control, and prior SARS and Ebola outbreaks are clear instances (43, 44). To manage this challenge, individual governments and media institutions should engage public health experts to dominate

the media landscape, especially those from the Centers for Disease Control to accurately provide relevant COVID-19 information to avoid fear among the general public (45). Mian and associate reiterated that if health institutions effectively manage public worries through regular education, the level of skepticism among populations often stirred by some social commentators, political opponents, and internet bloggers would be minimized. Local communities, civil society, media as well as other support groups are encouraged to provide accurate COVID-19 information. This goal can be achieved through strategic partnerships at community level so that authorized information is distributed (46).

Negative attitudes of some persons toward formulated public guidelines could be minimized by regular community monitoring by local task force teams. There is also the need to prioritize some special groups (e.g., the disabled, incarcerated persons, people living on the streets, illiterates and other marginalized groups [e.g., mentally challenged persons]) on the continent that are often left to live on the fringes during developmental issues. Considering the ailing health systems of most African countries, existing structures are unable to serve the needs of these groups adequately. Admittedly, these groups could also be at serious risk in times of pandemic outbreaks such as the COVID-19 due to their vulnerability. The COVID-19 precautionary messages, both text and audio, ought to be translated into all predominantly spoken local languages across the continent. The earlier audio-visual aids and braille versions of all precautionary measures are adopted, the better it will be for the continent to ensure the safety of these groups.

CONCLUSIONS

Many countries in Africa have instituted interventions to curtail COVID-19. However, these measures are not without challenges. Many health care systems in Africa are woefully inadequate characterized by under-resourced facilities. The World Health Organization (WHO) and donor agencies should partner local governments in the African region to establish new health infrastructure (e.g., referral laboratories) and equip existing ones with appropriate materials and requisite health logistics. African governments should adopt appropriate context-specific strategies that fit their contextual and geopolitical situations. To effectively deal with public health challenges like the current outbreak, training more human capacities in areas such as surveillance, rapid epidemic response, diagnostic testing, and crisis management should be compelling for governments as well as their private sector and international partners (e.g., WHO, UNICEF, IMF, World Bank). Besides, collaborating with local stakeholder groups (e.g., telecommunication companies-radio/television stations, religious and educational institutions) for regular public health educational campaigns to support the dissemination of COVID-19 information regarding prevention and control practices and creating awareness at the grassroots level are still required.

Although case-fatality rate (CFR) for COVID-19 on the African continent is lower than the global estimation, the scientific evidence about the virus could be inconclusive and may still evolve. Therefore, it is more likely other potential parameters (e.g., population heterogeneity- density, age distribution) may be unraveled in the months to come relative to other non-communicable diseases. It is imperative that compromised healthcare systems, including inadequate human capacity in Africa effectively should be managed effectively to overcome the current outbreak as well as future unforeseen ones. Future epidemiological studies to ascertain case fatality and mortality trends are warranted for policy directions.

AUTHOR CONTRIBUTIONS

JH, BA, A-AS, and EA conceived the work. JH, BA, A-AS, EA, and TS wrote and drafted the manuscript. All authors read and approved the final version of the manuscript.

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Clinical Utility of a Nomogram for Predicting 30-Days Poor Outcome in Hospitalized Patients With COVID-19: Multicenter External Validation and Decision Curve Analysis

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Aim: Early detection of coronavirus disease 2019 (COVID-19) patients who are likely to develop worse outcomes is of great importance, which may help select patients at risk of rapid deterioration who should require high-level monitoring and more aggressive treatment. We aimed to develop and validate a nomogram for predicting 30-days poor outcome of patients with COVID-19.

Methods: The prediction model was developed in a primary cohort consisting of 233 patients with laboratory-confirmed COVID-19, and data were collected from January 3 to March 20, 2020. We identified and integrated significant prognostic factors for 30-days poor outcome to construct a nomogram. The model was subjected to internal validation and to external validation with two separate cohorts of 110 and 118 cases, respectively. The performance of the nomogram was assessed with respect to its predictive accuracy, discriminative ability, and clinical usefulness.

Results: In the primary cohort, the mean age of patients was 55.4 years and 129 (55.4%) were male. Prognostic factors contained in the clinical nomogram were age, lactic dehydrogenase, aspartate aminotransferase, prothrombin time, serum creatinine, serum sodium, fasting blood glucose, and D-dimer. The model was externally validated in two cohorts achieving an AUC of 0.946 and 0.878, sensitivity of 100 and 79%, and specificity of 76.5 and 83.8%, respectively. Although adding CT score to the clinical nomogram (clinical-CT nomogram) did not yield better predictive performance, decision curve analysis showed that the clinical-CT nomogram provided better clinical utility than the clinical nomogram.

Conclusions: We established and validated a nomogram that can provide an individual prediction of 30-days poor outcome for COVID-19 patients. This practical prognostic model may help clinicians in decision making and reduce mortality.

Keywords: nomogram, poor outcome, COVID-19, CT score, clinical usefulness

INTRODUCTION

The rapid spread of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as a potentially fatal disease is a major and urgent threat to global health (1). As of July 9, 2020, there are more than 12.14 million confirmed cases by the World Health Organization (WHO) with 551,044 deaths (2). The clinical spectrum of COVID-19 ranges from mild to critically ill. Most COVID-19 patients had mild acute respiratory infection symptoms, such as fever, dry cough, and fatigue, but some could rapidly develop fatal complications, including respiratory failure, multiple organ dysfunction, shock, or even death (3). To date, no specific treatments were recommended for COVID-19 except for meticulous supportive care (4); therefore, early identification of patients with poor prognosis may facilitate the provision of proper supportive treatment in advance and reduce mortality.

The profusion of data requires machine learning to improve and accelerate COVID-19 diagnosis, treatment, and prognosis (5). Machine learning uses patterns in data to improve performance or make accurate predictions (6). It provides a powerful set of tools to unravel the relationship between the variables and outcomes, particularly when data are non-linear and complex (7). At present, some early warning models using machine learning for predicting COVID-19 patients at risk of developing a severe or critical condition have been reported (8–14). Such models are usually assessed with statistical measures for discrimination and calibration. Theoretically, a model with better discrimination and calibration indicates a better guide to clinical management, whereas statistical measures fall short when we want to determine whether the risk model improves clinical decision-making (15). Such measures cannot inform us whether it is beneficial to apply a model to make clinical decisions or which of two models lead to better decisions, especially if they have similar discrimination and calibration. As compared to traditional performance metrics, decision curve analysis (DCA) can assess the clinical utility of models for decision-making (16). DCA plots net benefit at a range of clinically reasonable risk thresholds (16). It identifies risk models that can help us make better clinical decisions.

Therefore, the purpose of this study was to develop and validate a prognostic machine-learning model based on clinical, laboratory, and radiological features of COVID-19 patients at hospital admission for 30-days poor outcome assessment during hospitalization. We also used the DCA and clinical impact curve (CIC) analysis to evaluate the clinical utility and net benefit of the predictive model in supporting clinical decisions. This model may serve as a tool for early identification of COVID-19 patients at high risk for poor outcomes during hospitalization.

MATERIALS AND METHODS

Patients

This study was approved by the institutional review board and the need for written informed consent was waived. A total of 233 confirmed COVID-19 from two designated hospitals of Wuhan, Hubei province of China were consecutively and retrospectively included between January 3 to March 20, 2020. The inclusion criteria were as follows: (1) patients with a laboratory-confirmed COVID-19, which was achieved by real-time reverse transcription-polymerase chain reaction (RT-PCR) assay of throat swab samples (at least two samples were taken, at least 24 h apart) for COVID-19 according to the protocol established by the WHO; and (2) patients received treatment at hospitals. The exclusion criteria were as follows: (1) patients with critical diseases at presentation. This exclusion allows unbiased analysis, for predicting deterioration in patients during their hospitalization; (2) time interval more than 2 days from admission to examinations because delayed testing may skew the inclusion set to a more critical status; and (3) unavailable clinical and laboratory data. The primary cohort was randomly divided into two datasets, 80% for training, and the remaining 20% for internal validation using 5-fold cross-validation. Two externally validation cohorts were under the same inclusion and exclusion criteria. The external validation cohorts included patients hospitalized for COVID-19 between January 3 and May 21, 2020. Finally, 110 and 118 COVID-19 patients were enrolled in the external validation cohort 1 (Tianmen, Hubei province) and cohort 2 (Dongguan, outside Hubei province), respectively.

Data Collection

After consultation with physicians in charge of COVID-19 patients and review of the recent literature regarding the prognosis of COVID-19 on the dataset of PubMed using the terms "COVID," "SARS-CoV-2," "prognosis," "poor outcome," "severe," and "critically ill," a set of clinical, laboratory, and radiological characteristics were identified and the data were collected from the electronic medical records. The clinical characteristics included demographics, comorbidities, and symptoms. Laboratory parameters were recorded, including complete blood count, D-dimer, C-reactive protein (CRP), cardiac enzymes, procalcitonin, liver function test, kidney function test, fasting blood glucose (FBG), and electrolyte. The data in source documents were confirmed independently by at least two researchers. We also calculated the neutrophil–lymphocyte count ratio as it is an important risk factor of disease severity. Imputation for missing variables was considered if missing values were $<15\%$. We used mean value to impute numeric features.

A semiquantitative CT scoring system was designed to assess the involvement degree or area of pneumonia for every single lobe (total five lung lobes): 0 for 0% involvement; 1 for 1–25% involvement; 2 for 26–50% involvement; 3 for 51–75% involvement; 4 for 76–100% involvement. The final CT score (range, 0–20) was assigned by summarizing the total scores of five lobes (17). CT images were reviewed independently by two radiologists with more than 10 years of experience, who were blinded to clinical and laboratory results. Any discrepancy was resolved by a consensus viewing. The detailed CT acquisition and reconstruction parameters are presented in the **Supplementary Document**.

Predictive Variable Selection and Clinical Score Development

Clinical variable selection and risk score development were only performed on the primary cohort as an independent process and ultimately evaluated by the external validation cohort 1 and 2. Pearson correction (PCC) analysis was first used to assess the correlation between variable pairs; a PCC of 0.9 was usually used to eliminate the redundancy in previous studies (18–20). However, most of the variables remained relatively independent in our work (PCC <0.9), with only one pair of variables' (serum creatinine and procalcitonin) coefficients exceeding 0.86. Considering the significance of PCC analysis for variable selection and modeling, we set the cutoff value to 0.86. If the PCC value of the variable pair was larger than 0.86, we calculated the correlation between these two variables and the label and removed one of the slightly unrelated variables. After this process, the dimension of the variable space was reduced, and each variable was independent of each other. Then, the least absolute shrinkage and selection operator (LASSO) logistic regression algorithm was used for further variable selection and development of the clinical score for the 30-days poor outcome prediction. The complexity and performance of the LASSO algorithm relies crucially on the choice of the tuning parameter λ, and the larger λ penalizes the linear model more, resulting in a model with fewer variables. The predictors with non-zero LASSO coefficients based on the 1 standard error rule and penalty parameter tuning were identified using 5-fold cross-validation and the minimum criteria of the area under the receiver-operator characteristic (ROC) curve (AUC). Afterward, the selected clinical variables were combined linearly to construct a clinical score.

Clinical and Clinical-CT Nomogram Construction and Validation

For the superior clinical variables, we conducted the clinical nomogram to visualize the relationship between the variables and predicted probabilities. In addition, a multivariable logistic regression analysis was applied to integrate the predictive clinical variables with the CT score to construct a clinical-CT nomogram. By applying 5-fold cross-validation on the primary cohort, the performance of two constructed nomograms was first internally validated using the AUC metric and then externally validated by the external validation cohort 1 and 2. The clinical score and clinical-CT score for each patient were computed, and the association between the score and COVID-19 30-days poor outcome was assessed with the Mann–Whitney *U*-test. Subsequently, the calibration properties reflecting goodness of fit of the nomograms generated were assessed by plotting the predicted probabilities against the observed event proportions, subjected to bootstrapping validation (1,000 bootstrap resamples) and the Hosmer–Lemeshow test. The degree of overlap between the calibration curve and the diagonal reflects the predictive accuracy of the proposed nomograms.

Clinical Usefulness of the CT Score, Clinical Nomogram, and Clinical-CT Nomogram

DCA can be used to estimate the net benefit of a model based on the difference between the number of true-positive and false-positive results, weighted by the odds of the selected threshold probability of risk (21). To assess the clinical usefulness of the predictive models, the decision curves for each model were then compared with those for the two default strategies where patients are managed without the use of a model: "treating all" or "treating none" (22). The "treat all" strategy assumes that doctors will treat all patients regardless of their risk estimates. The "treat none" assumes that all patients are at low risk that none of them is treated. The net benefit is dependent on the threshold probability that defines "high risk" of critical illness. A predictive model has clinical utility if its net benefit curve is above that of "treat all" or "treat none" for a range of reasonable risk thresholds. The model with higher net benefit for a certain risk or probability has more clinical utility. The clinicians could refer to this to determine whether clinical decision-making based on the models will do better than harm. On this basis, we further plotted the CIC of the models. The CIC shows the estimated number who would be declared high risk for each risk threshold and visually showed the proportion of those who were cases (true positives).

Definition of Clinical Endpoint

We defined the severity of COVID-19 according to the newest COVID-19 guidelines released by the National Health Commission of China (23) and the guidelines of the American Thoracic Society for community-acquired pneumonia (24). Thirty-day poor outcome is defined as meeting at least one of the following criteria within 30 days after admission to hospital: respiratory failure requiring mechanical ventilation, shock, intensive care unit (ICU) admission, multiple organ dysfunction, or death.

Statistical Analysis

Categorical variables were expressed as counts and percentages, while continuous variables are shown as median and interquartile range. Continuous variables were compared using the Mann–Whitney *U*-test, and categorical variables were compared using the chi-square test. PCC analysis, variable selection, clinical score development, and nomogram construction were conducted with R statistical software version 3.5.1 (http://www.R-project.org). The values difference of each selected superior variable within COVID-19 risk groups was compared by the Mann–Whitney *U*-test. Similarly, the test was applied to the comparison of clinical score and clinical-CT score between low-risk and high-risk groups. The performance of the CT score and nomograms were evaluated by AUC, sensitivity, specificity, accuracy, positive predictive value (PPV), as well as negative predictive value (NPV). ROC curves of the two nomograms were compared by the method of DeLong et al. using the MedCalc version 15.2.2 (MedCalc Inc., Mariakerke, Belgium). Note that a two-tailed $p < 0.05$ indicated statistical significance. All the

cutoff values of ROC curves were determined by the principle of the maximum Youden index within the primary cohort. Patients were stratified into low-risk or high-risk group according to the cutoff value. We reported our findings in accordance with the Guidelines for Standards for Reporting Diagnostic accuracy studies, Developing and Reporting Machine Learning Predictive Models in Biomedical Research, and Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis.

RESULTS

Demographic and Clinical Characteristics

Of 233 patients hospitalized for COVID-19 in the primary cohort, the mean age was 55.4 years ± 16.9 (interquartile range, 42–67 years), and 129 (55.4%) were male. Of 228 patients hospitalized for COVID-19 in two cohorts at test datasets, the mean age was 46.2 years ± 17.7 (interquartile range, 35–58 years) and 135 (59.2%) were male. The incidence of 30-days poor outcome in the primary cohort, external validation cohorts 1 and 2 was 20.2, 10.9, and 16.1%, respectively. Clinical characteristics and CT scores of the primary cohort and external validation cohorts 1 and 2 are summarized in **Table 1** and **Supplementary Table 1**, respectively.

Variable Selection and Clinical Score Development

Three laboratory parameters uric acid, creatine kinase, and creatine kinase MB with missing value >15% were excluded for the variable selection. Most of the variables remained relatively independent in this study (PCC < 0.9), with only one pair of variable coefficients exceeding 0.86 (serum creatinine and procalcitonin). Thus, procalcitonin with low correlation with 30-days poor outcome in hospitalized patients with COVID-19 was removed after PCC analysis, and the remaining 31 variables were initially selected. Then, eight superior variables with non-zero coefficients were identified across 5-fold cross-validation using the LASSO regression, with the optimization tuning parameter of 0.051 (**Figure 1**). **Supplementary Figure 1** depicts the value distribution of the identified eight superior clinical variables in patients with good or poor 30-days outcome across all the cohorts. In **Figure 2**, the clinical score constructed by linearly integrating the eight clinical variables achieved an AUC of 0.943 (95% CI: 0.927–0.957), sensitivity of 96.8%, specificity of 82.4%, accuracy of 85.2%, PPV of 58.0%, and NPV of 99.0% in the training cohort and an AUC of 0.934 (95% CI: 0.894–0.963), sensitivity of 93.6%, specificity of 80.7%, accuracy of 83.3%, PPV of 55.0%, and NPV of 98.0% in the internal validation cohort. When tested, the clinical score yielded excellent performance in the external validation cohort 1 (AUC, 0.946, 95% CI: 0.886–0.980; sensitivity, 100%; specificity, 76.5%; accuracy, 79.1%; PPV, 34.3%; and NPV, 100%), and external validation cohort 2 (AUC, 0.878, 95% CI: 0.805–0.931; sensitivity, 79.0%; specificity, 83.8%; accuracy, 82.2%; PPV, 46.9%; and NPV, 95.3%). The patients could be stratified into low-risk or high-risk group according to the optimal cutoff value of 0.190.

Predictive Performance of the Clinical and Clinical-CT Nomogram

A clinical nomogram was provided for the convenience of clinical score calculation (**Figure 3A**). **Figure 3B** shows the clinical-CT nomogram integrating the eight valuable clinical variables with the CT score. Akaike information criterion (AIC) of the two nomograms were −506.61 and −505.45, respectively. The clinical-CT nomogram yielded an AUC of 0.936 (95% CI: 0.917–0.951), sensitivity of 94.6%, specificity of 78.8%, accuracy of 81.8%, PPV of 52.0%, and NPV of 98.4% in the training cohort and AUC of 0.877 (95% CI: 0.825–0.918), sensitivity of 85.7%, specificity of 72.7%, accuracy of 75.2%, PPV of 43.4%, and NPV of 95.4% in the internal validation cohort. The clinical-CT nomogram achieved good performance in the external validation cohort 1 (AUC, 0.943, 95% CI: 0.882–0.979; sensitivity, 75.0%; specificity, 89.7%; accuracy, 88.1%; PPV, 47.4%; and NPV, 96.7%) and the external validation cohort 2 (AUC, 0.872, 95% CI: 0.796–0.928; sensitivity, 50.0%; specificity, 90.6%; accuracy, 83.9%; PPV, 43.8%; and NPV, 90.6%). However, AUCs of the clinical score and clinical-CT score were not significantly different in two external validation cohorts ($p = 0.807$ and 0.486) (**Supplementary Figure 2**). The cutoff value of clinical-CT score was 0.204, which could stratify patients into a low-risk or high-risk group. **Figure 4** shows the comparison of clinical score and clinical-CT score between the patients with good and poor outcome, with significant differences in all the cohorts (all $p <$ 0.0001). **Supplementary Figure 3** shows the calibration curves of the clinical nomogram and clinical-CT nomogram.

Clinical Usefulness of the CT Score, Clinical Nomogram, and Clinical-CT Nomogram

The area under the decision curves shows the clinical utility of corresponding strategies. The clinical-CT nomogram (blue) showed more net benefit than that of clinical nomogram (green) or CT score (yellow), which were better than the "treat all" (gray) or "treat none" (black) strategies, indicating better clinical application of the clinical-CT nomogram. The decision curve (**Figure 5A**) and CIC (**Figure 5B**) showed that the clinical-CT nomogram had superior standardized net benefit and impact on the outcome of COVID-19 patients.

DISCUSSION

Due to the challenges that arise during the ongoing COVID-19 pandemic, we call for robust tools to aid in making complex clinical decisions. Clinical management of COVID-19 requires frequent monitoring and re-assessment of patients who may suffer from deterioration. Our models provide a reliable and accurate tool for risk quantification for 30-days poor outcome among COVID-19 patients during hospitalization. Our models exhibited relatively good discriminatory power, and external verification was also satisfactory. Of note, our models were applicable for guiding clinical decision-making.

Several elements of the clinical nomogram have been either established as prognostic markers or identified as risk factors for

TABLE 1 | Clinical and laboratory characteristics among patients in the primary cohort with or without poor outcome.

	Total ($n = 233$)	30-days poor outcome		
		Yes ($n = 47$)	No ($n = 186$)	*P*-value
Age (years), *n* (%)	58.0 (42.0, 67.0)	67.0 (60.0, 78.0)	54.5 (40.8, 65.0)	<0.001
Sex, *n* (%)				
Male	129 (55.4)	32 (68.1)	97 (52.2)	0.050
Female	104 (44.6)	15 (31.9)	89 (47.8)	
Comorbidities				
Hypertension	53 (22.7)	18 (38.3)	35 (18.8)	0.004
Coronary heart diseases	19 (8.2)	7 (14.9)	12 (6.5)	0.073
Diabetes	28 (12.0)	12 (25.5)	16 (8.6)	0.001
Hepatitis	8 (3.4)	0	8 (4.3)	0.364
Chronic lung diseases	20 (8.6)	4 (8.5)	16 (8.6)	1.000
Symptoms and signs				
Fever	142 (60.9)	40 (85.1)	102 (54.8)	<0.001
Cough	93 (39.9)	23 (48.9)	70 (37.6)	0.157
Sputum	25 (10.7)	8 (17.0)	17 (9.1)	0.119
Headache	1 (0.4)	1 (2.1)	0 (0.0)	0.202
Sore throat	5 (2.1)	2 (4.3)	3 (1.6)	0.265
Fatigue	17 (7.3)	3 (6.4)	14 (7.5)	1.000
Myalgia	1 (0.4)	0	1 (0.5)	1.000
Chest pain/Chest distress	13 (5.6)	1 (2.1)	12 (6.5)	0.475
Shortness of breath	26 (11.2)	11 (23.4)	15 (8.1)	0.003
Diarrhea	4 (1.7)	2 (4.3)	2 (1.1)	0.182
Chills	2 (0.9)	1 (2.1)	1 (0.5)	0.363
Asymptomatic	52 (22.3)	0	52 (28.0)	<0.001
Laboratory findings, median (IQR)				
WBC ($\times 10^9$/L)	5.3 (4.1, 6.7)	6.0 (4.4, 9.4)	5.1 (4.1, 6.4)	0.011
Neutrophil ($\times 10^9$/L)	3.5 (2.6, 5.1)	5.1 (3.2, 8.7)	3.2 (2.5, 4.3)	<0.001
Lymphocyte ($\times 10^9$/L)	1.1 (0.7, 1.6)	0.7 (0.4, 1.0)	1.2 (0.9, 1.7)	<0.001
NLR	3.0 (1.8, 6.0)	8.0 (3.3, 14.8)	2.6 (1.7, 4.3)	<0.001
LDH (U/L)	204.0 (154.0, 287.5)	394.3 (277.0, 550.0)	185.0 (147.0, 244.8)	<0.001
Hemoglobin (g/L)	130.0 (119.5, 143.5)	134.0 (123.0, 146.0)	128.0 (119.0, 142.0)	0.217
Platelet (g/L)	200.0 (148.5, 252.2)	165.0 (125.0, 222.0)	210.5 (153.5, 260.3)	0.007
Albumin (g/L)	37.3 (32.4, 41.0)	31.9 (29.2, 35.5)	38.4 (34.5, 42.0)	<0.001
AST (U/L)	23.0 (16.5, 36.0)	38.0 (27.0, 58.0)	20.0 (15.0, 31.5)	<0.001
ALT (U/L)	22.0 (15.0, 36.5)	26.0 (18.0, 41.0)	21.0 (15.0, 35.0)	0.061
DBIL (μmol/L)	3.6 (2.6, 5.0)	4.2 (3.2, 6.9)	3.4 (2.5, 4.5)	0.002
IBIL (μmol/L)	7.3 (5.2, 10.2)	5.7 (4.5, 7.6)	7.8 (5.6, 10.8)	0.001
TBIL (μmol/L)	11.0 (8.3, 15.1)	9.8 (8.1, 14.6)	11.3 (8.4, 15.1)	0.444
APTT (s)	34.2 (31.8, 36.9)	35.3 (31.1, 38.4)	34.1 (32.0, 36.5)	0.236
PT (s)	13.3 (12.5, 14.3)	14.0 (13.0, 15.0)	13.2 (12.5, 14.0)	0.001
D-dimer (μg/ml)	0.3 (0.1, 0.9)	0.8 (0.3, 8.0)	0.2 (0.1, 6.3)	<0.001
Serum creatinine (μmol/L)	70.0 (58.0, 82.0)	79.0 (67.0, 99.0)	68.0 (57.0, 80.0)	<0.001
hs-CRP (mg/L)	12.2 (1.3, 36.0)	36.5 (32.2, 38.2)	3.7 (0.8, 27.2)	<0.001
Procalcitonin (ng/ml)	0.2 (0.1, 2.9)	0.5 (0.2, 4.9)	0.2 (0.1, 1.2)	<0.001
Potassium (mmol/L)	4.0 (3.7, 4.4)	3.9 (3.5, 4.5)	4.0 (3.7, 4.4)	0.174
Sodium (mmol/L)	139.0 (138.0, 141.0)	138.0 (134.0, 140.0)	140.0 (138.5, 142.0)	<0.001
Chloride (mmol/L)	105.0 (103.0, 107.0)	103.0 (99.0, 107.0)	106.0 (104.0, 107.0)	<0.001
FBG (mmol/L)	5.6 (4.8, 7.0)	7.6 (6.3, 11.9)	5.3 (4.8, 6.3)	<0.001

Data were median (interquartile range, IQR) or number (percentage). P-values were calculated by Mann–Whitney U-test, χ^2 test, or Fisher's exact test, as appropriate.
WBC, white blood cells; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; APTT, activated partial thromboplastin time; PT, prothrombin time; hs-CRP, high-sensitivity C-reactive protein; NLR, neutrophil–lymphocyte ratio; FBG, fasting blood glucose.

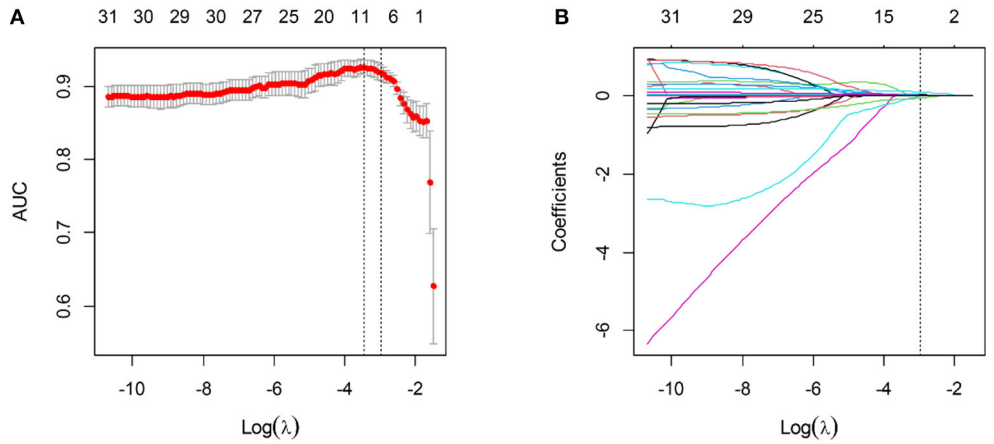

FIGURE 1 | Variable selection using the LASSO algorithm. **(A)** The optimal tuning parameter λ was selected based on the minimum criteria of AUC using 5-fold cross-validation. λ adjusts the regularization penalty to control the complexity of the clinical score, and the optimal value was 0.051. **(B)** LASSO coefficient profiles of the variables. The vertical line was drawn at the selected log (λ), where eight clinical variables with non-zero coefficients were selected.

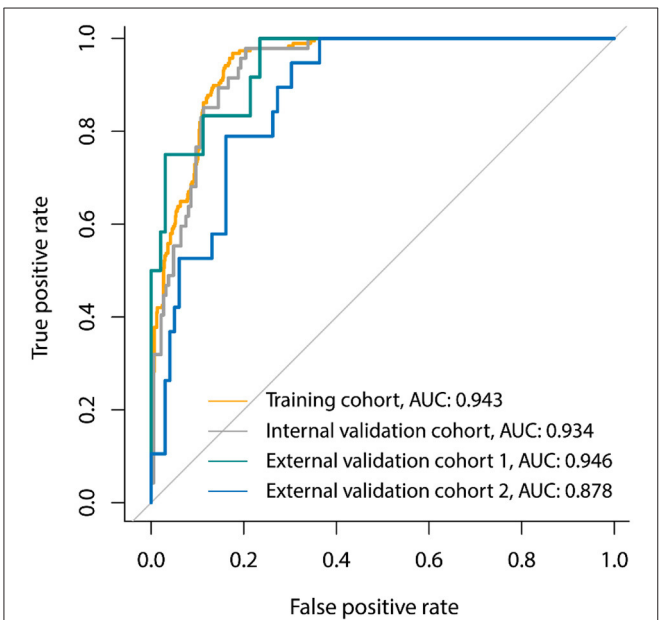

FIGURE 2 | Receiver-operator characteristic (ROC) curves of the clinical signature for 30-days poor outcome prediction in the primary cohort, internal validation cohort, and two external validation cohorts.

severe illness or death in patients with COVID-19. Elderly people are at higher risks for chronic diseases and more susceptible to COVID-19 infection (4). Older age is identified as a well-known risk factor for worse outcomes (e.g., respiratory failure and ICU admission) among patients with COVID-19 partially because age-related immune dysfunctions result from low-grade chronic inflammation (14). In addition, elderly patients were more likely to have underlying comorbidities, such as hypertension, diabetes, chronic lung disease, and cardiovascular disease, which complicated the treatment of COVID-19 and deteriorated the severity of disease.

Although the lung is the most affected organ by COVID-19, other organ dysfunction, including liver, kidney cardiac, and coagulation dysfunction, indicates poor survival outcomes (25). Although LDH is not a marker of a specific organ, the rise in LDH level indicates an increase in the activity and extent of lung injury, especially in critically ill patients with COVID-19 (26). AST elevation was common and may be due to cholangiocyte dysfunction and other causes, such as drug-induced and systemic inflammatory response-induced liver injuries (27). Liver injury was independently associated with the need for ICU admission, mechanical ventilation, and/or death in COVID-19 patients (27). Previous studies suggested a 3–11% incidence of acute kidney injury (AKI) in patients with COVID-19 (28). Around 9.6 and 13.7% of patients had elevated serum creatinine and blood urea nitrogen, respectively (28). The etiology of AKI in COVID-19 is thought to be multifactorial, and the mechanism of kidney involvement may include direct cellular injury due to the virus or sepsis leading to cytokine storm syndrome (29). AKI is closely associated with the severity and prognosis of COVID-19 patients (30). Coagulation dysfunction is more common in patients with severe and critically ill COVID-19. Elevation of PT and D-dimer indicated a hypercoagulable state in patients at the early stage, which was an independent predictor of requiring critical care support or in-hospital mortality (31, 32). The coagulation indicators such as D-dimer and PT should be monitored as early as possible in order to detect thrombotic complications. We strongly suggest that special care of multiple organ dysfunction should be included in the treatment of patients with COVID-19 during hospitalization.

While serum sodium has not yet been related to COVID-19, it has been independently and consistently associated with adverse outcomes in other populations (33) and disease states (34, 35). Previous studies showed that admission FBG was an independent predictor for poor prognosis of COVID-19

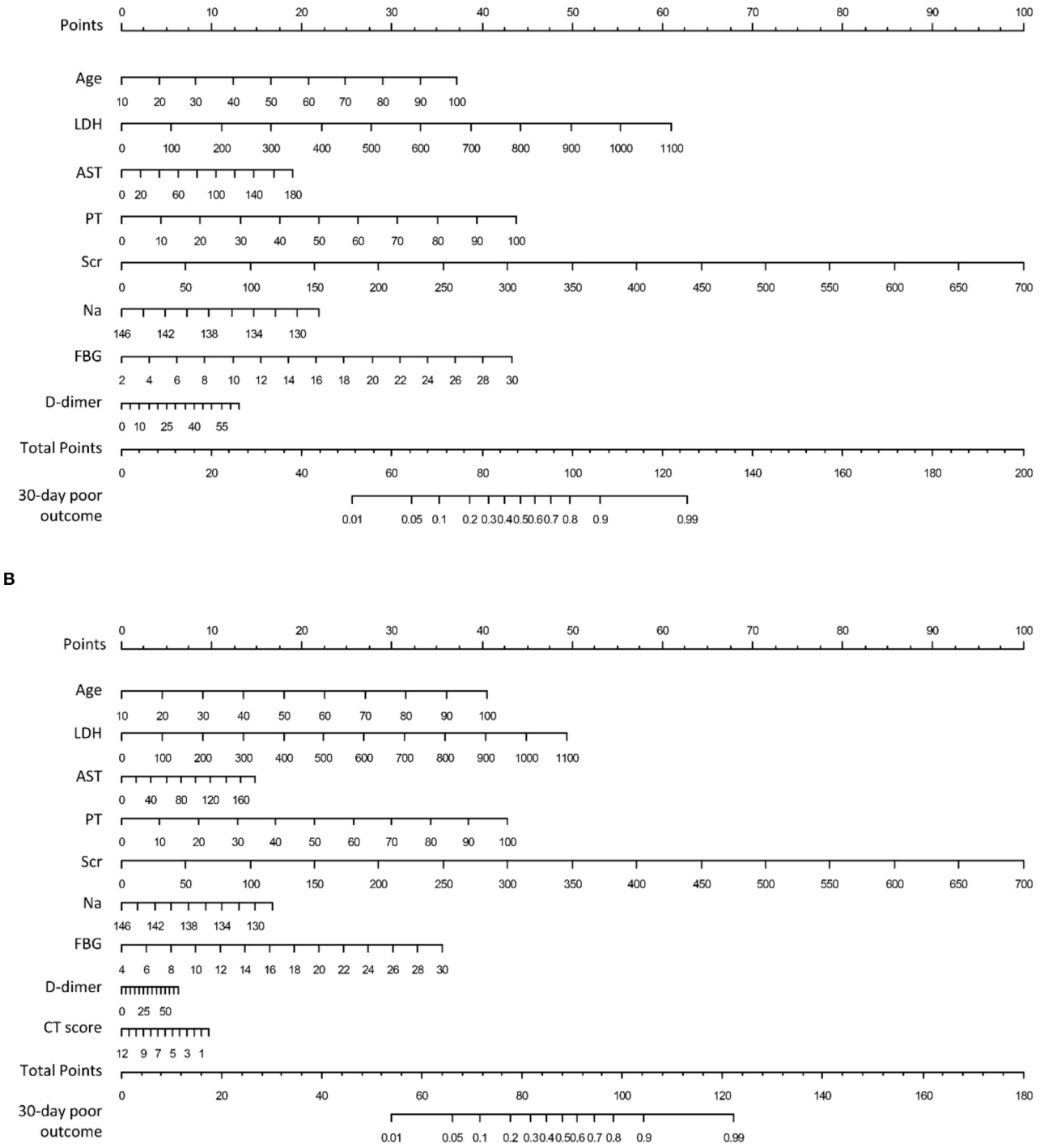

FIGURE 3 | Nomograms to predict 30-days poor outcome in patients hospitalized for COVID-19. **(A)** Clinical nomogram constructed by linearly integrating the eight clinical variables; **(B)** clinical-CT nomogram constructed by linearly integrating the eight clinical variables and CT score. LDH, lactic dehydrogenase; AST, aspartate aminotransferase, PT, prothrombin time; Scr, serum creatinine; Na, serum sodium; FBG, fasting blood glucose.

patients (36, 37). Hyperglycemia is mainly caused by pre-existing diabetes and stress-induced hyperglycemia. Diabetes has been identified as an important risk factor for mortality and progression in COVID-19 patients (38). In addition to pre-existing diabetes, elevation of FBG level at admission could also be due to stress hyperglycemia. Stress hyperglycemia is

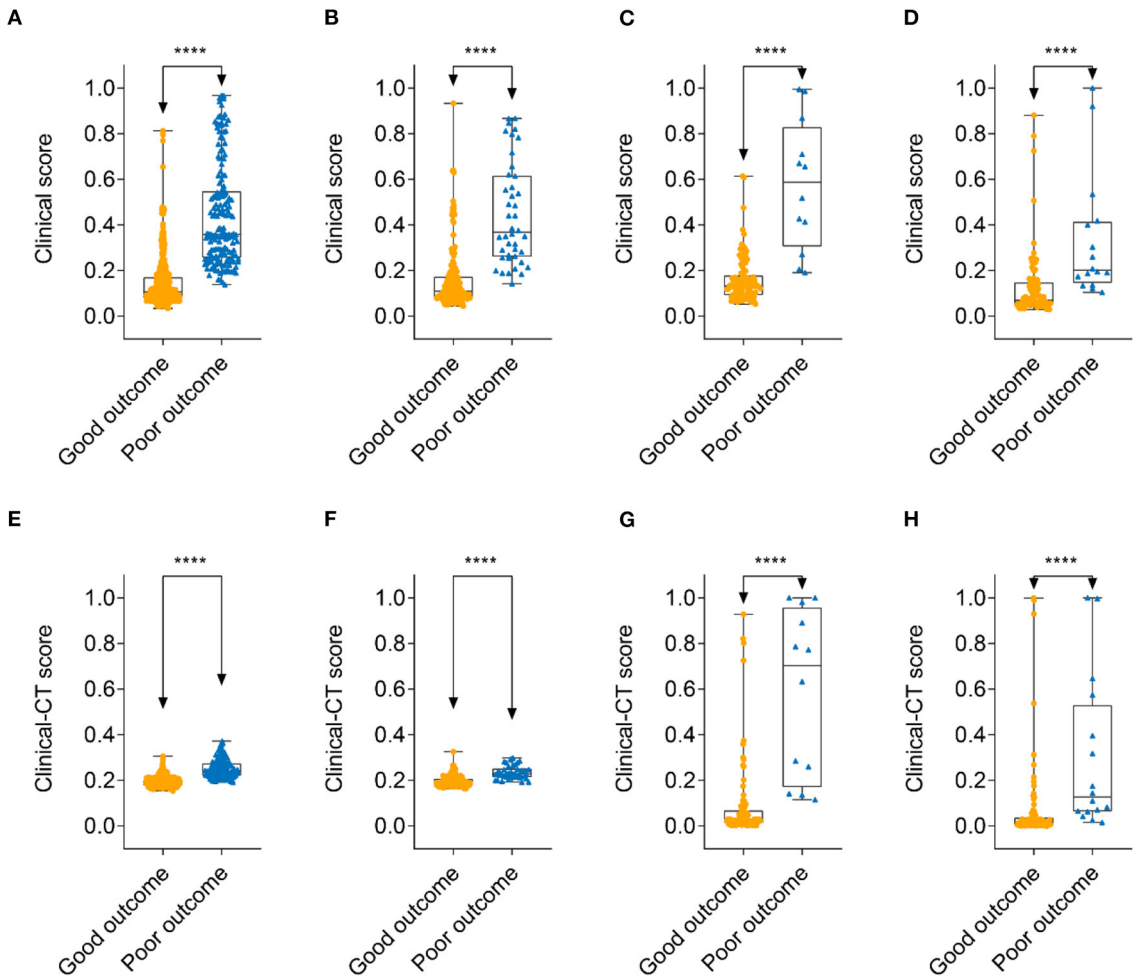

FIGURE 4 | Comparison of clinical score and clinical-CT score between patients with 30-days good outcome and poor outcome. Clinical score: **(A)** training cohort; **(B)** internal validation cohort; **(C)** external validation cohort 1; and **(D)** external validation cohort 2. Clinical-CT score: **(E)** training cohort; **(F)** internal validation cohort; **(G)** external validation cohort 1; and **(H)** external validation cohort 2. ****denotes $p < 0.0001$.

common in patients without diabetes, which is more concerning in clinical practice. Stress hyperglycemia may be induced by a decrease of both insulin secretion and the worsening of insulin resistance; it may produce organ damage by inducing endothelial dysfunction and thrombosis through the glycation process and oxidative stress generation (39). Glucose control helps prevent and control infections and their complications (40). Accordingly, well-controlled blood glucose may lead to improved outcomes of patients with COVID-19.

Chest CT plays an indispensable role in the detection, diagnosis, and follow-up of COVID-19 pneumonia. Visual CT score is a semi-quantitative marker that can assess the disease severity of COVID-19 according to lung involvement in the clinical setting (41). The index is simple, reproducible, and readily available in daily practice without image post-processing. However, it is usually visually calculated by radiologists, which is somewhat subjective with variability that unable to quantitatively assess the disease severity and is also time-consuming (42). Despite these limitations, some previous studies suggested that CT score was highly correlated with laboratory findings and disease severity, which could serve as a biomarker of predicting the outcome of COVID-19 patients (41, 43–51). However, our study might suggest that admission CT score is not a significant predictor for longer-term prognosis. Interestingly, although adding CT score to the clinical nomogram could not improve the predictive performance, the combination of both had more net benefit than clinical nomogram alone.

Based on the identified predictors, clinical nomogram was developed for doctors to quickly assess the risk with sample clinical and laboratory features and facilitated early decision making of COVID-19. Nomogram as the visualization of these models could serve as a simple tool for physicians and patients to calculate individual risk. Our model is able to stratify COVID-19 patients into low- and high-risk groups for developing 30-days poor outcome. The clinical usefulness of our models was tested, and the results showed that although the clinical nomogram and

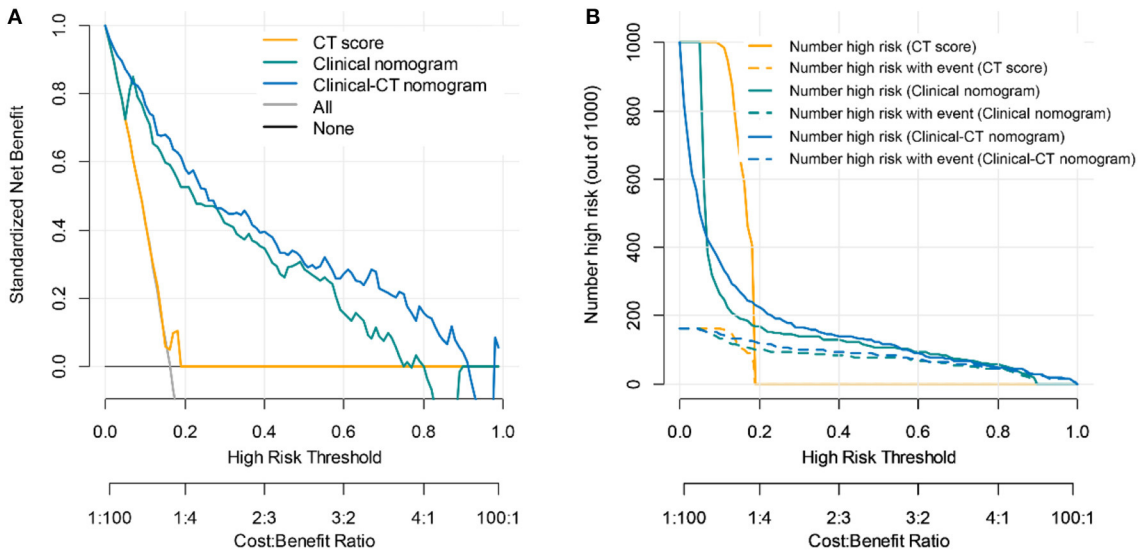

FIGURE 5 | Clinical utility evaluation of the clinical score, CT nomogram, and clinical-CT nomogram for 30-days poor outcome prediction. **(A)** Decision curve analysis. Net benefit curves are plotted across risk or probability thresholds for an event (critical illness) for five options: "treat all" as if they are critically ill, "treat none" considering none is critically ill, treat according to critical illness by CT score, clinical nomogram, and clinical-CT nomogram. **(B)** Clinical impact curve of the CT score, clinical nomogram, and clinical-CT nomogram plotted the number of COVID-19 patients classified as high risk, and the number of cases classified high risk with severe COVID-19 at each high-risk threshold. The dotted yellow, green, and blue curves (number of high-risk individuals with outcome) denote the number of true positives at each threshold probability. The solid yellow, green, and blue lines (number of high-risk individuals) indicate the number of people who are classified as positive (high risk) by the CT score, clinical nomogram, and clinical-CT nomogram at each threshold probability.

clinical-CT nomogram shared similar discrimination power, the latter had a better clinical application. Several previous studies also showed the clinical usefulness of prognostic models in the management of COVID-19 patients (52–60).

LIMITATIONS

This study also has some potential limitations. First, we included the retrospective nature of the sample that may introduce potential risks of bias in the data particularly if this involved convenience sampling or potentially crucial predictors were not available. Second, the training and validation of the model are restricted to several populations in China; further validation using external populations would improve the generalizability of the model. Third, CT score is somewhat subjective with large intra- and inter-observer variability obtained from the initial CT examination; CT-based radiomics or deep learning and follow-up CT scan may provide more prognostic information. Fourth, indicators of cardiac injury such as creatine kinase and creatine kinase MB were not analyzed due to insufficient data. This may limit the model fit and introduce bias if the data were not missing at random. Fifth, the potential duration of infection prior to presentation need not be indicated, which may be useful to assess rate of progression of infection in patients independently of biomarkers used in this study. Sixth, this model was not applicable for patients with critical illness at admission, which may result in inclusion bias. Seventh, COVID-19 triaging might lead to less severe cases having delayed testing, thereby skewing the inclusion set to a more critical status; thus, the performance of model may be overestimated. Eighth, self-medication of patients before admission may affect the clinical and laboratory results, but it should have no major effect on the models as long as these medications were random. Finally, the identification of predictors depends on available features, feature selection method used, and sample size of studies.

CONCLUSION

This study developed and externally validated a simple predictive model of 30-days poor outcome for hospitalized patients with COVID-19 based on objective data that are routinely used in clinical setting. Clinical nomogram integrated eight optimal predictors of 30-days poor outcome, including age, lactic dehydrogenase, aspartate aminotransferase, prothrombin time, D-dimer, serum creatinine, serum sodium, and fasting blood glucose. We found that older age, multiple organ dysfunction, hyponatremia, and hyperglycemia were key prognostic factors of COVID-19 patients. Although the addition of CT score to the clinical nomogram could not enhance its predictive performance, the combination of eight clinical predictors and CT score might be more clinically useful than clinical nomogram alone. Early detection of patients who are likely to develop poor outcome is of great importance, which may help select patients at risk of rapid deterioration who should require high-level monitoring and more aggressive treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The First Affiliated Hospital of Jinan University. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

SZ, QZ, BZ, and QL designed the study. XZ, SL, and WC analyzed and interpreted the data. ML, ZC, LvC, and YD collected the data. JY, QC, and LuC supervised the data. BZ and QL wrote the original draft of the manuscript. All authors contributed to the final editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.590460/full#supplementary-material

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Association Between Clinical Characteristics and Short-Term Outcomes in Adult Male COVID-19 Patients With Mild Clinical Symptoms: A Single-Center Observational Study

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Majority of patients with 2019 novel coronavirus infection (COVID-19) exhibit mild symptoms. Identification of COVID-19 patients with mild symptoms who might develop into severe or critical illness is essential to save lives. We conducted an observational study in a dedicated make-shift hospital for adult male COVID-19 patients with mild symptoms between February and March 2020. Baseline characteristics, medical history, and clinical presentation were recorded. Laboratory tests and chest computed tomography were performed. Patients were observed until they were either transferred to a hospital for advanced care owing to disease exacerbation or were discharged after improvement. Patients were grouped based on their chest imaging findings or short-term outcomes. A total of 125 COVID-19 patients with mild symptoms were enrolled. Of these, 7 patients were transferred for advanced care while 118 patients were discharged after improvement and showed no disease recurrence during an additional 28-day follow-up period. Eighty-five patients (68.0%) had abnormal chest imaging findings. Patients with abnormal chest imaging findings were more likely to have disease deterioration and require advanced care as compared to those with normal chest imaging findings. Patients with deteriorated outcomes were more likely to have low peripheral blood oxygen saturation and moderately-elevated body temperature. There were no significant differences between patients with deteriorated or improved outcomes with respect to age, comorbidities, or other clinical symptoms (including nasal congestion, sore throat, cough, hemoptysis, sputum production, shortness of breath, fatigue, headache, nausea or vomiting, diarrhea). Abnormal chest imaging findings, low peripheral blood oxygen saturation, and elevated temperature were associated with disease deterioration in adult male COVID-19 patients with mild clinical symptoms.

Clinical Trial Registration: https://register.clinicaltrials.gov/prs/app/action/SelectProtocol?sid=S0009RA3&selectaction=Edit&uid=U0003F4L&ts=2&cx=-ajpsbw, identifier NCT04346602.

Keywords: COVID-19, chest imaging study, peripheral blood oxygen saturation, observational study, mobile cabin hospital

INTRODUCTION

In December 2019, an outbreak of novel coronavirus pneumonia was reported in Wuhan, China. Subsequently, the outbreak rapidly spread in China and across the world (1). The World Health Organization (WHO) named this new coronavirus as "2019 Novel Coronavirus (2019-nCoV)" (2). This novel coronavirus belongs to the same family as the 2003 severe acute respiratory syndrome coronavirus (SARS-CoV). The human disease caused by this novel coronavirus is referred to as COVID-19, which was declared as a pandemic by WHO (3). As of May 3 2020, the cumulative number of confirmed COVID-19 cases has surpassed 3 million globally, with more than 200,000 deaths reported. The ongoing pandemic has imposed an enormous burden on the society and individual patients. Based on the clinical presentation, laboratory investigations, and imaging findings, patients with COVID-19 can be classified into mild, moderate, severe, or critical illness (4). Several studies have investigated the epidemiology, pathogenesis, and the management of COVID-19. However, most of these studies involved hospitalized patients with moderate or critical illness (5–8). Few studies have investigated patients with mild or moderate illness, despite the fact that more than 80% of patients with COVID-19 exhibit mild or moderate illness (9). It is important to identify patients who have mild initial presenting symptoms, but who are at high risk of developing severe illness. This would help optimize the use of medical resources and reduce the associated morbidity and mortality.

In China, all patients diagnosed with COVID-19 were admitted to a dedicated healthcare facility. This was done to provide centralized care for infected patients and to minimize the risk of community spread of the virus (10). Patients with mild symptoms were admitted to makeshift hospitals referred to as mobile cabin hospitals; these temporary hospitals were typically set up at sports stadiums or convention centers (11, 12). This provided us an opportunity to study the clinical characteristics and short-term outcomes of COVID-19 patients who had mild initial presenting symptoms.

Here, we report the clinical characteristics COVID-19 patients with mild initial presenting symptoms. We further studied the association between the short-term outcomes of these patients and their chest imaging findings and laboratory indices.

MATERIALS AND METHODS

Study Design and Participants

We performed a prospective observational study at a mobile cabin hospital dedicated for treatment of adult male COVID-19 patients with mild symptoms in Wuhan, China between February and March 2020. The study protocol was approved by the hospital ethics committee. Written informed consent was obtained from all subjects prior to their enrolment.

Inclusion Criteria

Male patients who had the body temperature remaining below 37.3°C, did not experience the symptoms such as cough, expectoration, dyspnea or diarrhea, did not or only experienced mild throat pain, and reported a positive nucleic acid test over the 1 week prior to admission.

Exclusion Criteria

Patients who had the body temperature exceeding 37.3°C and experienced significant dyspnea and/or diarrhea over the 1 week prior to admission.

Study Protocol

Baseline demographic information and medical history were collected from the subjects before starting treatment on the day of admission. Their clinical presentation was recorded. Laboratory tests included complete blood counts, serum biochemistry, and hepatic and renal function tests. All patients also underwent chest computed tomography (CT).

During their stay at the mobile cabin hospital, patients were closely monitored, including daily physical examination, recording of vital parameters, and pulse oximetry. This was continued until they were either transferred to a hospital for advanced care due to deterioration of their illness or were discharged home after clinical improvement. The criteria for transfer to an advanced care hospital were: (1) exacerbation of any underlying disease, such as hypertension, diabetes, or other illnesses; (2) persistent fever; (3) shortness of breath; (4) oxygen saturation <93% (by pulse oximetry). The criteria for discharge were: (1) resolution of fever for >3 days; (2) improvement in respiratory symptoms; (3) improvement in chest imaging findings; (4) negative nucleic acid test for SARS-CoV-2 in at least two consecutive samples collected more than 24 h apart.

Patients who were discharged were followed up for an additional 28 days and 12 weeks for any signs of disease recurrence.

Statistical Analysis

Continuous data are presented as mean ± standard deviation or as median and interquartile range (IQR), as appropriate. Categorical data are presented as frequencies and percentages. Patients were grouped based on their chest imaging findings or short-term outcomes. Comparisons between patients with normal or abnormal chest CT findings as well as between patients with deteriorated or improved outcomes were performed using the sum rank test or Chi squared analysis (Fisher exact test), as appropriate. All statistical analyses were performed using SPSS (IBM, USA). *P*-values <0.05 were considered indicative of statistical significance.

RESULTS

A total of 309 patients were admitted to our mobile cabin hospital during the study reference period. Of these, 125 patients qualified the inclusion criteria and were enrolled in the study. Complete records pertaining to clinical evaluation and follow-up outcomes were available for all patients. Seven patients were transferred for advanced care due to disease exacerbation. The remaining 118 patients were discharged home and were followed up for 28 days.

All subjects had a recent history of exposure to COVID-19. The median age of patients was 35 years (IQR, 30–49) (**Table 1**).

TABLE 1 | Clinical characteristics of the study population.

Variables	All patients (*N* = 125)
Age, median (IQR), years	35 (30–49)
Smoking history, N (%)	
Never smokers	38 (30.4%)
Ex-smokers	5 (4.0%)
Current smokers	82 (65.6%)
Comorbidities, N (%)	14 (14.1%)
COPD	1
Diabetes	8
Hypertension	8
Hepatitis B	1
Fever, N (%)	8 (6.4%)
Highest temperature during hospital stay, N (%)	
<37.5°C	2 (1.6%)
37.5–38.0°C	3 (2.4%)
38.1–39.0°C	2 (1.6%)
>39.0°C	1 (0.8%)
SpO_2, median (IQR)	98 (96–100)
Respiratory symptoms during admission, N (%)	
Nasal congestion	0 (0%)
Sore throat	0 (0%)
Cough	2 (1.6%)
Hemoptysis	0 (0%)
Sputum production	0 (0%)
Shortness of breath	2 (1.6%)
Other symptoms, N (%)	
Fatigue	0 (0%)
Headache	0 (0%)
Nausea or vomiting	0 (0%)
Diarrhea	1 (0.8%)

COPD, chronic obstructive pulmonary disease; SpO_2, peripheral oxygen saturation. Non-normally distributed continuous variables were presented as median and interquartile range (IQR): median (IQR). Categorical variables were presented as number and percentage: number (percentage).

TABLE 2 | Chest imaging findings and laboratory indices.

Variables	All patients (*N* = 125)
Chest computed tomography	
Total abnormalities, N (%)	85 (68.0%)
Ground-glass opacity	70 (56.0%)
Local patchy shadowing	18 (14.4%)
Bilateral patchy shadowing	10 (8.0%)
Interstitial abnormalities	4 (3.2%)
Complete blood counts	
Leukocyte count	6.4 (5.3–7.4)
$>10 \times 10^9$/L	2 (2.1%)
$<4 \times 10^9$/L	6 (4.8%)
Lymphocyte count	2.0 (1.6–2.3)
$<1.5 \times 10^9$/L	21 (16.8%)
Platelet count	216 (172–261)
$<150 \times 10^9$/L	15 (12%)
Hemoglobin level, g/L	145 (139–152)
Hepatic and renal functions	
AST > 40 U/L, N (%)	14 (11.2%)
AST > 80 U/L, N (%)	1 (0.8%)
ALT > 40 U/L, N (%)	60 (48.0%)
ALT > 80 U/L, N (%)	16 (12.8%)
TB 17.1 μmol/L, N (%)	7 (0.56%)
Creatinine ≥ 133 μmol/L, N (%)	0 (0%)
Blood biochemistry	
Sodium, mmol/L	140.3 (138.4–141.8)
Potassium, mmol/L	4.65 (4.34–5.0)
Chloride, mmol/L	100.3 (99.1–101.5)
CRP ≥ 10 mg/L, N (%)	10 (8.0%)
LDH ≥ 250 U/L, N (%)	1 (0.8%)
CK ≥ 200 U/L, N (%)	4 (0.32)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; TB, total bilirubin; CRP, C-reactive protein; LDH, lactose dehydrogenase; CK, creatine kinase. Non-normally distributed continuous variables were presented as median and interquartile range (IQR): median (IQR). Categorical variables were presented as number and percentage: number (percentage).

A large number of patients (82 patients, 65.6%) were current smokers. Most common comorbidities were hypertension (8 patients, 6.4%) and diabetes (8 patients, 6.4%). Two patients (1.6%) had cough and 2 patients (1.6%) had mild shortness of breath. Eight patients (6.4%) had fever. Only one patient (0.8%) had mild diarrhea. A total of 85 (68.0%) patients had abnormal chest CT findings at admission (**Table 2**). The most common abnormality was ground-glass opacities in the lung (70 patients, 56.0%). A small number of patients had decreased leukocyte counts (6 patients, 4.8%, $<4 \times 10^9$/L) and lymphocyte counts (21 patients, 16.8%, $<1.5 \times 10^9$/L). Platelet count was reduced in 15 (12%) patients. There was mild elevation of hepatic enzyme levels. The renal function was intact in all patients.

Comparison Between Patients With Normal or Abnormal Chest CT Findings

Out of 125 patients, 40 patients showed normal chest CT findings, while 85 patients exhibited abnormal CT findings (**Supplementary Figure 1**). Patients with normal chest CT findings were significantly younger than those with abnormal CT findings. There were no significant between-group differences with respect to baseline characteristics, clinical presentation, or medical history (**Table 3**). Among the laboratory parameters, a greater number of patients in the abnormal CT group had blood leukocyte count lower than 4×10^9/L (**Table 4**).

Comparison Between Patients With Deteriorated or Improved Outcomes

Based on the outcomes (discharged to home or referred for advanced healthcare), the 125 patients were assigned to improved outcome group (118 patients) or deteriorated outcome group (7 patients). None of the 118 patients who were discharged home showed recurrence of their clinical symptoms during the 28-day follow-up period. And in fact, a telephone follow-up at 12 weeks after the patient was discharged from the mobile cabin hospital

TABLE 3 | Comparison of baseline characteristics, medical history, and clinical presentation between COVID-19 patients with normal or abnormal chest CT findings.

Variables	Normal CT (*N* = 40)	Abnormal CT (*N* = 85)	*P*-value
Age, median (IQR), years	33.5 (27–42)	40 (30–52.5)	0.02
Smoking history, N (%)			
Never smokers	15 (37.5%)	23 (27.1%)	0.24
Ex-smokers	2 (5.0%)	3 (3.5%)	0.66
Current smokers	23 (57.5%)	59 (69.4%)	0.19
Comorbidities, N (%)	6/40 (15%)	8/85 (9.4%)	0.60
COPD	1	0	0.32
Diabetes	3	5	1.00
Hypertension	3	5	1.00
Hepatitis B	1	0	0.32
Fever, N (%)	0 (0%)	8 (9.4%)	0.11
Highest temperature during hospital stay, N (%)			
<37.5°C	0 (0%)	2 (2.4%)	1.00
37.5–38.0°C	0 (0%)	3 (3.5%)	0.55
38.1–39.0°C	0 (0%)	2 (2.4%)	1.00
>39.0°C	0 (0%)	1 (1.2%)	1.00
SpO_2, median (IQR)	98 (97–100)	97 (95–100)	0.54
Respiratory symptoms during admission, N (%)			1.00
Nasal congestion	0 (0%)	0 (0%)	
Sore throat	0 (0%)	0 (0%)	
Cough	1 (2.5%)	1 (1.2%)	
Hemoptysis	0 (0%)	0 (0%)	
Sputum production	0 (0%)	0 (0%)	
Shortness of breath	1 (2.5%)	1 (1.2%)	
Other symptoms, N (%)			1.00
Fatigue	0 (0%)	0 (0%)	
Headache	0 (0%)	0 (0%)	
Nausea or vomiting	0 (0%)	0 (0%)	
Diarrhea	1 (2.5%)	0 (0%)	

CT, computed tomography; COPD, chronic obstructive pulmonary disease; SpO_2, peripheral oxygen saturation; IQR, interquartile range. Non-normally distributed continuous variables were presented as median and interquartile range (IQR): median (IQR). Categorical variables were presented as number and percentage: number (percentage).

TABLE 4 | Comparison of laboratory indices between COVID-19 patients with normal or abnormal chest CT findings.

Variables	Normal CT (*N* = 40)	Abnormal CT (*N* = 85)	*P*-value
Complete blood counts			
Leukocyte count	6.1 (5.1–7.3)	6.5 (5.3–7.5)	0.45
$>10 \times 10^9$/L	2 (5%)	0 (0%)	0.10
$<4 \times 10^9$/L	0 (0%)	6 (7.1%)	0.02
Lymphocyte count	1.9 (1.7–2.2)	2.0 (1.6–2.5)	0.26
$<1.5 \times 10^9$/L	4 (10.0%)	17 (20.0%)	0.21
Platelet count	217 (180.8–259.8)	215 (168–268)	0.93
$<150 \times 10^9$/L	3 (7.5%)	12 (14.1%)	0.38
Hemoglobin level, g/L	145 (139–155)	146 (139–152)	0.60
Hepatic and renal functions			
AST > 40 U/L, N (%)	2 (5%)	12 (14.1%)	0.22
AST > 80 U/L, N (%)	0 (0%)	1 (1.2%)	1.00
ALT > 40 U/L, N (%)	19 (47.5%)	41 (48.2%)	1.00
ALT > 80 U/L, N (%)	5 (12.5%)	11 (12.9%)	0.95
TB 17.1 μmol/L, N (%)	2 (5%)	5 (5.9%)	1.00
Creatinine ≥ 133 μmol/L, N (%)	0 (0%)	0 (0%)	1.00
Blood biochemistry			
Sodium, mmol/L	139.8 (138.2–141.4)	140.3 (138.5–142.0)	0.54
Potassium, mmol/L	4.6 (4.3–4.9)	4.7 (4.4–5.0)	0.18
Chloride, mmol/L	100.5 (99.1–101.4)	100.1 (99.1–101.5)	0.96
CRP ≥ 10 mg/L, N (%)	0 (0%)	10 (11.8%)	0.10
LDH ≥ 250 U/L, N (%)	0 (0%)	1 (1.2%)	1.00
CK ≥ 200 U/L, N (%)	1 (2.5%)	3 (3.5%)	1.00

AST, aspartate aminotransferase; ALT, alanine aminotransferase; TB, total bilirubin; CRP, C-reactive protein; LDH, lactose dehydrogenase; CK, creatine kinase. Non-normally distributed continuous variables were presented as median and interquartile range (IQR): median (IQR). Categorical variables were presented as number and percentage: number (percentage).

showed that all patients (125 patients) were in good general condition at that time.

Compared with the improved outcome group, patients in the deteriorated group were more likely to develop fever (**Table 5**). There were no other significant between-group differences with respect to age, clinical presentation, or medical history.

Patients with improved outcomes were less likely to have abnormal chest CT findings and more likely to have had better pulse oximetry readings (**Table 6**). In addition, the blood leukocyte count in the deteriorated group was significantly higher than that in the improved group.

In order to further evaluate the effects of body temperature, WBC, peripheral blood oxygen saturation, and CT changes on the prognosis, we analyzed the correlation (by Spearman Rank Correlation) between the prognosis and various indicators, and the results confirmed that body temperature, leukocyte counts, and peripheral blood oxygen saturation (SpO_2) are closely correlated with the prognosis. CT changes showed some correlation with the prognosis, which however was not statistically significant (**Supplementary Table 1**).

DISCUSSION

The ongoing pandemic of COVID-19 has imposed an enormous burden on the societies and individuals across the world (13–15). Early studies have categorized the clinical spectrum of COVID-19 into mild, moderate, severe, or critical illness (4). More than 80% of patients tend to recover with supportive care (9). Fewer than 20% of patients require hospital admission for advanced healthcare. Previous studies have identified several risk factors for severe or critical illness; these include age, presence of comorbidities (such as hypertension, obesity, and diabetes), and certain laboratory indices (6, 8, 16–18). However, young patients with no comorbid conditions who initially exhibit mild illness

TABLE 5 | Comparison of baseline characteristics, clinical presentation, and medical history between COVID-19 patients with deteriorated or improved outcomes.

Variables	Deteriorated outcome (*N* = 7)	Improved outcome (*N* = 118)	*P*-value
Age, median (IQR), years	43 (25–53)	30 (35–49)	0.92
Smoking history, N (%)			
Never smokers	2 (28.6%)	36 (30.5%)	1.00
Ex-smokers	0 (0%)	5 (4.2%)	1.00
Current smokers	5 (71.4%)	77 (65.3%)	1.00
Comorbidities, N (%)	1 (14.3%)	13 (11.0%)	1.00
COPD	1 (14.3%)	0 (0)	0.06
Diabetes	0 (0)	8 (6.8)	1.00
Hypertension	0 (0)	8 (6.8)	1.00
Hepatitis B	0 (0)	1 (0.8)	1.00
Fever, N (%)	4 (57.1%)	4 (3.4%)	< 0.01
Highest temperature during hospital stay, N (%)			
<37.5°C	0 (0%)	2 (1.7%)	1.00
37.5–38.0°C	1 (14.3%)	2 (1.7%)	0.16
38.1–39.0°C	2 (28.6%)	0 (0%)	< 0.01
>39.0°C	1 (14.3%)	0 (0%)	0.06
SpO_2, median (IQR)	94 (92–98)	98 (96–100)	0.04
Respiratory symptoms during admission, N (%)			1.00
Nasal congestion	0 (0%)	0 (0%)	
Sore throat	0 (0%)	0 (0%)	
Cough	0 (0%)	2 (1.7%)	
Hemoptysis	0 (0%)	0 (0%)	
Sputum production	0 (0%)	0 (0%)	
Shortness of breath	2 (28.6%)	0 (0%)	
Other symptoms, N (%)			1.00
Fatigue	0 (0%)	0 (0%)	
Headache	0 (0%)	0 (0%)	
Nausea or vomiting	0 (0%)	0 (0%)	
Diarrhea	0 (0%)	1 (0.8%)	

COPD, chronic obstructive pulmonary disease; SpO_2, peripheral oxygen saturation. Non-normally distributed continuous variables were presented as median and interquartile range (IQR): median (IQR). Categorical variables were presented as number and percentage: number (percentage).

TABLE 6 | Comparison of chest CT findings and laboratory indices between COVID-19 patients with deteriorated or improved outcomes.

Variables	Deteriorated outcome (*N* = 7)	Improved outcome (*N* = 118)	*P*-value
Chest computed tomography scan			
Total abnormalities, N (%)	7 (100%)	78 (66.1%)	0.10
Ground-glass opacity	7 (100%)	63 (53.4%)	0.02
Local patchy shadowing	6 (85.7%)	12 (10.2%)	<0.001
Bilateral patchy shadowing	5 (71.4%)	5 (4.2%)	<0.001
Interstitial abnormalities	4 (57.1%)	0 (0%)	<0.001
Laboratory findings			
Complete blood counts			
Leukocyte count	7.9 (6.6–9.5)	6.3 (5.2–7.3)	0.04
$>10 \times 10^9$/L	1 (14.3%)	1 (0.8%)	0.11
$<4 \times 10^9$/L	1 (14.3%)	5 (4.2%)	0.30
Lymphocyte count	2.2 (1.6–2.8)	2.0 (1.6–2.3)	0.51
$<1.5 \times 10^9$/L	1 (14.3%)	20 (17.0%)	1.00
Platelet count	224 (157–303)	215.5 (172.5–261.0)	0.76
$<150 \times 10^9$/L	1 (14.3%)	14 (11.9%)	1.00
Hemoglobin level, g/L	151 (138–157)	145 (139.0–152.3)	0.52
Hepatic and renal functions			
AST > 40 U/L, N (%)	0 (0%)	14 (11.9%)	1.00
AST > 80 U/L, N (%)	0 (0%)	1 (0.8%)	1.00
ALT > 40 U/L, N (%)	2 (28.6%)	48 (40.7%)	0.70
ALT > 80 U/L, N (%)	0 (0%)	16 (13.6%)	0.60
TB 17.1 μmol/L, N (%)	0 (0%)	7 (5.9%)	1.00
Creatinine ≥ 133 μmol/L, N (%)	0 (0%)	0 (0%)	1.00
Blood biochemistry			
Sodium, mmol/L	140 (139.1–142.4)	140.3 (138.2–141.8)	0.70
Potassium, mmol/L	5.07 (4.2–5.3)	4.64 (4.4–5.0)	0.26
Chloride, mmol/L	99.0 (98.3–101.8)	100.4 (99.2–101.5)	0.33
CRP ≥ 10 mg/L, N (%)	1 (14.3%)	9 (7.6%)	0.45
LDH ≥ 250 U/L, N (%)	0 (0%)	1 (0.8%)	1.00
CK ≥ 200 U/L, N (%)	0 (0%)	4 (3.4%)	1.00

CT, computed tomography; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TB, total bilirubin; CRP, C-reactive protein; LDH, lactose dehydrogenase; CK, creatine kinase. Non-normally distributed continuous variables were presented as median and interquartile range (IQR): median (IQR). Categorical variables were presented as number and percentage: number (percentage).

may rapidly develop severe or critical illness, and may even die (19). Therefore, it is important to identify patients who have mild symptom at onset, but who are at a high-risk of developing critical illness. This can help optimize the use of healthcare resources and facilitate early intervention for high-risk patients.

COVID-19 may affect multiple organ systems in the body; however, it most commonly causes pneumonia (20). Respiratory involvement is also the most common cause of death of patients with COVID-19 (21). Therefore, we first investigated the chest imaging findings in COVID-19 with mild clinical symptoms. Our results indicate that a sizable proportion of patients (68.0%) with mild clinical symptoms may have abnormal chest imaging findings. Ground-glass opacity was the most common chest imaging abnormality. We further compared the differences between patients with improved or deteriorated outcomes during the stay at the mobile cabin hospital. Patients with deteriorated outcomes were significantly more likely to exhibit abnormal chest imaging findings than patients who had improved outcomes. Very few patients in our cohort had cough (2 patients) or shortness of breath (2 patients), even though a majority of patients (85, 68.0%) had abnormal chest imaging findings. This is consistent with a previous report that documented typical abnormal chest imaging findings in asymptomatic patients with COVID-19 (22). Therefore, we recommend chest imaging study

of all patients with COVID-19, including those with mild symptoms. This may help identify patients who are at high risk of developing severe illness.

Chest imaging helps in the direct evaluation of pulmonary involvement in patients with COVID-19. However, chest imaging, especially CT scan, entails the risk of radiation exposure. It is also inconvenient to frequently perform repeat chest imaging. Peripheral oxygen saturation measurement is another method to indirectly assess the pulmonary ventilation and oxygenation status (23). Moreover, it is a convenient and safe procedure. In our study, patients with deteriorated outcomes were significantly more likely to have low peripheral oxygen saturation (**Supplementary Figure 2**) than patients with improved outcomes. This suggested that monitoring of peripheral oxygen saturation is a useful method for identification of patients who may develop severe illness. Early initiation of oxygen therapy may reverse or save their lives.

In our study, patients with deteriorated outcomes were more likely to have moderately elevated temperature and elevated blood leukocyte count. Fever is the most common presenting feature of COVID-19 (24). Studies have shown that SARS-CoV may attack leukocytes resulting in reduced leukocyte counts (25, 26). Further studies are required to assess the predictive value of body temperature and leukocyte count in COVID-19 patients with mild symptoms.

Several studies have identified comorbid conditions such as hypertension, diabetes, and chronic pulmonary disease as risk factors for severe and critical COVID-19 illness (6, 8, 16–18). In our study, we did not observe any significant association of comorbidities with chest imaging findings or outcomes. Age was also found to be a risk factor for increased morbidity and mortality in COVID-19 patients (27). In our study, older patients were more likely to have abnormal chest CT findings. However, age was not associated with the short-term outcome. We believe that the risk factors may differ depending on the initial presentation (mild or severe symptoms) of COVID-19 patients.

COVID-19 may impair the functioning of multiple organs and systems in the body. In addition to lung injury, liver and kidney are also frequently affected in these patients (28). Some of the patients in our cohort exhibited mild elevation in liver enzyme levels; however, there was no significant difference between patients with deteriorated or improved outcomes in this respect. None of our patients showed any sign of kidney injury.

The single-center scope of our study and the relatively small sample size are some of the limitations of our study. We were only able to study adult male patients, since our mobile cabin hospital was exclusively dedicated for hospitalization of adult male COVID-19 patients. Lastly, this was an observational study; therefore, we could not control the treatment modalities, such as oxygen supplementation or administration of certain traditional Chinese medicines.

In summary, we observed an association of chest imaging findings, peripheral blood oxygen saturation, and body temperature with disease deterioration in adult male COVID-19 patients with mild clinical symptoms. Close monitoring of these indices may facilitate identification of patients who are high risk of developing severe or critical illness. This can help optimize the use of healthcare resources and facilitate early interventions to reduce morbidity and mortality in high-risk patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee of First Hospital of Jilin University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LP and DL conceived, designed the study, and had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. BY and YW conducted the primary analysis and prepared the first draft of the manuscript. LS and JG reviewed the draft for intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.571396/full#supplementary-material

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Communication and Cooperation Between the Medical Academy, Medical Association, and Local Government: Health Counseling Program After Recovery From Coronavirus Disease 2019 (COVID-19) in Daegu

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We are currently experiencing the disaster of the COVID-19 pandemic. Since the first case of Coronavirus disease 2019 (COVID-19) was confirmed in South Korea on January 20, the number of COVID-19 cases in South Korea has been rapidly increasing until early March due to a local spread in Daegu, which is one of the eight metropolitan cities in South Korea with a population of 2.5 million. As the medical academy has social accountability as professionals, Daegu-Gyeongbuk branch of the Korean Academy of Family Medicine (Daegu-Gyeongbuk branch) developed the health counseling program for discharged COVID-19 patients. The Daegu-Gyeongbuk branch communicated with Daegu Medical Association and Daegu city for this program and incorporated available resources and capabilities as a leader of this program. This newly developed counseling program consists of medical consultations, sending healthcare brochures and medical supplies, and the appraisal at the end of the program. Not only COVID-19 related symptoms but also other psychological problems are also dealt with during consultations. This program started on March 18, and over 1,700 recovered patients have been receiving counseling as of April 28. Communication and cooperation between the medical academy, medical association, and government are essential to overcome the COVID-19 pandemic. Besides, we expect to apply this health counseling program and our model of setting this program cooperating with medical association and government to different infectious pandemic crisis.

Keywords: COVID-19, medical academia, medical association, local government, consultation

INTRODUCTION

Twenty-seven cases of pneumonia of unknown origin were reported by the China National Health Commission on December 31, 2019 in Wuhan, Hubei province (1). This unidentified pneumonia was later revealed to be due to a new coronavirus (2019-nCov) (2), which has been spreading rapidly, reaching 1,210,956 confirmed cases and 67,594 deaths worldwide as of April 6, 2020 (3). Since the first case of 2019-nCov disease, named Coronavirus disease 2019 (COVID-19) was confirmed in South Korea on January 20 (4), the 31st case of COVID-19 was confirmed in Daegu, which is one of the eight metropolitan cities in South Korea with a population of 2.5 million as first local case on February 18 (5). Since then, the number of COVID-19 cases in Daegu has been rapidly increasing until early March mostly related to religious events. As of March 9, the total number of confirmed cases in South Korea was 7,382 and 5,571 in Daegu which accounts for about 67% of overall cases in South Korea (6). Considering this rapid increase of confirmed cases in Daegu and the possible assumption that many patients will be discharged accordingly, an emergency board meeting of the Daegu-Gyeongbuk branch of the Korean Academy of Family Medicine (Daegu-Gyeongbuk branch) was held to determine how to cope with COVID-19, on March 9 and 17th. Among several suggestions during the meeting, the publication of healthcare brochures and the health counseling program for discharged COVID-19 patients were accepted. In this perspective, we aimed to introduce how we cooperated with medical association and local government to set this health counseling program in COVID-19 pandemic. This study was approved by the institutional review board of Daegu Catholic University Medical Center (IRB approval number: CR-20-164-L).

COOPERATION BETWEEN THE MEDICAL ACADEMY, MEDICAL ASSOCIATION, AND LOCAL GOVERNMENT

The health counseling program after recovery from COVID-19 is a newly developed medical counseling program by family physicians at the Daegu-Gyeongbuk branch based on phone consultations. The purposes of the health counseling program focus on the management of medical and psychological problems and early recognition of coronavirus reactivation. Thus, not only medical symptoms associated with COVID-19 but also other psychological problems such as depression, anxiety, and even family relations are included in the counseling process. In addition, physicians provide emotional support to individuals in every counseling session. This program was scheduled to be held for a month for each participant.

As the first step to start this program, the Daegu-Gyeongbuk branch proposed this program to the Daegu Medical Association and Daegu city. In the emergent situation of rapid spread of COVID-19, the Daegu-Gyeongbuk branch communicated and cooperated closely with Daegu city and Daegu Medical Association reaching an agreement that management for patients who recovered from COVID-19 is necessary. As a rapid response to the proposal of this program, Daegu city was responsible for introducing this newly developed program to discharged Daegu citizens, getting the consent for program participation, and providing individual information including the date of isolation and discharge to the Daegu-Gyeongbuk branch. Daegu city also provides resource assistance, such as mobile phones for consultations. Daegu Medical Association has helped in terms of communication and coordination between Daegu city and the Daegu-Gyeongbuk branch. Furthermore, the Korean Academy of Family Medicine provided donations to the Daegu-Gyeongbuk branch. As a leader of this program, the Daegu-Gyeongbuk branch contributed by coordinating each organization's role as well as suggesting this program. Besides, the Daegu-Gyeongbuk branch distributed funding resources to program participants based on the principles of justice. Regarding physicians, the Daegu-Gyeongbuk branch recruited volunteers to counsel the recovered COVID-19 patients, and a total of 20 family physicians decided to participate in the program. All these processes are briefly demonstrated in **Figure 1**. Finally, this program started on March 18 for COVID-19 patients who recovered and resided in Daegu city.

As shown in **Figure 2**, the program consists of two regular consultations at the beginning and the end of the program. During the first consultation, physicians introduce the program and obtain baseline information such as underlying diseases, first symptoms of COVID-19, and current symptoms. Sleep, mood, stress, and family relations are also dealt with during consultations. According to a counselee's condition, physicians provide proper medical recommendations and emotional support individually, and consecutive consultations might be conducted based on the participant's needs. Healthcare brochures and medical supplies such as a set of masks and disposable thermometers are also sent to the participants with funding resources from the Korean Academy of Family Medicine mentioned above. In addition, a mobile questionnaire survey regarding sleep, depression, anxiety, stress, quality of life, and family relations has also been planned during this program under the Daegu-Gyeongbuk branch's supervision.

OVERALL FINDING

A total of 1,706 recovered patients have been receiving counseling, which means 25% of all recovered patients from COVID-19 in Daegu have had medical consultation. Most of the participants were female (66.2%), and the mean age was 39.6 years (range: 0–79 years). This program ended at the 1st week of June considering new confirmed cases in Daegu have declined since mid-April. During this program, we assessed recovered patients' depression, anxiety, stress, quality of life, and family relations along with clinical presentations of COVID-19.

Here is an example of actual consultation; A 26-year-old woman who was confirmed as COVID-19 on March 4 and released from quarantine on March 19 had first consultation on March 24. She has had asthma and still complained of mild dyspnea in the first consultation. She also complained of mild

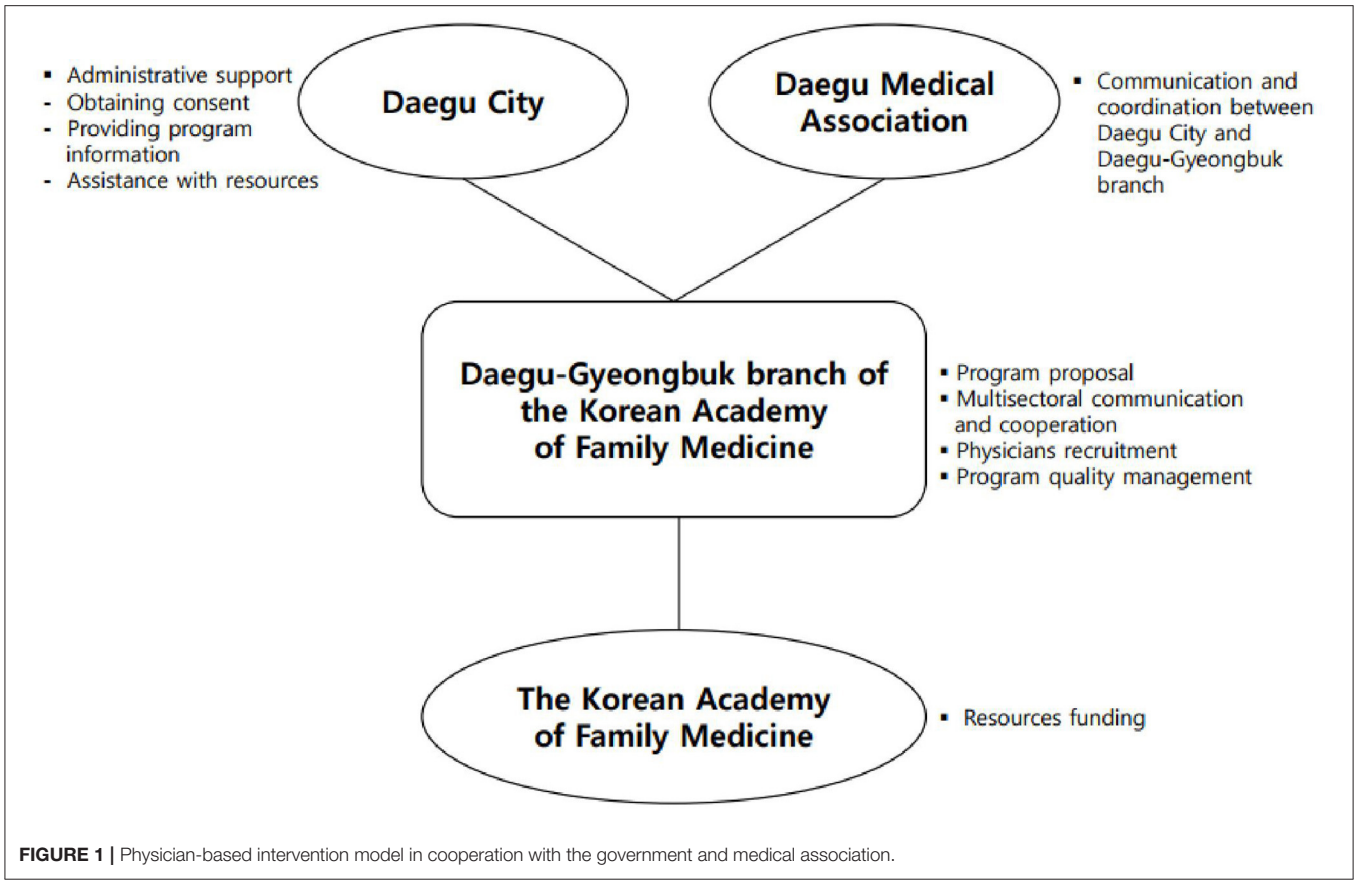

FIGURE 1 | Physician-based intervention model in cooperation with the government and medical association.

anxiety and depression from stigma attached with COVID-19. Her father and her colleagues treated her as an infection source even after discharge with twice negative on COVID-19 tests. Counseling physician recommended watchful waiting for mild dyspnea since she did not have other related symptoms and its course was getting better per participant. However, she seemed to need further consultation for anxiety and depression. So, the physician recommended follow up consultation a week later, and introduced COVID-19 mental health hot line which is available for 24 h a day. On the 2nd consultation, the participant said she felt much better for her breathing, but still anxious to meet other people. Though, she mentioned that she felt somewhat relieved after having conservation with the physician. In the final consultation, the participant said she went back to work without any symptoms, but she was still cautious when talking with other people even with her mask on. The physician ended the counseling program congratulating her recovery from COVID-19, and emphasizing the health education again such as hand hygiene and wearing mask when going for work.

According to the participant satisfaction survey at the end of the program, about 78% of participants were satisfied with the consultation, 16% reported neither positive nor negative, and only 6% were dissatisfied with the consultation. As reasons for satisfaction with this consultation, health education including hygiene education to prevent COVID-19 and early recognition against COVID-19, and emotional support such as active listening and showing empathy was mainly selected. On the other hand, a lack of empathy was presented as a reason for dissatisfaction. According to the physician satisfaction survey, 7 physicians among 20 reported their routine work was disturbed a little. However, most of the physicians reported they felt overwhelmed and proud to have been part of this newly developed program as medical professionals in the COVID-19 pandemic. One of limitations that physicians pointed was immediate testing or prescribing was impossible due to phone-based consultation.

DISCUSSION

We are currently experiencing the disaster of the COVID-19 pandemic. Rapid diagnosis, treatment, and isolation of confirmed or suspected cases are essential to cope with this global health crisis. Along with these actions, the management strategy for patients who have recovered from COVID-19 is also crucial. To the best of our knowledge, this is the first health counseling program for patients who have recovered from the COVID-19 pandemic, conducted by physicians. This phone-based counseling program surveyed not only current remain symptoms which might be the early signs of coronavirus reactivation, but also each participants' mental health. However,

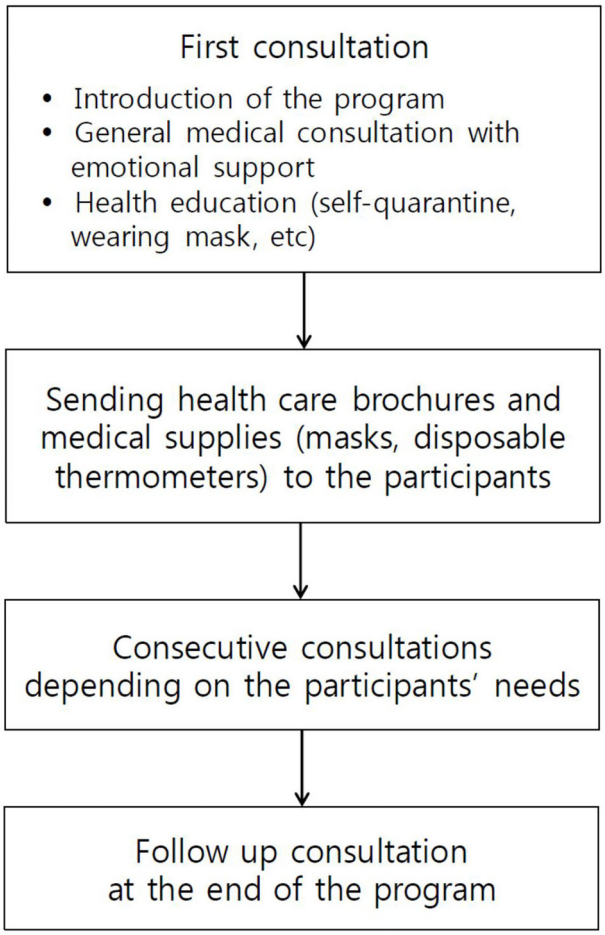

FIGURE 2 | Flowchart of the health counseling program after recovery from the Coronavirus disease 2019 (COVID-19).

there are some limitations in this program. First, this health counseling program might not be well-organized since it was urgently designed, and promptly performed responding to COVID-19 outbreak in Daegu. The performance evaluation for both physicians and participants has been completed and being analyzed in regard to planning, action, and overall satisfaction. Second, we could not involve all patients recovered from COVID-19, and this program was limited to only Daegu citizens. Thus, individualized adaptation should be required when this program is applied to different government or country. Last, we could not evaluate this program in terms of comparison with other counseling programs since the organized counseling program by physician in COVID-19 pandemic has not been reported yet. Considering that COVID-19 is still ongoing and over hundreds of thousands of patients will be discharged worldwide, communication and cooperation between the medical academy, medical association, and government are essential to overcome the COVID-19 pandemic. Moreover, as the medical academy and medical association have social accountability as professionals, they should provide leadership and cooperation between national authorities and other medical associations to incorporate available resources and capabilities during this pandemic crisis. Besides, we expect to apply this health counseling program and our model of setting this program cooperating with medical association and government to different infectious pandemic crisis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board of Daegu Catholic University Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GHL developed the research questions, provided critical revision of the article, and provided final approval of the version to publish. KML, HJK, DWL, and ASK provided substantial contributions to the program management and supervision. YAK contributed to writing and submitting the manuscript. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Sensitivity of SARS-CoV-2 Detection With Nasopharyngeal Swabs

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Background: SARS-CoV-2-infected subjects have been proven contagious in the symptomatic, pre-symptomatic and asymptomatic phase. The identification of these patients is crucial in order to prevent virus circulation. No reliable data on the sensitivity of nasopharyngeal swabs (NPS) are available because of the lack of a shared reference standard to identify SARS-CoV-2 infected patients. The aim of our study was to collect data on patients with a known diagnosis of COVID-19 who underwent serial testing to assess NPS sensitivity.

Methods: The study was a multi-center, observational, retrospective clinical study with consecutive enrollment. We enrolled patients who met all of the following inclusion criteria: clinical recovery, documented SARS-CoV-2 infection (≥1 positive rRT-PCR result) and ≥1 positive NPS among the first two follow-up swabs. A positive NPS not preceded by a negative nasopharyngeal swab collected 24–48 h earlier was considered a true positive. A negative NPS followed by a positive NPS collected 24–48 h later was regarded as a false negative. The primary outcome was to define sensitivity of SARS-CoV-2 detection with NPS.

Results: Three hundred and ninety three NPS were evaluated in 233 patients; the sensitivity was 77% (95% CI, 73 to 81%). Sensitivity of the first follow-up NPS ($n = 233$) was 79% (95% CI, 73 to 84%) with no significant variations over time. We found no statistically significant differences in the sensitivity of the first follow-up NPS according to time since symptom onset, age, sex, number of comorbidities, and onset symptoms.

Conclusions: NPS utility in the diagnostic algorithm of COVID-19 should be reconsidered.

Keywords: COVID-19, sensitivity, swab analysis, diagnosis, false negative (FN)

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BACKGROUND

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible of coronavirus disease 2019 (COVID-19) (1). SARS-CoV-2-infected subjects have been proven contagious in the symptomatic, pre-symptomatic, and asymptomatic phase (2, 3). The identification of these patients is crucial in order to prevent virus circulation. The ideal diagnostic test should be easily

accessible, not invasive, with quick results and possibly cheap. Presently, clinicians rely on real time reverse transcription polymerase chain reaction (rRT-PCR) tests performed on various biological specimens (4). Lower respiratory tract specimens display the highest sensitivity (5), however their collection is not feasible at large scale. The most accessible diagnostic test is rRT-PCR on upper respiratory tract samples, such as nasopharyngeal swabs. Assuming a 100% specificity (6), no reliable data on the sensitivity of nasopharyngeal swabs are available because of the lack of a shared reference standard to identify SARS-CoV-2 infected patients. In fact, rRT-PCR-based tests imply known pre-analytical and analytical vulnerabilities (7). Composite reference standards including clinical and radiological features have been used (8, 9), none of which are however pathognomonic of COVID-19. RNA-positivity of biological specimens has been shown to outlast symptom resolution (10). For these reasons we decided to collect data on patients with a known diagnosis of COVID-19 who underwent serial testing to assess nasopharyngeal swab sensitivity.

METHODS

Study Design and Population

The study was a multi-center, observational, retrospective clinical study with consecutive enrollment. Participating centers included IRCCS Fondazione Ca' Granda Ospedale Maggiore Policlinico and ASST Santi Paolo e Carlo, Università degli Studi di Milano, both based in Milan. All patients who sequentially referred to the two participating centers for follow-up outpatient testing with nasopharyngeal swabs between 05/03/2020 and 20/05/2020 were screened for enrollment. We enrolled patients who met all of the following inclusion criteria: clinical recovery (apyrexia and no need for supplemental oxygen therapy for 3 consecutive days), documented SARS-CoV-2 infection ($\geq$1 positive rRT-PCR result) and $\geq$1 positive nasopharyngeal swab among the first two follow-up swabs. We excluded patients whose first two follow-up swabs delivered negative results (viral clearance).

Nasopharyngeal Swab Technique

All patients, once clinically recovered, were to undergo the first follow-up swab after 14 days since hospital discharge. In case of a positive result, the test was to be repeated after 7 days; in case of a negative result, a second swab was performed 24–48 h later. If this was also negative, patient isolation was ended (11); if positive, the test was to be repeated after 7 days. No other kind of respiratory specimen was collected for follow-up purposes. Nasopharyngeal swabs were performed, stored and delivered to the testing laboratory as recommended by the CDC and ECDC. Nasopharyngeal swabs were performed following a standardized procedure (12). Briefly, GeneFinder™ COVID-19 PLUS RealAmp Kit has been used for detection of SARS-CoV-2 virus through reverse Transcription and Real-Time Polymerase Chain Reaction from RNA extracted from nasopharyngeal swab (ELITe InGenius® system; ELITechGroup, Puteaux, France). The extraction volume was 200 μL. One-Step Reverse Transcription Real-Time polymerase chain reaction is used to confirm the presence of COVID-19 by amplification of RdRp, E, and N genes. The cut-off Ct value of GeneFinder COVID-19 Plus RealAmp Kit (ELITechGroup, Puteaux, France) assay is 40 and the analytical sensitivity of the assay is 1 copy/ μL.

Definitions

A positive nasopharyngeal swab not preceded by a negative nasopharyngeal swab collected 24–48 h earlier was considered a true positive (TP). A negative nasopharyngeal swab followed by a positive nasopharyngeal swab collected 24–48 h later was regarded as a false negative (FN) (**Figure 1**).

Outcomes

The primary outcome was to define sensitivity of SARS-CoV-2 detection with nasopharyngeal swabs. The secondary outcome was to evaluate nasopharyngeal swab sensitivity over time and the association between the sensitivity of the first nasopharyngeal follow-up swab and the patient's age, sex, comorbidities, and onset symptoms.

Statistical Analysis

We estimated that the enrollment of 230 patients with documented SARS-CoV-2 infection would enable to estimate a 63% sensitivity with an acceptable precision, as quantified by the 95% CI (56 to 69%). Patient data have been recorded on Microsoft Excel and analyzed with STATA 15 (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC). Data were expressed as means ± standard deviation (SD) or medians with interquartile ranges (IQR) as appropriate and sensitivities were compared using the Chi-squared test.

RESULTS

Patients

Seven hundred and six patients were screened for enrollment after referral to the two participating centers for serial nasopharyngeal swab outpatient testing between 05/03/2020 and 20/05/2020. Four hundred and seventy-three patients reached viral clearance after the first two follow-up nasopharyngeal swabs, and were therefore excluded. Two hundred and thirty-three patients met all inclusion criteria. All patients had $\geq$1 positive rRT-PCR test collected at the time of diagnosis of COVID-19. All patients received the first follow-up swab once clinically recovered, in most cases 14 days after hospital discharge. The patients' median age was 55.3 years (interquartile range, 43.4 to 64.4), 39% of the patients were women, 74% were Caucasian, 15% were Hispanic, 7% were Maghrebian, Middle Eastern or Arab, and 3% were Asian. The ethnicity of 4 patients was unknown. Clinical data were available for 222 patients. Forty-nine patients (22%) had no comorbidities, while 169 (78%) had $\geq$1. One hundred and eighty-seven patients (84%) had pneumonia. The most frequent onset symptoms were fever (96%), cough (80%), dyspnea (47%), fatigue (45%), ageusia (41%), and anosmia (34%).

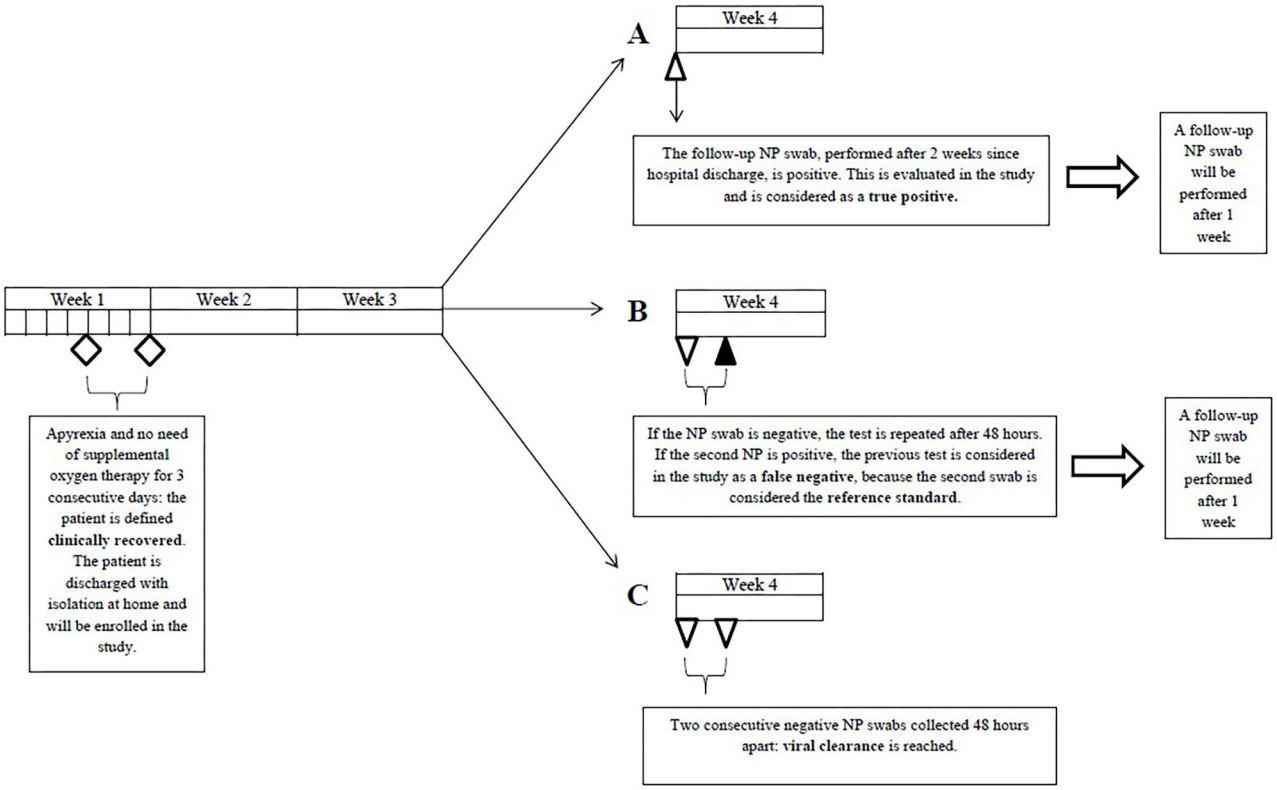

FIGURE 1 | Definition of true positive and false negative nasopharyngeal swabs. NP, nasopharyngeal.

All patients underwent ≥1 follow-up swab. The median number of TP and FN nasopharyngeal swabs per patient was 1 (range, 1 to 6). Of the 233 patients included in our analysis, 182 (78%) reached viral clearance. At the end of the study period data collection was still ongoing for the remaining 51 (22%). The median time to viral clearance was 45.0 days (interquartile range, 38.0 to 52.7) since symptom onset.

Sensitivity

The total number of TP and FN follow-up nasopharyngeal swabs performed in our patient population was 393. Total TP swabs were 303; total FN swabs were 90. Of the 233 first follow-up swabs of our data set, 184 were TP and 49 were FN. Overall nasopharyngeal swab sensitivity was 77% (95% CI, 73 to 81%). Sensitivity of the first follow-up nasopharyngeal swabs ($n = 233$) was 79% (95% CI, 73 to 84%) with no significant variations over time (**Figure 2**). We found no statistically significant differences in the sensitivity of the first follow-up nasopharyngeal swab according to time since symptom onset, age, sex, number of comorbidities, and onset symptoms.

DISCUSSION AND CONCLUSIONS

Because of the unavailability of a shared reference standard for COVID-19 diagnosis there are no reliable data on nasopharyngeal swab sensitivity. We decided to assess nasopharyngeal swab sensitivity in patients with known SARS-CoV-2 infection, based on the presence of symptoms and of ≥1 positive rRT-PCR test, who underwent serial testing. In our patient population sensitivity was 77% (95% CI, 73 to 81%). Wang et al. evaluated SARS-CoV-2 detectability in different biological specimens in a similar cohort of COVID-19 patients and found a nasopharyngeal swab sensitivity of 63% (5); however, this result was based on 8 samples. Kucirka et al. pooled data from 7 relevant studies on both nasal and throat swabs, and found that the probability of a false negative result was as high as 21% even at the optimal testing window (3 days after symptom onset) (13). Two of the 7 analyzed studies included both rRT-PCR confirmed cases and probable cases, identified through clinical criteria alone.

Our sensitivity assessment might have been overestimated. Firstly, most of the patients included in our study had ≥1 positive nasopharyngeal swab at time of diagnosis. Secondly, positive follow-up nasopharyngeal swabs weren't followed by a second swab 24–48 h later, unlike negative swabs; this might have led to the underestimation of the number of FN nasopharyngeal swabs. On the other hand, we didn't perform further nasopharyngeal swabs once viral clearance was reached; this, too, might have contributed to the underestimation of total FN swabs.

Our study has some limitations. The fact that nasopharyngeal swab sensitivity varies throughout disease course limits the external validity of our findings. Although we might have

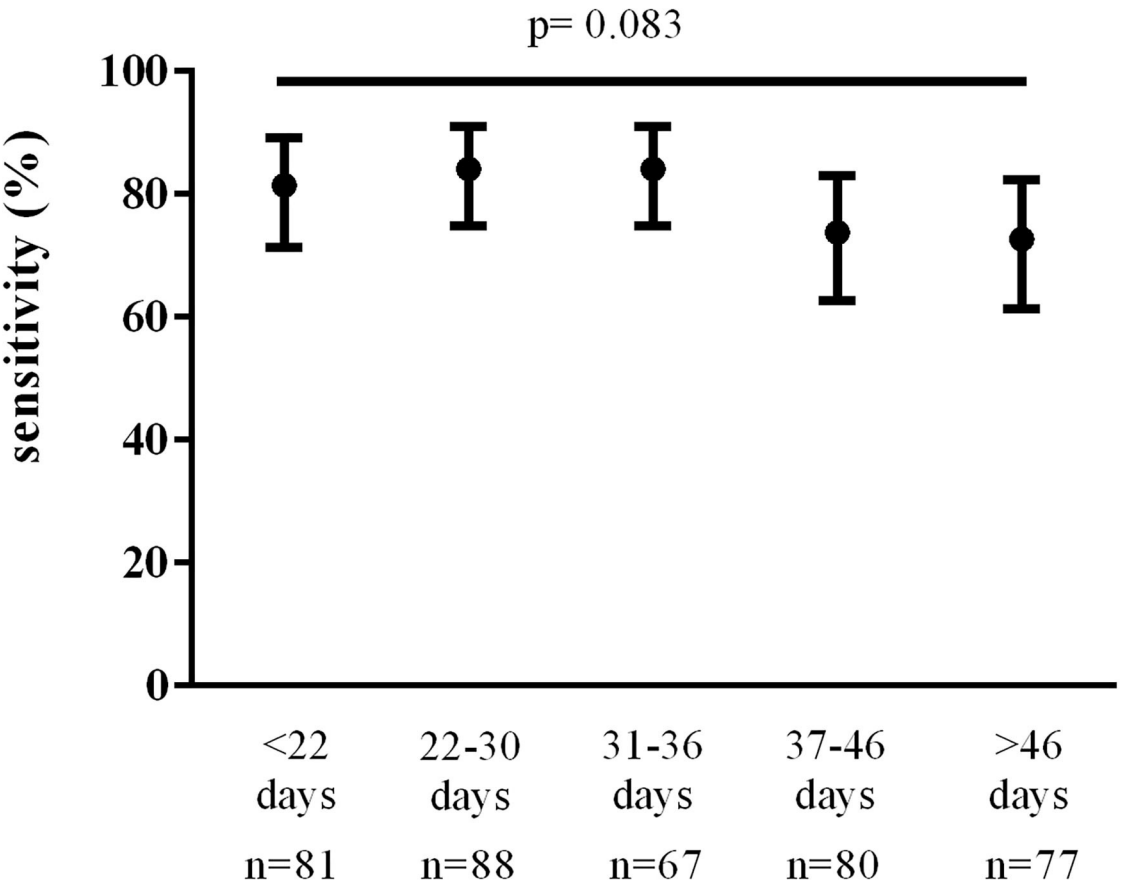

FIGURE 2 | Nasopharyngeal swab sensitivity over time since symptom onset. Data were analyzed using the Chi-square test.

overestimated true nasopharyngeal swab sensitivity for the aforementioned reasons, sensitivity may be likely higher at the beginning of the disease, when viral shedding is greater. We did not investigate the association between the antiviral treatment received during hospitalization and sensitivity of the first follow-up swab. Lastly, as recommended by the WHO (11), we didn't perform further rRT-PCR-based tests after viral clearance was reached. We cannot exclude that subsequent tests might have shown the recurrence of positive nasopharyngeal swabs at least in some patients. Finally, it is possible that the sensitivity values of the nasopharyngeal swabs are influenced by pre-analytical and/or analytical variables; therefore, we cannot exclude that different RT-PCR methods may be sources of variability in the sensitivity of the nasopharyngeal swabs.

In conclusion, in our large cohort of COVID-19 patients, sensitivity of SARS-CoV-2 detection with rRT-PCR on nasopharyngeal swabs was 77% (95% CI, 73 to 81%). For the purposes of our study, we assumed specificity to be 100%. It is however safe to say that a positive nasopharyngeal swab indicates SARS-CoV-2 infection. Conversely, with consideration of the 77% sensitivity we found, a negative result alone cannot rule out infection when this is suspected on clinical or epidemiological grounds. This has led to serial retesting in clinical practice. We think that such a strategy should not be encouraged since it is time-consuming, requires complex organization and leads to facilities overcrowding and delay in treatment initiation. An alternative diagnostic strategy is urgently needed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Milano Area. All subjects signed a written informed consent to personal data treatment, which allowed the anonymous use of clinical data for research purposes.

AUTHOR CONTRIBUTIONS

AM, FB, DP, MO, GM, and VC provided clinical data and revised the manuscript. GMP conceived the study and wrote

the manuscript. BC wrote and edited the manuscript. GCo conceived and designed the study and revised the manuscript. GCa performed the analysis. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Joint Investigation of 2-Month Post-diagnosis IgG Antibody Levels and Psychological Measures for Assessing Longer Term Multi-Faceted Recovery Among COVID-19 Cases in Northern Cyprus

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Following the outbreak of COVID-19, multidisciplinary research focusing on the long-term effects of the COVID-19 infection and the complete recovery is still scarce. With regards to long-term consequences, biomarkers of physiological effects as well as the psychological experiences are of significant importance for comprehensively understanding the complete COVID-19 recovery. The present research surveys the IgG antibody titers and the impact of COVID-19 as a traumatic experience in the aftermath of the active infection period, around 2 months after diagnosis, in a subset of COVID-19 patients from the first wave (March-April 2020) of the outbreak in Northern Cyprus. Associations of antibody titers and psychological survey measures with baseline characteristics and disease severity were explored, and correlations among various measures were evaluated. Of the 47 serology tests conducted for presence of IgG antibodies, 39 (83%) were positive. We identified trends demonstrating individuals experiencing severe or critical COVID-19 disease and/or those with comorbidities are more heavily impacted both physiologically and mentally, with higher IgG titers and negative psychological experience compared to those with milder disease and without comorbidities. We also observed that more than half of the COVID-19 cases had negative psychological experiences, being subjected to discrimination and verbal harassment/insult, by family/friends. In summary, as the first study co-evaluating immune response together with mental status in COVID-19, our findings suggest that further multidisciplinary research in larger sample populations as well as community intervention plans are needed to holistically address the physiological and psychological effects of COVID-19 among the cases.

Keywords: COVID-19, SARS-CoV-2, recovery, immune response, antibody, psychological impact, stigma, long COVID

INTRODUCTION

Coronavirus disease of 2019 (COVID-19), resulting from SARS-CoV-2 infection, was declared a pandemic by the World Health Organization on 11 March 2020. As of 29 July 2020, more than 16,000,000 COVID-19 cases were identified, and more than 650,000 deaths were reported due to the disease (1). Although the scientific community has responded rapidly to detect the transmission mechanisms and develop vaccines, multidisciplinary research focusing on the long-term effects of the COVID-19 infection is still scarce, and not much is known on how the human body responds to COVID-19 infection, both biologically and psychologically during the "longer term recovery" period after discharge from the hospital/isolation. With regards to long-term effects, biomarkers of physiological effects as well as the psychological experiences are of significant importance for a comprehensive understanding of the COVID-19 recovery period (2). COVID-19 as a life-threatening infection can act as an acute stressor (3) and stress can have a down-regulatory effect on the immune system (4). The present research surveys the IgG antibody titers and the impact of COVID-19 as a traumatic experience both during and in the aftermath of the active infection period.

There is insufficient information on the immune response to COVID-19 (e.g., prevalence of different antibodies against the infection over time and development of long-term immunity). It is essential to better understand the timeline of immune response including the appearance of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies, their lifespan and whether they are protective, at least partially, against a second infection. Preliminary research shows that detectable IgG antibodies generally start appearing after the 1st week after symptom onset, reach a peak around 2–3 weeks, and stay at detectable blood levels at least for a duration of 2–3 months even in milder cases, similar to previous observations in other SARS infections (5–7). Moreover, the psychological effects of having the infection are also complex. The potential life-threatening impact of having severe COVID-19, the overall disease burden, along with many unknowns about its short- and long-term effects increase the stigma attached to the infection and the related anxiety among the public. These factors, in turn, make COVID-19 cases more vulnerable to post-traumatic stress as well as targets for harassment and discrimination (8). It is presumed that the period of complete physiological and psychological recovery from the infection depends on disease severity and other physiological and socioeconomic factors. However, given all the elaborate aspects of COVID-19 yet to be investigated and understood, the multi-faceted complete recovery period is still far from being deciphered.

From a psychological point of view, initial findings suggest that both the disease itself and the negative consequences of the lockdown imposed by governments to curb the spread of the disease could result in negative coping behavior which includes but is not limited to panic, anxiety, stigmatization, and post-traumatic stress disorder (PTSD) (3). As scarce research shows, these reactions can also be influenced by contextual factors such as a history of war, famine, natural disasters, man-made accidents and the size of the population. More specifically, while smaller nations might appear to have the upper hand in rapid enforcement of measures, contextual factors such as the increased connectivity of the individuals in smaller societies, or negative collective experiences of war and famine in the past might increase the prevalence of negative coping behaviors and stigma induced depression (9).

A particular case in point is Northern Cyprus, governed by a state that remains internationally unrecognized, and hence, not included in the global epidemiological COVID-19 statistics. In the first wave of the COVID-19 outbreak in Northern Cyprus, 108 cases were diagnosed between 10 March and 16 April 2020. The authorities responded promptly and lockdown was imposed on March 11 (9) effectively halting education and government offices, and all other services except those considered essential. In addition to the global concern over the pandemic, the small community setting in Northern Cyprus (an estimated total population of around 400,000) with a history of war and trauma (10) further caused intensified anxiety and fear in an already sensitive population. Panic engulfed the small nation and there was widespread stigma toward those who tested positive or considered high-risk for transmitting the disease, i.e. Turkish Cypriots living abroad, who were brought home and quarantined (11). Videos of individuals under duress as a result of being quarantined were widely circulated in the social media, and there were news of occasional small-scale protests in neighborhoods where quarantine hotels were chosen due to the perceived infection threat (12). Similarly, those who were tested positive recounted psychological trauma as their names made public and have been targeted (13). Therefore, there are sufficient grounds to assume that in addition to the physiological impact of the disease, those who tested positive for the COVID-19 have also experienced psychological distress during and after the active infection period. In fact, in an earlier study conducted in Wuhan (China), the prevalence of significant post-traumatic stress symptoms associated with COVID-19 was estimated as 96.2% among clinically stable COVID-19 cases at discharge from quarantine (14). Taken together, these observations suggest that assessing the biological markers of physiological effects vis-à-vis negative psychological experiences of the COVID-19 cases is important for holistic management of COVID-19 patients from diagnosis to potentially complete physiological and psychological recovery. The present research surveys the immune response (IgG antibody titers) and negative psychological experiences among the COVID-19 cases in the complete recovery period in the small society setting of Northern Cyprus.

SAMPLE POPULATION AND METHODOLOGY

Participants and Study Design

We performed a joint investigation of the immune response and mental status of the COVID-19 cases at an average time of 2 months after diagnosis. Within the scope of our study, these two main outcomes of interest comprise the assessments toward

the complete recovery of the cases. Of the 108 cases diagnosed, 32 were tourists on the island: two died with the disease, and the remaining 30 individuals returned to their country after discharge from hospital/isolation. Dependent on the severity of the disease, COVID-19 cases were either monitored in the hospital or isolation hotels designated by the health authority. Of the remaining 76 individuals residing in Northern Cyprus, two died with the disease. A total of 74 individuals were invited to participate in the post-discharge assessment of antibody development and psychological impact. For the psychological evaluation, eight individuals under the age of 18 as well as three individuals who did not speak Turkish or English fluently were excluded from the study. Hence, a total of sixty-three individuals were eligible to participate in the psychological evaluation.

All subjects were informed about both components of the study, provided informed consent acknowledging voluntary participation, option to withdraw from study at any time, and the confidentiality of their antibody results and their responses to the survey.

Eligibility Criteria

General Inclusion Criteria: Confirmed (i.e., with positive polymerase chain reaction test result) COVID-19 infection in Northern Cyprus between the dates of 10 March – 17 April and residence in northern Cyprus.

Exclusion Criteria for Antibody Development Analysis: Refusal to give informed consent, or contraindication to venipuncture.

Exclusion Criteria for Psychological Survey: Refusal to give informed consent, inability to understand/speak Turkish or English fluently, or being under the age of 18.

Blood Collection and Transfer

Blood samples were taken by trained nurses during home visits. Venipuncture was used to collect blood. 10 mL complete gel barrier formation tubes were used for blood collection (See **Supplementary Text** for the details).

Serology Testing

The Abbott SARS-CoV-2 IgG assay is a chemiluminescent microparticle immunoassay (CMIA) intended for both the quantitative and qualitative detection of IgG antibodies to the nucleocapsid protein of SARS-CoV-2 in human blood serum and plasma. Assay specifications indicate that the SARS-CoV-2 IgG assay is intended for use as an aid in identifying individuals with an adaptive immune response to SARS-CoV-2, indicating recent or prior infection. This assay is only for use under the United States Food and Drug Administration's Emergency Use Authorization. Per the assay's recommended definition, we defined positive IgG response in the study as a titer level $\geq$ 1.4 index signal/cutoff (s/co) (15). Assays were run on Abbott's ARCHITECTPlus $i2000_{SR}$ System.

The reported positive predictive agreement (PPA) for the assay at $\geq$14 days post-symptom onset was 100.0% (95% confidence-interval (CI): 95.9–100%) while the negative predictive agreement (NPA) was 99.6% (95% CI: 99.1–99.9%). Performance characteristics of the assay were independently evaluated in a study conducted in Boise, Idaho, where specificity and sensitivity were reported as 99.90 and 100% (starting at day 17 after symptom onset), respectively (16).

Psychological Measures

We designed a questionnaire-based survey to assess the negative psychological experiences of the cases. Whenever possible, we adapted and used tested and validated measures for known psychological processes. More specifically, we assessed the extent of experiencing COVID-19 as a life changing trauma (CALCT), negative emotions, perceived importance of preventive measures, awareness and habits, initial reaction to diagnosis, evaluation of general health, stigma, perceived discrimination, post-traumatic anxiety, and evolving perspectives after discharge via the survey response measures. Ordinal response scales with five levels (with corresponding scores of 1–5) were used for each measure. Multiple measures on the same psychological process were combined to create one composite scale process measure by computing the average score per individual. Higher computed scores indicated stronger experience of COVID-19 as a life changing trauma, perceived higher importance of preventive measures, stronger initial reaction to diagnosis, more positive evaluation of general health, more perceived discrimination, higher post-traumatic anxiety, and stronger anticipation of future COVID-19 related anxiety.

We verified the internal reliability of our multi-item process measures via Chronbach's alpha ($\alpha > 0.70$). Experiencing COVID-19 as a life-changing trauma was measured with three items ($\alpha = 0.84$) adapted from (17). Negative emotions during the recovery were assessed by four items ($\alpha = 0.79$). Perceived discrimination on the basis of being COVID-19 positive was measured by six items ($\alpha = 0.90$) adapted from (18). We also measured anxiety related to anticipated stigma in the future as a result of COVID-19 diagnosis with two items ($\alpha = 0.82$). We measured subjective evaluation of health before the diagnosis and after the discharge with a single item each. Willingness to help others by sharing information was measured by a single item and perceived importance of protective measures by four items ($\alpha = 0.96$). Full list of the items can be found in the **Supplementary Material** - Psychological Survey.

Statistical Analysis

Analysis of quantitative IgG titers and CALCT psychological process scores was conducted via non-parametric tests: Wilcoxon rank-sum test (for factors with two levels) and Kruskal-Wallis test (for factors with three or more levels). Due to small group sample sizes, these rank-based non-parametric tests that do not make any assumptions regarding the underlying distribution of the data were preferred for group comparisons (19, 20). We computed descriptive statistics for the socio-demographics factors and summary measures [mean score (M) with standard deviation (SD)] for psychological processes, and conducted Pearson correlation tests to explore whether the selected psychological processes were associated with each other. All single-item survey questions and multi-item process measures use five-point Likert scales (one lowest, five highest) and have a mid-level at 2.5. Disease severity was defined as critical (requiring intensive care), severe (requiring oxygen therapy, but otherwise stable) and

TABLE 1 | Baseline characteristics and disease severity by endpoint.

		Serology ($N = 47$)	Psychological survey ($N = 41$)[a]
Sex	Women	28 (60%)	23 (56%)
	Men	19 (40%)	18 (44%)
Age	0–29	9 (19%)	7 (17%)
	30–59	25 (53%)	21 (51%)
	60+	13 (28%)	13 (32%)
Education Completed	Elementary School	11 (23%)	10 (24%)
	Middle/High School	15 (32%)	13 (32%)
	University or Higher	18 (38%)	18 (44%)
	Not Reported	3 (6%)	
Any Symptom Reported at the Time of Diagnosis	No	10 (21%)	8 (20%)
	Yes	37 (79%)	33 (80%)
Fever/History of Fever Reported at the Time of Diagnosis	No	25 (53%)	23 (56%)
	Yes	22 (47%)	18 (44%)
Comorbidity[b]	No	32 (68%)	28 (68%)
	Yes	15 (32%)	13 (32%)
Disease Severity[c]	Mild/Moderate	38 (81%)	33 (80%)
	Severe/Critical	9 (19%)	8 (20%)

[a] *Of the 47 individuals who provided blood samples for serology testing, four cases were not invited to respond to the survey (one <18 years old, and three not fluent in local language) and two declined to participate in the survey.*

[b] *Most frequently reported chronic diseases were hypertension (N = 9) and diabetes (N = 5, two with concurrent hypertension).*

[c] *Disease severity was defined as critical (requiring intensive care), severe (requiring oxygen therapy, but otherwise stable) and mild/moderate (all other cases including asymptomatic cases).*

mild/moderate (all other cases). *P*-values <0.001 were displayed as "$p < 0.001$." Statistical significance was defined as $p < 0.05$. Multivariate analyses were not carried out due to small sample size. Analyses were performed using SAS version 9.4 (SAS, Cary, NC, USA).

RESULTS

Baseline Characteristics

Of the 74 cases eligible for serology testing, 47 (64%; 60% women and 40% men) accepted the invite and provided blood for testing. Median [interquartile range (IQR)] time from initial COVID-19 diagnosis to blood draw for serology testing was 66 [63.5–73] days with min-max of 50–86 days. Of the 63 cases eligible for responding to the psychological survey, 41 (65%) responded to survey questions (**Table 1**).

For the serology testing, 19% were <30 years of age, 53% were between 30 and 60 years old, and 28% were ≥60 years of age. At the time of COVID-19 diagnosis, 79 and 47% of the serology analysis participants reported "at least one symptom" and "fever history," respectively. Thirty-two percent had at least one comorbidity – most frequently hypertension ($N = 9$) and diabetes ($N = 5$, two with concurrent hypertension). COVID-19 disease severity was severe or critical for 9 (19%) cases and mild/moderate for the remaining 38 (81%). For the psychological survey, distributions of participant baseline and disease severity characteristics were similar to those of the blood serology analysis (**Table 1**). Detailed cross-tabulation of baseline characteristics and disease severity by age group is displayed in **Supplementary Table 1**.

Serology

Of the 47 serology tests conducted for IgG antibody development, 39 (83%) were positive and 8 (17%) were negative. All of the negative results came from individuals who experienced mild/moderate disease. Overall median [IQR] titer level was 4.38 [2.05–5.88]. Median [IQR] titer level among positives and negatives were 4.95 [3.79–6.09], and 0.61 [0.16–0.72], respectively.

Figure 1 and **Supplementary Table 2** display the distribution of IgG antibody titers by baseline characteristics and disease severity. The factor that had the most impact on IgG titer at a median follow-up of 2 months post-diagnosis was disease severity. Nine subjects who had severe/critical disease had median [IQR] IgG titer of 6.09 [5.88–6.24] vs. 3.94 [1.70–5.52] reported for thirty-eight subjects with mild/moderate disease (Wilcoxon rank-sum test; $p = 0.001$). Among the baseline factors, fever/history of fever reported at the time of diagnosis yielded median [IQR] IgG titer of 5.56 [4.11–6.20] vs. 3.57 [1.47–5.13] reported for those without fever/history of fever (Wilcoxon rank-sum test; $p = 0.01$). Having a comorbidity also produced higher median [IQR] IgG titers of 5.52 [4.31–6.09] vs. 3.87 [1.25–5.56] in those without a comorbidity (Wilcoxon rank-sum test; $p = 0.03$).

The distributions of IgG titers by cross-tabulation of baseline characteristics and disease severity are displayed in **Supplementary Table 3**. In the mild/moderate disease severity group, a significantly higher level of IgG titer was observed in individuals with comorbidities (median [IQR]: 5.02 [3.92–5.67]) compared to those without (median [IQR]: 3.43 [0.88–4.87]) (Wilcoxon rank-sum test; $p = 0.03$).

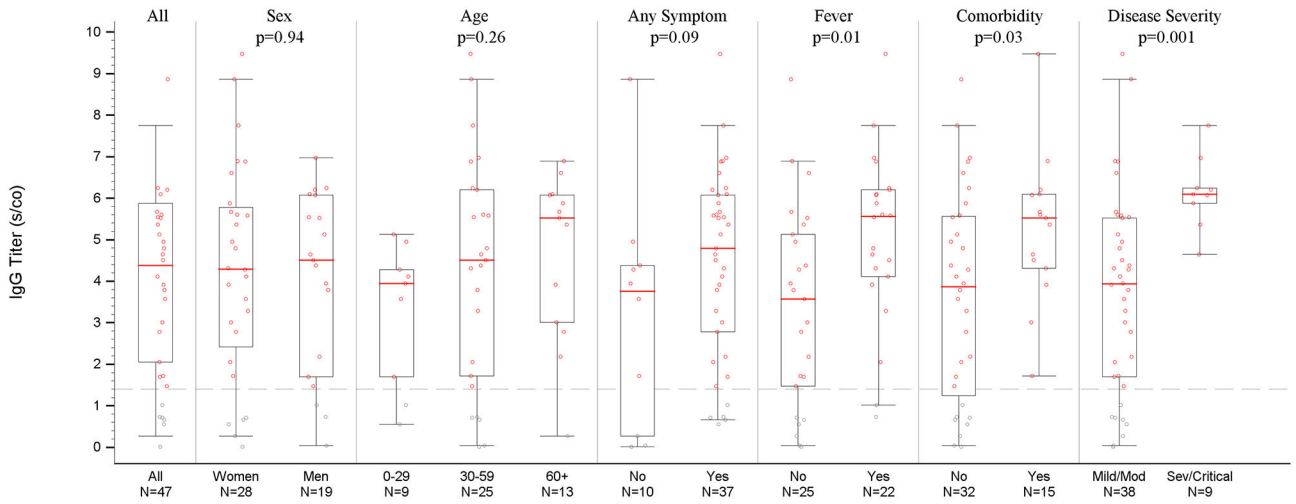

FIGURE 1 | IgG Levels Overall, by Baseline Factors and Disease Severity. IgG levels were measured at a median [IQR] time of 66 [63.5–73] days from initial COVID-19 diagnosis. See **Supplementary Table 2** for detailed summary statistics. Mod, Moderate; Sev, Severe.

TABLE 2 | Descriptive statistics and correlations between the measured psychological processes.

Process	M	SD	1	2	3	4	5	6	7	8
1. COVID-19 as Life-changing Trauma (CALCT)	3.17	1.41								
2. Negative Emotions	2.61	1.25	0.54**							
3. Perceived Discrimination	2.48	1.30	0.54**	0.24^{ns}						
4. Global Health before Diagnosis	4.45	0.72	-0.02^{ns}	-0.07^{ns}	0.09^{ns}					
5. Global Health after Diagnosis	4.21	0.83	-0.15^{ns}	-0.20^{ns}	-0.17^{ns}	0.42**				
6. Pro-social Tendencies	4.39	0.97	0.25^{ns}	0.28^{ns}	0.18^{ns}	-0.20^{ns}	-0.16^{ns}			
7. Perceived Importance of Protective Measures	4.42	1.00	0.24^{ns}	0.09^{ns}	0.09^{ns}	0.14^{ns}	-0.12^{ns}	0.41**		
8. Future Stigma Related Anxiety	1.99	1.06	0.54**	0.05^{ns}	0.80**	-0.07^{ns}	-0.06^{ns}	0.13^{ns}	-0.02^{ns}	

M, Mean; SD, Standard Deviation.
Standardized coefficients are shown. A psychological process score for a participant reflects the corresponding average score of the survey responses (in the ordinal scale of 1–5) used to measure that psychological process. Higher computed score indicated stronger experience of COVID-19 as a life changing trauma, perceived higher importance of preventive measures, stronger initial reaction to diagnosis, more positive evaluation of general health, more perceived discrimination, higher post-traumatic anxiety, or stronger anticipation of future COVID-19 related anxiety.
***$p < 0.001$; ns, non-significant ($p \geq 0.05$).*

Negative Psychological Experiences

We report the descriptive statistics and the associations between negative psychological processes in **Table 2**.

Perception of COVID-19 diagnosis as a life changing traumatic event revealed a mean score of 3.17 [SD 1.41], which is above the mid-level. **Figure 2** displays the distribution of CALCT scores by baseline characteristics and disease severity. Similar to the IgG titer analysis, the factors that have shown trends for the most impact on CALCT scores at a median follow-up of 2 months post-diagnosis was disease severity, followed by presence of a comorbidity. Mean (SD) CALCT scores in mild/moderate and severe/critical disease groups were 3.01 (1.38) and 3.95 (1.42), respectively (Wilcoxon rank-sum test; $p = 0.10$). For individuals with a comorbidity, mean (SD) CALCT score was 3.53 (1.48) as compared to 3.02 (1.39) in those without (Wilcoxon rank-sum test; $p = 0.30$).

Among the individual questions measuring the negative psychological experiences, 24 (59%) respondents indicated a change in their perspective on life and their priorities due to the COVID-19 infection. All 6 (100%) responders to the question with severe/critical disease and 18 out of 33 (55%) responders with mild/moderate disease indicated a change in their perspective on life and their priorities due to the COVID-19 infection. Nineteen (46%) individuals indicated that they have become a more worried/anxious person because of the infection, and 20 (49%) perceived the infection period as a turning point in their lives (42 and 75% of the individuals with mild/moderate and severe/critical disease, respectively) (**Supplementary Table 4**).

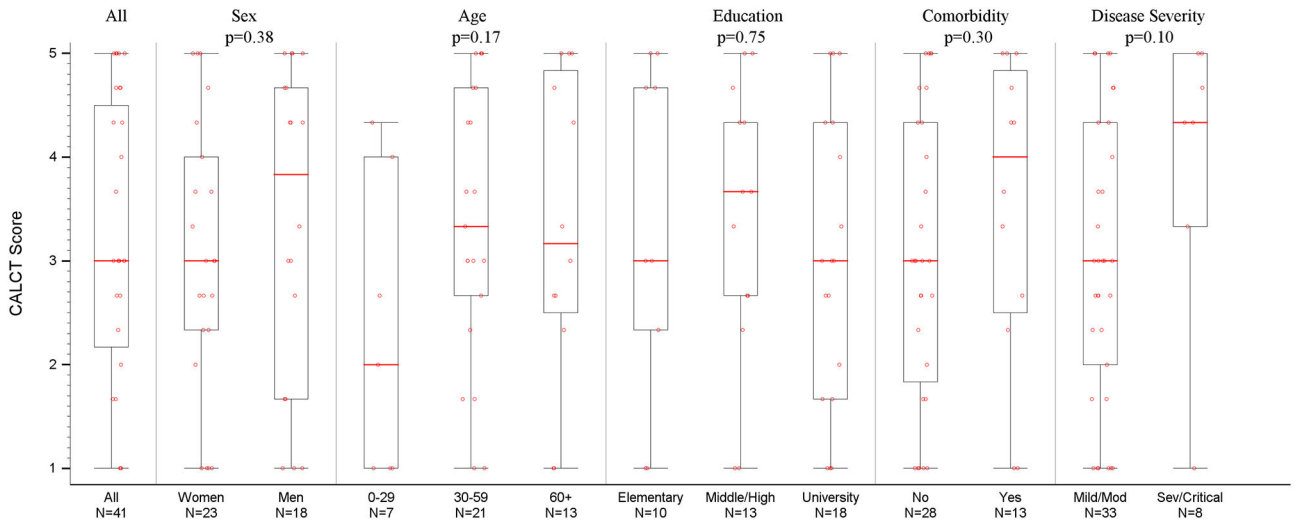

FIGURE 2 | COVID-19 as Life-changing Trauma (CALCT) Scores Overall, by Baseline Factors and Disease Severity. Psychological measures were assessed around 2 months from initial COVID-19 diagnosis. See **Supplementary Table 4** for distribution of responses to the three CALCT survey items. Mod, Moderate; Sev, Severe.

The mean score for the negative emotions due to COVID-19 diagnosis was 2.61 (SD 1.25) and also above the mid-level of the scale (2.5). As for the individual emotions, felt as an initial reaction to COVID-19 diagnosis, worry ranked the first with 71% of respondents having felt it moderately, a lot or quite a lot, followed by helplessness (47%), fear of death (31%) and guilt due to not being sufficiently self-protected (19%). Fear of death and helplessness were both reported moderately or above by 27% and 50% of individuals in the mild/moderate and severe/critical disease severity groups, respectively (**Supplementary Table 5**).

Additional analyses of our psychological measures revealed that perceiving COVID-19 as a life changing trauma is strongly and positively associated with experiencing negative emotions ($r = 0.54$, $p < 0.001$), perceived discrimination ($r = 0.54$, $p < 0.001$); and future stigma related anxiety ($r = 0.54$, $p < 0.001$). Similarly, perceived importance of protective measures is again strongly and positively associated with pro-social tendencies ($r = 0.41$, $p < 0.001$). Last but not least, perceived discrimination at present is strongly and positively associated with future stigma related anxiety ($r = 0.80$, $p < 0.001$) (**Table 2**).

CONCLUSIONS

We detected IgG antibodies in 39 (out of 47; 83%) of cases after a median of 66 days, which was a considerably longer follow-up period compared to the previous serological studies on IgG (on average up to ∼30 days; 21–23). This observation confirms that IgG antibodies are still detectable in the blood in most COVID-19 cases around 2 months post-diagnosis. However, further studies are necessary to determine the neutralizing activity of these antibodies and whether they provide any immunity against a second infection. Moreover, severe/critical COVID-19 cases most of whom were older and/or with comorbidities had higher IgG titers, and also showed trends for the most impact mentally. Overall, we conclude that more specialized attention should be paid to this group for providing further monitoring and treatment post-discharge because of their higher healthcare needs related to comorbidities as well as the psychological impact in order to expedite the full recovery period after the infection.

Our analyses replicated the previous observations that disease severity is an important predictor of blood IgG levels (21–23), and confirmed that this observation holds true in the longer follow-up period we examined. Furthermore, among individuals with mild or moderate disease, we observed that those with comorbidities had significantly higher IgG levels (**Supplementary Table 3**). Similarly, Liu et al. observed that besides the severe COVID-19 cases who tended to have a more vigorous IgG response, a subset of the cases with mild disease had a robust IgG antibody response, and suggested that age and comorbidities may impact the timing and magnitude of the immune response (23). Fever reported at the time of diagnosis also hinted at a possible association with post-discharge IgG levels, but studies with larger case numbers are needed to evaluate these potential predictors of IgG levels with respect to potential confounders such as age, sex, different types of co-morbidities (e.g., autoimmune and endocrine-related diseases) and disease severity via multivariate models. All these factors with potential association to higher IgG titers are correlated with each other, and reflect increased disease burden during diagnosis and post-discharge (**Supplementary Table 1**). It is known that severity of COVID-19 is associated with a dysregulated immune response, and hence, further investigation of how dysregulated immune response is reflected in the long-term blood antibody levels may provide insights into the biological mechanism of the disease and support development of effective vaccines that are based on long-term immune response (24, 25).

In line with previous research, one in every two individuals with severe/critical disease felt fear of death and helplessness

while one in every four individuals with mild/moderate disease felt these two emotions. Worry was the most commonly expressed emotion among the four negative emotions queried, with 71% of respondents having felt it moderately or more (**Supplementary Table 5**). Based on the responses to the psychological survey about 2 months after diagnosis, we infer that most cases have not yet recovered from the mental impact of the disease. Participants experienced COVID-19 as a life-changing trauma, experienced negative emotions, perceived themselves as discriminated against and experienced anxiety due to anticipated stigma in the future. In addition to replicating previous research on the negative psychological consequences of being tested positive for an infectious disease and that pandemics have a lasting negative impact on mental health among the general population (26–28), our findings also show that a subset of cases experienced anxiety as a result of anticipated stigma. This is a novel finding which reveals that pandemics like COVID-19 have long-term negative mental health effects. Future research could replicate and extend these findings via longitudinal designs.

Post-traumatic stress is an important part of this disease due to its overall severity, global impact and stigma attached to it. About half of the survey respondents reported being a more worried person due to the infection, and perceiving the infection as a turning point in their life. About one in four individuals also reported concern that their relationship with their workplace and family/friends will deteriorate due to infection. Hence, community resources for provision of psychological support to the COVID-19 cases post-discharge is very important to minimize the long-term impact of the disease and maintain mental health in these individuals. In Northern Cyprus, a number of organizations and universities have already taken action and set up psychological counseling hotlines, free for use by the public (9). These initiatives are very important and need to be expanded throughout the countries, regions and globally. However, more tailored intervention programs are needed especially for COVID-19 cases to combine mental check-ups with regular health check-ups at regular intervals. About one in ten individuals thought they could still transmit the disease. This provides another example of importance of using up-to-date medical info about the disease, and the person's current status in providing tailored therapy to the person for getting over pre-conceived notions about fear of continuing disease in the individual.

Overall perception about the disease as a threat varied with disease severity (**Supplementary Table 12**). While more than half of the cases with mild/moderate disease deemed the infection was nothing to be afraid of, only one in four thought the same among the severe/critical cases. Therefore, a consistent public communication strategy is needed to ensure public perception of the disease will not change over time from a conscious alertness to the disease being "nothing to be afraid of" due to sharing of experiences/perceptions by an estimated 80% of the cases in the mild/moderate severity group among the community.

The study is subject to a number of limitations. Although our study participation rates of 64% (serology) and 65% (psychology) among discharged COVID-19 cases are acceptable for an exploratory study such as this one, there may be some differences between individuals willing and unwilling to participate in the study, especially with respect to psychological endpoints. Actually, we observed lower participation rates in the study by cases from a rural region that was more severely impacted by the outbreak and had to go under a regional quarantine for an extended time. Disease stigma, continuing worry, suspicion and mistrust likely led to lower participation rates, and these factors are directly related to psychological endpoints studied here. To facilitate a more practical implementation in the field, it was not possible to use a consistent time point for evaluation of the outcomes of interest. Nevertheless, timing of blood draw and survey response showed limited variability around a 2-month time point post-diagnosis, with median [IQR] and range time being 66 [63.5–73] and 50–86 days, respectively. Due to limited resources, it was not possible to conduct the study longitudinally via multiple time points to evaluate trends in further detail. There were possibly correlated responses for either or both endpoints as we allowed participation of multiple family or household members in the study. There were eight families that were represented in the study with 2–3 members each. Finally, compared to continuous IgG titer measures, categorical nature of the survey responses produced higher variability in calculated psychological process scores, and hence, lower statistical power in detecting any associations with baseline factors and disease severity.

Another major limitation of our study is absence of any data collection on clinical signs, symptoms or measures that are potentially associated with continuing recovery process. At the time we conducted our study, little was known on the long-term impact of COVID-19 and how it manifested in the cases. In recent months, there has been evolving information regarding numerous defined and undefined conditions associated with COVID-19, including long-term organ damage, nervous system damage and immune system dysregulations. The most commonly reported general symptoms post acute COVID-19 have been fatigue and dyspnea, followed by joint pain and chest pain. As part of the organ-specific dysfunction, myocardial injury/inflammation has been detected via increased troponin levels and cardiac magnetic resonance imaging, and pulmonary dysfunction via radiologic abnormalities, decreased diffusion capacity for carbon monoxide and diminished respiratory muscle strength. The most common neurologic symptoms reported have been headache, vertigo, anosmia and ageusia, with encephalitis, seizures, major mood swings and "brain fog" also having been reported (29). A refined and detailed assessment of complete recovery process in future studies should include monitoring for these conditions occurring mostly post-discharge via their associated symptoms, laboratory test results and/or medical imaging findings.

In conclusion, this is the first study jointly evaluating post-discharge blood antibody levels and psychological status at a median time of 2 months after diagnosis. Severe/critical COVID-19 cases had higher blood IgG antibody levels as well as the highest long-term mental impact. Holistic and a more personalized approach is needed for post-discharge monitoring and treatment of COVID-19 cases, with a focus on older age, comorbidity status and disease severity. Recognizing the long-term impact of the disease [coined as "long COVID" (30)],

collaborating globally to accumulate detailed standardized long-term psychological and physiological data (31) and continuing to re-define and publicize the importance of complete recovery are key in addressing the long-term health consequences of COVID-19 via awareness, monitoring and timely intervention.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are available upon request submitted to the corresponding author.

ETHICS STATEMENT

The study has been reviewed and approved by the International Cyprus University Ethics Committee in Nicosia, Cyprus. Written informed consent to participate in this study was provided by the participant or participant's legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

BY, BB, and FS planned the study and its implementation. BB, HÇ, BY, and FS designed the psychological survey. FS coordinated the fieldwork for collection of blood samples, administration of psychological surveys and compilation of survey data. GO coordinated processing of blood samples, running of immunoassays and compilation of the assay data. BB and HÇ performed the statistical analyses. BB wrote the first draft. TK and HÇ conducted critical review and editing for the major revisions. BB, TK, and HÇ conceptualized, revised and finalized the article. All authors have reviewed the article, provided feedback and approved the article for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.590096/full#supplementary-material

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BRIEF RESEARCH REPORT
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Hemorrhagic Fever With Renal Syndrome in Vladivostok City, Russia

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Hemorrhagic fever with renal syndrome (HFRS) is a public health problem in Vladivostok city, Russia. From 1997 to 2019, a study of hantaviruses in Norway rats (*Rattus norvegicus*), a natural reservoir of Seoul virus (SEOV), and in HFRS patients was conducted. We demonstrated the presence of SEOV in the local population of Norway rats and detected SEOV in 10, Amur virus (AMRV) in 4 and Hantaan virus (HTNV) in 1 out of 15 HFRS patients. Genetic analysis based on partial S, M and L segment sequences revealed that the Russian SEOV strains were related most closely to strains from Cambodia and Vietnam. We postulate that the SEOV strains found in the port city of Vladivostok have been spread from South-East Asia as a result of distribution of rats during standard shipping trade activities. Moreover, we suggest that city residents may have acquired AMRV and HTNV infection during visits to rural areas.

Keywords: hantavirus, Seoul virus, *Rattus norvegicus*, hemorrhagic fever with renal syndrome, Russia, Vladivostok

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INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) is endemic around the world. The disease is caused by viruses belonging to the genus *Orthohantaviruses*, that includes Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava/Belgrade virus (DOBV), Puumala virus (PUUV) and Tula virus (TULV) (1, 2). Hantaviruses (family *Hantaviridae*) possess a negative-sense, single-stranded, tripartite RNA genome, comprising L, M, and S segments, which encode an RNA-dependent RNA polymerase (RdRp), envelope glycoproteins (Gn and Gc) and a nucleocapsid (N protein), respectively. HFRS-associated hantaviruses are transmitted by aerosolized excreta of their natural hosts, rodents belonging to the Muridae and Cricetidae families.

SEOV is the only hantavirus with a worldwide distribution: Asia (South Korea, Japan, China, Indonesia, Cambodia, Singapore, Vietnam), both Americas (USA, Brazil, Argentina), Africa (Egypt), Eurasia (Russia), and Europe (France, Belgium, United Kingdom, the Netherlands and Sweden) (3). SEOV is carried by wild Norway rats (*Rattus norvegicus*) and is classified as causing mild to moderate clinical forms of HFRS. SEOV has also been detected in other species of rats, e.g., *Rattus rattus* and *Rattus losea* (4). Phylogenetic analysis has identified at least seven distinct genetic lineages of SEOV (5). It has been suggested that, unlike other hantavirus species, which followed natural on-land migrations of their hosts, the main reservoir of SEOV, Norway rats, is omnipresent due to global trade and human migration (6, 7). For example, SEOV strains from France and Belgium do not show geographic clustering but are closely related to strains from Vietnam, Cambodia, Singapore, and Indonesia (5, 8, 9).

Russia included HFRS in the official reporting system of the Ministry of Public Health in 1978, and 131,590 cases of HFRS have been registered from 2000 to 2017 (10). In European Russia, reported cases are caused by PUUV and two types of DOBV, Kurkino virus and Sochi virus. HTNV, HTNV-related virus Amur (AMRV), and SEOV have been identified by us as causative agents of HFRS in Far Eastern Russia (11). SEOV has been detected in wild rats in the Asian part of Russia, in Primorsky, Amursk, and Sakhalin regions (12, 13). However, human cases of SEOV infection have only been seen in Vladivostok city (Primorsky region) since 1992 (11). The virus in Vladivostok was shown to cause generally mild to moderate HFRS, while 5.7% cases had severe clinical manifestations (12). Serologic differential diagnosis based on neutralization tests have demonstrated that the major cause of HFRS in Vladivostok city is SEOV (14). The genetic evidence for SEOV infection in both rodents and humans in Vladivostok city and the high percent of SEOV-associated HFRS cases (>60%) showed the importance of this hantavirus for public health in this part of Russia (15). Here, we investigate SEOV infection in wild rats and characterize etiologic agents of HFRS among the citizens of Vladivostok during a period of 23 years.

MATERIALS AND METHODS

Norway rats were trapped throughout Vladivostok city according to well-established protocols (16). Hantavirus infection in rats was analyzed by detection of virus-specific antibodies (Ab) or/and hantavirus antigen (Ag). Sera were screened for IgG and/or IgM anti-hantavirus Ab by an indirect immunofluorescence assay (IFA) using Vero E6 cells infected with PUUV, HTNV and SEOV as antigens (17). Lung tissues were analyzed for whole hantavirus Ag by ELISA using "HANTAGNOST" kit (Russia).

Blood samples of suspected HFRS patients from Vladivostok city were collected between 1997 and 2019. Clinical diagnosis of HFRS was laboratory-confirmed by the presence of IgG and/or IgM anti-hantavirus Ab, detected by IFA using "HFRS diagnostic kit" (Federal State Unitary Enterprise on Manufacture of Bacterial and Viral Preparations of Chumakov Institute of Poliomyelitis & Viral Encephalitis). For each HFRS patient a 4-fold increase in antibody level over a 2-week interval was registered. For genetic analysis, moderate and severe clinical HFRS cases were selected. The clinical disease severity of HFRS patients was subdivided into mild, moderate, or severe following the standard Russian criteria (length of febrile phase, minimal

TABLE 1 | Annual dynamic of *Rattus norvegicus* infection rate and HFRS morbidity in Vladivostok city, Russia (1997–2019).

Year	Number hantavirus positive/tested rats	Infection rate* in rats (%)	95% CI**	Incidence per 100,000 human population***	Number HFRS patients
1997	14/71	19.7	10.5–28.9	5.9	37
1998	–	–	–	8.4	53
1999	–	–	–	7.6	48
2000	22/70	31.4	20.6–42.2	7.7	48
2001	14/63	22.2	12.0–32.4	8.1	48
2002	41/406	10.1	7.2–13.0	6.9	41
2003	7/35	20.0	6.7–33.3	7.7	49
2004	4/16	25.0	3.8-46.2	6.4	38
2005	4/26	15.4	1.5–29.3	6.3	43
2006	24/277	8.7	5.4–12.0	4.9	30
2007	48/166	28.9	22.0–35.8	5.4	33
2008	11/83	13.3	2.1–24.5	5.0	31
2009	18/95	18.9	11.1–26.7	5.0	31
2010	13/168	7.7	3.7–11.7	5.0	31
2011	2/98	2.0	0–4.8	5.0	31
2012	5/132	3.8	0.5–7.1	3.5	22
2013	16/147	10.9	5.9–15.9	4.2	26
2014	10/50	20.0	8.8–31.2	4.8	30
2015	–	–	–	2.7	17
2016	–	–	–	2.1	13
2017	–	–	–	1.4	7
2018	–	–	–	3.9	22
2019	–	–	–	3.4	21
Total	253/1,903	13.3	11.8–14.8	5.3	750

**Infection rates are number hantavirus antibody and/or antigen positive rats/number tested.*

***CI - confidence interval, ***incidence was calculated for current number of city residents (mean number= 601,500); "–" not tested.*

TABLE 2 | Hantavirus strains identified in *Rattus norvegicus* and HFRS patients from Vladivostok city, Russia.

Virus	Strain	Source	Clinical case*	Year	S	M	L
SEOV	Vlad18992/Rn/1997	*R. norvegicus*		1997	MW073474	MW073489	MW088971
	Vlad18995/Rn/1997			1997	MW073475	MW073490	MW088972
	Vlad28935/Rn/2006			2006	MW073476	MW073491	–
	Vlad29910/Rn/2006			2006	–	MW073492	MW088973
	Vlad127/HU/2000	Human	Severe	2000	–	–	MW088974
	Vlad220/HU/2000		Severe	2000	–	–	MW088975
	Vlad725/HU/2001		Moderate	2001	MW073477	MW073493	MW088976
	Vlad2464/HU/2004		Severe	2004	MW449254	MW073494	MW088977
	Vlad4341/HU/2008		Moderate	2008	MW073478	MW073495	MW088978
	Vlad5163/HU/2011		Severe	2011	MW073479	MW073496	MW088979
	Vlad7139/HU/2018		Severe	2018	MW073480	–	MW088980
	Vlad7259/HU/2018		Severe	2018	MW073481	MW073497	MW088981
	Vlad7352/HU/2019		Severe	2019	MW073482	MW073498	–
	Vlad7364/HU/2019		Severe	2019	MW073483	–	–
HTNV	Vlad7308/HU/2018		Severe	2018	MW073484	–	–
AMRV	Vlad7194/HU/2018		Severe	2018	MW073485	–	MW088982
	Vlad7274/HU/2018		Severe	2018	MW073485	–	–
	Vlad7300/HU/2018		Severe	2018	MW073487	–	–
	Vlad7487/HU/2019		Severe	2019	MW073488	–	–

*"–" not tested, *clinical case for human HFRS patients.*

blood pressure in the hypotonic phase, extent of hemorrhagic symptoms, minimal urine production, serum creatinine level, and extent of proteinuria) (18). Signed informed consent was obtained from each patient (article 20, Federal Law "Protection of Health Right of Citizens of Russian Federation" N323-FZ, 11.21.2011).

RNA was extracted from rat lung tissues and human blood clots using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using Revert Aid premium reverse transcriptase (Thermo Scientific). Samples were screened for hantaviruses by nested RT-PCR, targeting the conserved regions of the viral genome. Three sets of nested primers to recover partial S (nt 610-936), M (nt 2737-2980) or L (nt 175-511) segment sequences were used (**Supplementary Table 1**).

The distance-based neighbor-joining and maximum likelihood methods, supported by MEGA 5 (19), were used to construct phylogenetic trees.

RESULTS

During the period 1997–2019, 750 human HFRS cases were laboratory-confirmed in Vladivostok city. 24.4% patients had a mild clinical course, 62.7% were moderate, and 12.9% had severe forms of disease. HFRS incident rate varied from 4.2 to 8.4 per 100,000 population (**Table 1**). At the same time, active circulation of hantavirus was detected in urban population of *R. norvegicus*, the primary reservoir for SEOV. A total of 1,903 Norway rats were captured in Vladivostok city in 1997 and between 2000 and 2014 (**Table 1**). Hantavirus-infected animals were found every year. The overall infection rate of *R. norvegicus* varied from 2.0% in 2011 to 31.4% in 2000. The highest incident rate in humans and a high infection rate among rats was registered during 1997–2007.

For molecular typing of Russian SEOV strains, lung samples of Ag-positive rats, captured in 1997 and 2006, and human blood samples from Ab-positive HFRS patients, infected in 2000–2001, 2004, 2008, 2011, 2018–2019 were tested. Samples from patients with moderate and severe clinical forms of HFRS were selected for analysis. Hantaviral RNA (328, 244, and 337 base pairs for S, M, and L segments, respectively) was identified in 4/4 rat samples and in 15/28 human samples (**Table 2**). All recovered viral sequences were deposited in GenBank (accession numbers: MW073474-MW073498, MW088971-MW088982).

Phylogenetic analysis of partial S and M segment sequences recovered from *R. norvegicus* showed similarity to that of the SEOV strains previously identified in HFRS patients and rats from Vladivostok city and also in rats from south-eastern Asia (Cambodia, Vietnam, Indonesia, Singapore) (**Figure 1**). Phylogenetic analysis of sequences recovered from HFRS patients identified three different hantaviruses: SEOV in 10/15, AMRV in 4/15, and HTNV in 1/15 samples. The nucleotide sequence divergence among the newly identified SEOV strains were 0–1.4%, 0–0.9%, and 0–1.9% for the S, M, and L segments, respectively, and deduced amino acid sequences were identical. Compared with previously published SEOV S and M nucleotide sequences from south-eastern Asia, divergence did not exceed 1.6%, while the differences with the SEOV strains from other regions (China, Korea, Japan, USA) reached 5.7%. Partial S segment sequences from two rats, captured in 1997, and three HFRS patients infected in 2001, 2004, and 2008 were identical to those of strains 24D12 from Vietnam and strain Rn152 from Cambodia (20, 21). The partial amino acid sequences of the

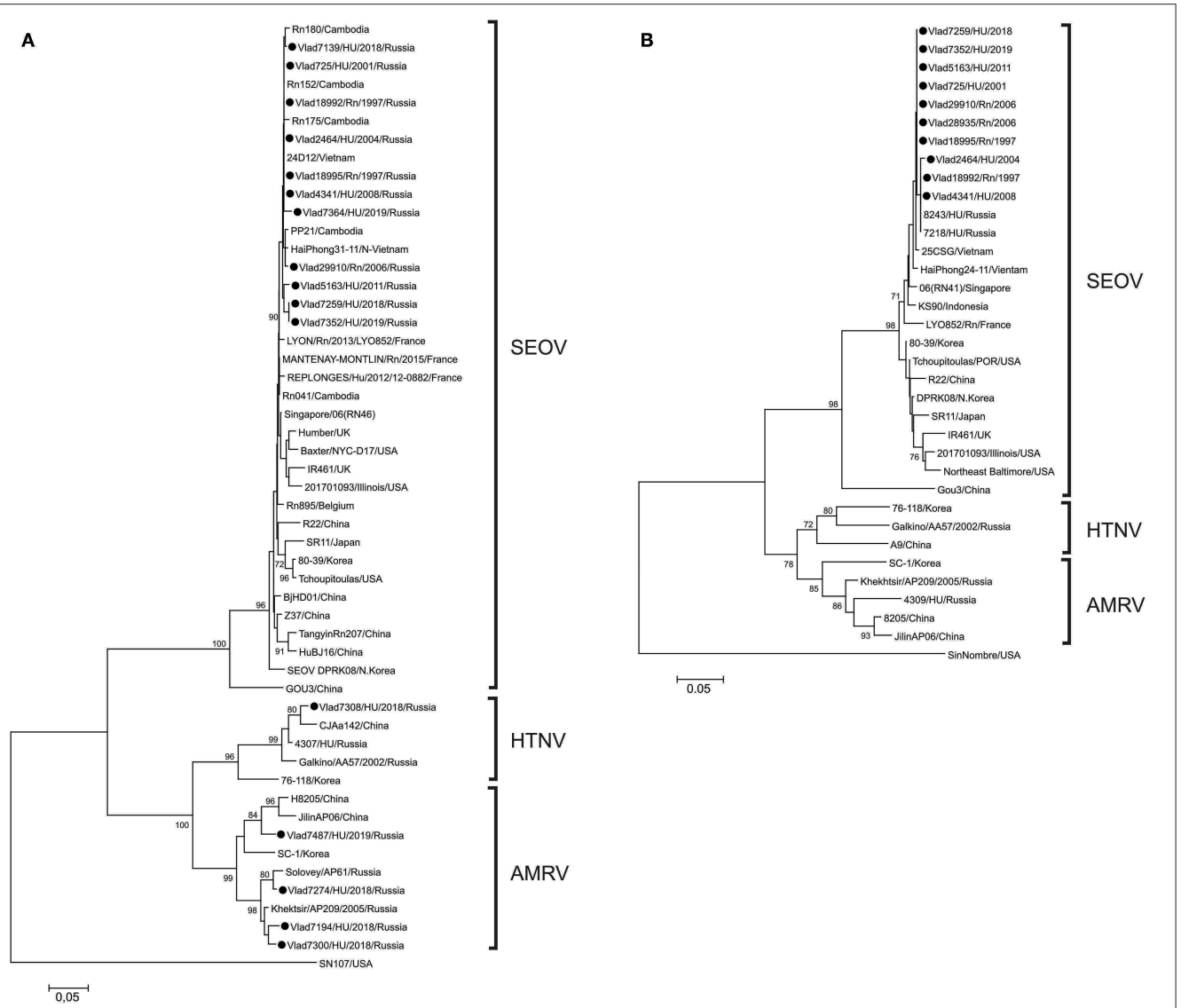

FIGURE 1 | Phylogenetic trees of orthohantaviruses based on partial sequences of the **(A)** S (nt 610-936) and **(B)** M (nt 2737-2980) segment sequences. All analyses (neighbor-joining method) were performed using the MEGA software (19). Sin Nombre virus was used as the outgroup. Bootstrap values were calculated from 1,000 replicates; only values >70% are shown at the branch nodes. The trees constructed by using the maximum-likelihood method (not shown) had similar topology. Circles indicate sequences of strains detected in Norway rats and from the patients in this study.

RdRp, Gc, and N proteins of the SEOV strains from Vladivostok were identical with that of SEOV strains from south-eastern Asia and strains found in France, USA and Belgium.

Analysis of AMRV partial S segment sequences showed that patients from Vladivostok were infected by three genetic lineages of virus (**Figure 1**). Two of these lineages joined previously identified strains from far-eastern Russia and partial S segment divergence between sequences within each lineage did not exceed 5.2%. The third lineage was formed by a novel Russian strain and strains from the neighboring Jilin province of China.

As expected, the Vladivostok HTNV sequence was more closely related to HTNV strains from far-eastern Russia and the neighboring north-eastern area of China. The partial S sequence divergence between strain from Vladivostok and strains from north-eastern China (strains CJAa142, Fuyuan-Aa-26, Bao14, ShenyangAa13) was 2.7–4.3%. All AMRV- and HTNV-infected human patients had severe clinical manifestations of HFRS.

DISCUSSION

The results of genetic analysis of SEOV strains from rats and HFRS patients over two decades demonstrate the high stability of SEOV strains circulating in Vladivostok city. Phylogenetic analysis of the short sequences of SEOV that we obtained

does not allow further differentiation of the lineages. However, the complete identity of the partial S segment sequences from Vladivostok with those from Cambodia and Vietnam for five strains isolated during 10 years leads us to define the origin of the virus and its phylogenetic placement into lineage 7, joining strains from Cambodia, Vietnam, Indonesia, Singapore and imported strains in France and Belgium (5, 8). In the Primorsky region, SEOV was detected in Norway rats and HFRS patients only in Vladivostok city, while circulation of a closely related genetic variant of SEOV among *R. norvegicus* was registered in large areas of South-East Asia, including the southern and central highland area of Vietnam, and the southern area of Cambodia. So, we suggest that SEOV in Vladivostok port city originated as a result of movement of its natural host from South-East Asia during intensive shipping trade activities between Vietnam, Cambodia and Russia.

Among serologically confirmed HFRS cases associated with SEOV, 31.5% patients had a mild clinical course and 62.8% were moderate, while 5.7% had severe forms of HFRS (12). Our direct genetic evidence shows that the SEOV strains from Vladivostok can cause severe clinical manifestations of HFRS. Also, some patients in Vladivostok were infected with AMRV and HTNV (4/15 and 1/15, respectively) and these finding are consistent with serologic data from a previous report (14). Phylogenetic analysis demonstrated that AMRV cases belong to three genetic lineages of AMRV. So, most probably, these HFRS patients were infected in different parts of the Primorsky region, likely located in the suburbs of Vladivostok city. City residents may acquire AMRV and HTNV infection from *Apodemus peninsulae* (a natural host of AMRV) and *A. agrarius* (a natural host of HTNV) during visits to their vegetable gardens in neighborhoods of Vladivostok or on trips to forests for nut harvesting and hunting.

In our study, a limited number of strains were genetically typed and information regarding the location of exposure for some patients from Vladivostok was not available. However, our data are in accordance with previously published results. In the Primorsky region, SEOV circulation was registered only in Vladivostok city but not in rural areas (12, 22). As the same genetically stable variant of SEOV, distinct from strains in neighboring China, was detected both in rats (4 strains) and human patients (10 strains) in Vladivostok, we inferred that patients who acquired this SEOV strain did so in the city. Identification of genetically divergent AMRV (4 strains) and HTNV (1 strain) from HFRS patients supports our hypothesis that some city residents likely acquired AMRV and HTNV infection from areas outside Vladivostok, where these strains circulate. Also, the proportion of severe HFRS cases (12.9%) among residents of Vladivostok that we detected, exceed those (5.7%) among serologically confirmed SEOV-associated patients, as published previously (12).

In summary, our study indicates that the main causative agent of HFRS in Vladivostok is SEOV, originating from infected Norway rats traveling by ship from south-east Asian countries. The other HFRS pathogens, AMRV and HTNV, showed clear spatial association with local geography.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of Somov Institute of Epidemiology and Microbiology, Vladivostok, Russia. The patients/participants provided their written informed consent to participate in this study. Ethical review and approval was not required for the animal study because Wild Norway rats were trapped according to well-established protocols (16).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.620279/full#supplementary-material

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BRIEF RESEARCH REPORT
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Phylogenetic Characteristics of West Nile Virus Isolated From *Culex modestus* Mosquitoes in West Kazakhstan

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West Nile virus is widespread in southern Russia, where the fever appears annually. Since Western Kazakhstan borders on southern Russia, we examined mosquitoes in this region for the presence of West Nile virus. Virus was detected in a small proportion of *Culex modestus* mosquitoes (3/239 pools) and isolates are related to strains from Volgograd, Russia. A screen for West Nile virus IgG was conducted and ∼5% of the local human population tested positive.

Keywords: E segment, phylogenetic analysis, West Kazakhstan region, seroconversion, West Nile virus, West Nile virus (WNV)

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INTRODUCTION

West Nile Virus (WNV) is a virus of arthropods that is classified within the *Flaviviridae* family. It is a member of the Japanese encephalitis virus (JE) virus group, whose members include Japanese encephalitis virus, Saint Louis encephalitis virus (SLE) and Murray Valley encephalitis virus (MVE) (1). WNV is widespread in Africa, Asia, Europe, and Australia and has been responsible for several significant epidemics [Israel (1950), France (1962), South Africa (1974) and Romania (1996)] (2–5). In 1999, WNV caused two epidemics. One occurred in Volgograd, Russia, and another in the New York area, USA, where there were 62 confirmed human cases, with six deaths (2, 6–8). WNV continues to be a rising health care problem in the twenty-first century. In 2018, for example, southern and southeastern Europe saw a large increase in cases; a plausible cause for this is climate change, with early Spring and a hot Summer leading to increased mosquito activity (9–11).

West Nile fever has been diagnosed in 16 regions of the Russian Federation (12), several bordering on NW Kazakhstan. No West Nile cases have been reported (or looked for) in Kazakhstan but, given the situation in these neighboring areas of Russia where cases of WNV have been reported (Volgograd, Astrakhan, Omsk, Samara, Chelyabinsk, Orenburg) (13, 14) there is cause for concern. We therefore conducted a study of local mosquitoes in West Kazakhstan oblast as possible carriers of WNV. The main *objectives* of our study were to determine: (1) whether WNV circulated in west Kazakhstan and (2) if so, what were the dominant species of mosquito involved in the region. We employed RT-PCR for viral RNA, followed by partial sequencing and phylogenetic

analyses of isolates to determine how Kazakh strains may be related to strains from Russia and other parts of the world.

METHODS

Mosquitoes for the study were collected using human landing catch approaches on collecting scientists, usually with a Mondchadskiy Bell (a 2 × 1.4 m calico cone held above the collector, acting as bait). Collections took place on 28 different occasions in all 11 districts of WKO in the area between 49.0401 and 51.2520 degrees North and 47.3325 and 54.0851 degrees East between May 25 and July 09, 2015. This area is ∼100 km south of Oral, Kazakhstan and ∼600 km east of Volgograd, Russia. **Supplementary Figure 1** shows the geographic location of the collecting area inside a black-bordered oval at the top left-hand corner of the map. Each mosquito was identified visually by qualified, experienced entomologists using Kazakh national identification criteria. We did not tabulate males vs. females but, given the collection method, a substantial number is likely to be female. Individual insects were pooled with 35–40 copies of the same species; these are standard numbers for official collections in this part of Kazakhstan. These were stored frozen (−70°C) as whole insects. To each frozen mosquito pool, 700 μl of PBS was added prior to immediate treatment in MK28R tissue homogenizing tubes using a Bead Beater homogenizer (Retsch MM400). To isolate viral RNA, 100 μl of the suspension was used with the "AmpliPraym Ribo-Sorb" kit (Russia). The first step in the process involves isothiocyanate denaturation/stabilization.

To detect viral RNA in mosquito pools, qRT-PCR analysis using viral E (envelope) gene sequences was carried out with a hybridization-fluorescent detection method, using the "AmpliSens WNV-FL variant FRT" kit (Russia). A Rotogene 6000 cycler was employed. This is a "single-tube" method, whereby RNA to cDNA to PCR product is carried out all in the same reaction mix.

Two samples showing Ct scores of 26–28 were chosen for sequence analysis. The third positive sample had a Ct score of 36 and was not used.

For sequence analysis, the above two samples were then subjected to further RT-PCR to generate a ∼900b fragment from the E gene prior to sequencing. We designed primers for nested PCR with the BioEdit Sequencing Alignment Editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html) using 70 published genome sequences of WNV. Targeting conservative areas of the viral E gene with minimal nucleotide polymorphism, we selected four degenerate primers as follows:

WNV f1 1330 GGACTGTTTGGRAARGGAAGCATTGA;
WNV f2 1400 CATCCAGAARGAGAAYATCAAGTA;
WNV r1 2444 ACGGGCATTGATKCCCATCCACA;
WNV r2 2305 AACTGAYCCAAARTCCCAAGC.

These nested primers were then used in a standard two-step amplification procedure, using HotStarTaq Plus DNA polymerase (QIAGEN), as follows:

Master Mix:

Component name		Final conc	First round of PCR (μl) ($n = 1$)	Second round of PCR (μl) ($n = 1$)
PCR Buffer with 15 mM MgCl2	10x	1x	3	3
Q-Solution	5x	1x	6	6
MgCl2	25 mM	1 mM	1.2	1.2
HotStarTaq Plus DNA Polymerase	5 u/μl	2 units/reaction	0.4	0.4
dNTP	2 mM of each	0.2 mM of each	3	3
WNV f1 1330	18 μM	600 nM	1	–
WNV r1 2444	18 μM	600 nM	1	–
WNV f2 1400	18 μM	600 nM	–	1
WNV r2 2305	18 μM	600 nM	–	1
cDNA			5	–
PCR product of the first stage			–	3
RNase-free water			9.4	11.4

Run Parameters:

Step name	First round of PCR			Second round of PCR		
	Temp (°C)	Time (min:s)	Cycles	Temp (°C)	Time (min:s)	Cycles
Denature	95	05:00	1	95	05:00	1
Denature	95	0:30	35	95	0:30	35
Aneling	60	0:50		52	0:50	
Amplify	72	01:30		72	01:30	
Amplify	72	10:00	1	72	10:00	1

After checking a small sample of the reaction mix on a 1.5% agarose gel that the product was the expected ∼900 base band, purification of this PCR fragment from primer residues and dNTPs was performed by precipitation in 20% PEG. Sequencing was performed using Big Dye Terminator v 3.1. (Thermo Scientific) according to the manufacturer's instructions. Sequencing reactions were performed on an automated DNA Analyzer 3730xl (Applied Biosystems). Nucleotide sequences of about 800 bases were obtained and the sequences were placed in GeneBank (GeneBank accession numbers KX129740.1; KX129741.1). Sequences obtained in two rounds of PCR were combined into a common sequence using SeqScape 2.6.0 software (AppliedBiosystems). Reference strain sequences deposited in the international NCBI database (http://www.ncbi.nlm.nih.gov/) were used as comparison. Dendrograms were constructed using Mega 6.0 software, alignment of nucleotide sequences was carried out using the Muscle algorithm and phylogenetic trees were constructed using Maximum Likelihood (**Figure 1**). Multiple sequence alignments, using all known sequences of the

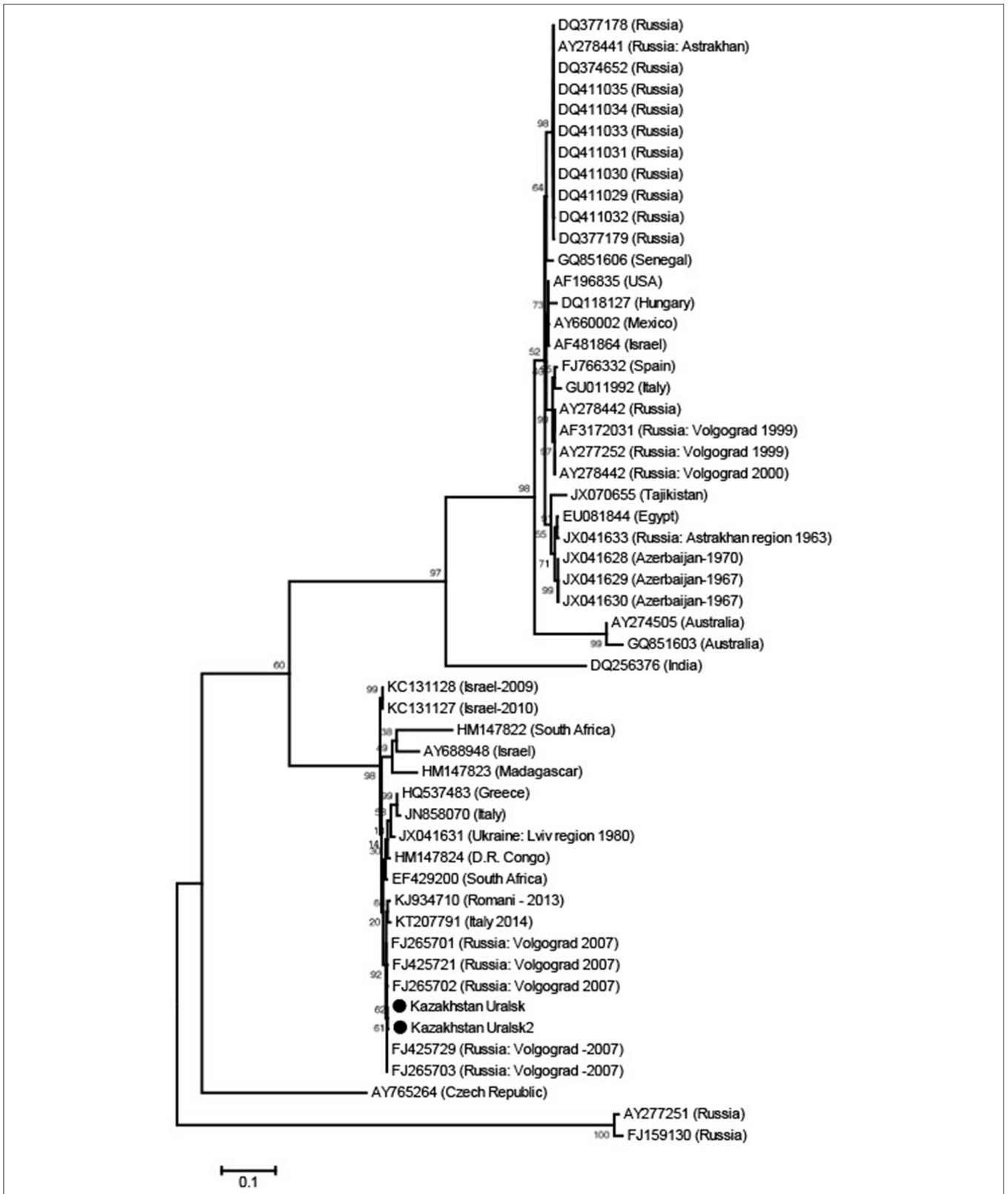

FIGURE 1 | Phylogenetic tree from WNV E gene sequences using Maximum Likelihood. The sequences were obtained from *C. modestus* mosquitoes collected in NW Kazakhstan.

WNV E gene, were performed with Muscle in MEGA 6.0 software (15).

The analysis details are as follows:

Phylogeny reconstruction using Maximum Likelihood
Phylogeny Test: Bootstrap method with 1,000 replications
Substitution Model: Type-nucleotide, method—Kimura 2-parameter model
Rates/Patterns: Rates among sites—Gamma distributed; 5 discrete categories
Data subset to use gaps/missing data—complete deletion

Tree inference options:

ML heuristic method—Nearest Neighbor Interchange
Initial tree for ML—Make automatically (default—NJ/BioNJ)
Branch swap filter—very strong
System resource usage—1st+2nd+3rd + non-coding
No. of seqs—53
No. of sites—692
No. of bootstrap reps—1,000

Following the mosquito findings, we ran a screen of 454 sera from inhabitants in the WKO, selected randomly. These serum samples had been collected by the Kazakh health authority in west Kazakhstan as part of routine screening activities and were kindly made available to us as anonymous numbered material (i.e., no identifying marks). A commercial kit was used (VectoNile-IgG, Vector Best, Russia), following the manufacturer's instructions. These kits contain positive and negative controls and have the CE seal of approval from the EU. Briefly, 1:10 and 1:100 dilutions of sera were used in 96 well plates, in duplicate, and the assays were carried out twice. Final readings were the average of these four readouts.

RESULTS

Mosquitoes were collected from 11 districts of the West Kazakhstan Oblast (WKO) and were identified by experienced entomologists, using Kazakh local morphology criteria. A total of 9,500 mosquitoes was combined into 239 pools. We identified insects in three genera and seven species. We did not differentiate between males and females.

The dominant potential vectors were of the genus *Culex*, representing 56.2% of the total collection, and all in one species—*Culex modestus*. The composition of mosquitoes of the genus *Aedes* was more diverse and was represented by four species—*Aedes flavescens, Aedes caspius, Aedes vexans,* and *Aedes cinereus*. Mosquitoes of the genus *Anopheles* were less common in the region, with 1.8% of the total number of captured insects, represented by two species—*Anopheles maculipennis* (we did not differentiate between sl and ss) and *Anopheles hyrcanus*. In this study, only *Culex modestus* harbored WNV. Real-time analysis by RT-PCR revealed 3 positive samples for WNV RNA in *Culex modestus* found in the Akzhaiksky area (**Table 1**). This area of Kazakhstan is between Oral in the north and the Caspian Sea in the south and is about 80 km from the Russian border, between Saratov and Volgograd. Comparing the Kazakh virus amino acid sequences with other strains in the NCBI database, it is clear that viruses from the WKO have a close relationship to strains from Volgograd. The high aggressiveness and anthropophilia of *Culex modestus* lend credence to the finding that it is an active carrier of WNV (16).

Our IgG screen of human serum samples for WNV IgG, showed that about 5% of the samples (21 of 454) we were given were positive. Thus, while there have been no formal reports of WN fever in WKO, as stated earlier, it seems that a) the virus circulates in WKO and b) that people are being infected by it.

DISCUSSION

The main findings in this paper are that WNV circulates in NW Kazakhstan and is associated with *C. modestus* mosquitoes, but not in any others in our sample of positive insects.

This mosquito is a known WNV vector, is prevalent in south and southeastern Europe and seems to be moving North, perhaps in response to climate change, as mentioned earlier.

The WNV strains we identified are very similar to those in Volgograd, Russia, about 600 km away from the collecting region in Kazakhstan.

The question arises concerning the origins of positive human serum samples collected in this region and whether those were individuals who had perhaps been infected in Russia, while visiting. While this cannot be ruled out, it is unlikely that this would explain all of the cases. The area where the positive sera originated is, in fact, not directly bordering Russia (several 100 km away) and there is limited travel between it and NW Kazakhstan. It seems more likely that people are being infected by resident *C. modestus* mosquitoes. As a comparison, analogous reports of WNV prevalence in mosquitoes during outbreaks of disease in New York and Louisiana, USA, revealed mosquito infection rates similar to those found in this study (17, 18).

It would have been useful for us to have been able to further test the individuals who were positive for WNV IgG, perhaps by looking for virus in blood, or in urine where the virus seems to persist for considerable times in certain cases (19). This hopefully would have yielded virus sequences for comparison. However, as pointed out earlier, the serum samples to which we had access had been collected separately from this study and we had no means of accessing these individuals for further testing. It will be important to build on these preliminary human sample findings and we plan additional work on IgM, as well as possible serum and urine analyses for the presence of WNV genomes.

Detection of WNV in *Culex modestus* in the northwest of Kazakhstan points to a focus of infection in this region. The public health authorities in the Republic will now hopefully mount careful monitoring of the mosquito population (particularly *C. modestus*) in WKO as well screening the population for signs of West Nile fever. Several other areas of Kazakhstan border with areas of Russia with known WNV cases, many relatively unpopulated, but some with cities (e.g., Aktobe in the North and Atyrau in the West) where continued exploratory work on WNV in Kazakhstan would be an important future public health activity.

TABLE 1 | The table provides data on the mosquito numbers captured by district, number of pools, and species of mosquito.

Districts of the WKO	*Culex modestus*	*Aedes flavescens*	*Aedes caspius*	*Aedes vexans*	*Aedes cinereus*	*Anopheles maculipennis*	*Anopheles hyrcanus*
Bokeyordinsky	201/4/0	–	–	–	–	–	–
Zhanibeksky	103/3/0	–	–	–	–	46/2/0	–
Zhangalinsky	1696/34/0	–	530/16/0	–	–	–	100/3/0
Kaztalovsky	12/2/0	138/5/0	118/3/0	2/1/0	–	–	–
Akzhaiksky	1080/24/3	11/1/0	259/9/0	–	–	68/5/0	74/2/0
Terektinsky	1359/28/0	–	–	387/8/0	–	50/1/0	–
Karatobinsky	376/8/0	–	20/1/0	–	19/1/0	–	–
Chingerlausky	442/11/0	1248/35/0	–	–	–	50/1/0	–
Burlinsky	–	–	–	–	–	160/4/0	–
Zelenovsky	73/2/0	–	–	–	–	–	–
Syrymsky	–	–	–	878/25/0	–	–	–
Total:	5342/116/3	1397/41/0	927/29/0	1267/34/0	19/1/0	374/13/0	174/5/0
Total (%)	56,2/48,5/ 1.2	14,7/17,2/0	9,8/12,2/0	13,4/14,2/0	0,2/0,4/0	3,9/5,4/0	1,8/2,1/0

WKO, West Kazakhstan Oblast.
Number of mosquitoes/Number of tested pools/WN virus positive pools.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Republic of Kazakhstan. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.575187/full#supplementary-material

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frontiers in Medicine

CASE REPORT
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Case Report: Post-mortem Histopathological and Molecular Analyses of the Very First Documented COVID-19-Related Death in Europe

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In Europe, the first case of coronavirus disease (COVID-19) and the first COVID-19-related death were reported in France on January 24th and February 15th, 2020, respectively. Officially, the first case of COVID-19 infection in the Republic of Serbia was registered on March 6th. Herein, we presented the first case of retrospective detection of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in the post-mortem-obtained vitreous humor (VH), which took place on February 5th, 2020. This is the first death in Europe proven to be caused by COVID-19 by means of post-mortem histopathological and molecular analyses. Based on this finding, it appears that SARS-CoV-2 has been spreading faster and started spreading much earlier than it had been considered and that COVID-19 was probably the cause of the much-reported pneumonia of unknown origin in January and February 2020.

Keywords: COVID-19, SARS-CoV-2, vitreous humor, autopsy, Europe (central)

INTRODUCTION

In December 2019 and January 2020, doctors around the world encountered an unusual situation—a significantly higher number of patients suffering from pneumonia of unknown origin compared to previous years (1, 2). In December 2019, it was obvious that Wuhan and other cities in China had faced a new public health challenge, recognized as coronavirus disease (COVID-19). The causative agent, identified from throat swab samples on January 7th, 2020, was named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and quickly became a serious problem worldwide with a high mortality rate and the need for quarantine (3, 4). In Europe, the first case and the first death caused by COVID-19 were reported in France on January 24th and February 15th, 2020, respectively (5). Officially, the first case of COVID-19 in the Republic of Serbia was registered on March 6th (6). Herein, we presented the first case of retrospective detection of SARS-CoV-2

in the post-mortem-obtained vitreous humor (VH), more than a month before the first case was officially registered in the Balkan region (6)[1]. In addition, this is the first death caused by COVID-19 in Europe proven by post-mortem diagnostics.

CASE DESCRIPTION

On February 5th 2020, a 56-year-old man presented to the hospital with fever, cough, and shortness of breath, which started 5 days before. The socio-epidemiological questionnaire showed that the patient worked in a construction company, was living in a Belgrade suburb, had not traveled abroad for a long time, had no chronic conditions, but was slightly overweight (BMI 25.2 kg/m^2). On admission, the patient had a high fever (39.2°C) and hypoxia (PaO2 78%) and was hypotensive (blood pressure 100/50 mm/Hg). Chest radiography revealed massive bilateral pneumonia. The biochemical and blood gas analyses revealed the following (reference ranges are shown in parentheses): white blood cell 1.9 × 10^9/L (4.40–11.50), platelet 127 × 10^9/L (150–400), D-dimer 3.9 g/L (2–4), C-reactive protein 229 mg/dL (0–7), pO2 25 (80–100), pCO2 33 (35–45), and $CHCO_3$ 21 (22–28). The patient was intubated and connected to mechanical ventilation; unfortunately, he died within a few hours after admission to the hospital. The autopsy was performed the next day, according to

[1]https://www.who.int/docs/default-source/coronaviruse/real-time-rt-pcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf (accessed on September 10, 2020).

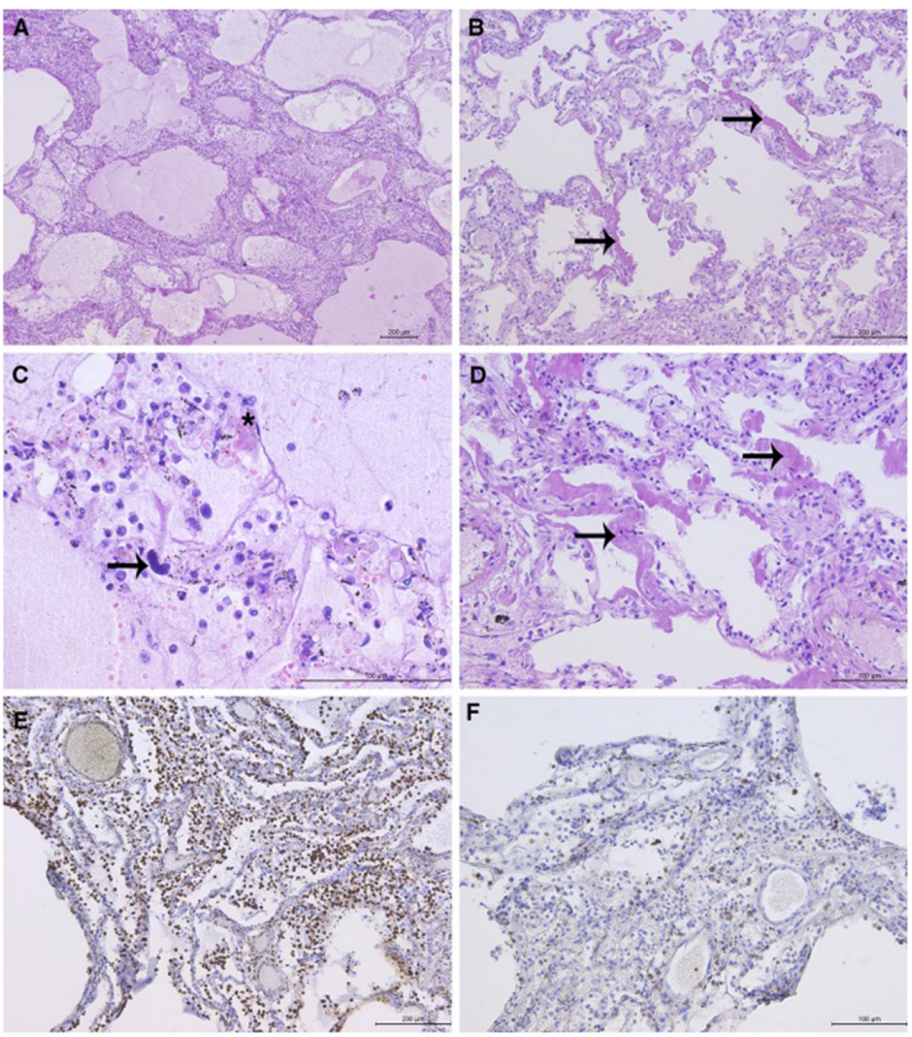

FIGURE 1 | Representative lung sections showing diffuse alveolar damage (DAD): **(A)** DAD with associated pneumonia. Intraalveolar protein-rich edema and inflammatory cells in the alveolar lumen and interstitium. **(B)** Hyaline membranes (arrows) with widening of the alveolar septae. **(C)** Alveolar protein-rich edema. Mixed inflammatory infiltrate with predominance of macrophages. Scattered granulocytes can be observed. Megakaryocyte in the capillary (arrow). Hyaline microthrombus (asterisk). **(D)** Hyaline membranes (arrows), light interstitial fibrosis, and proliferation of type 2 pneumocytes. **(E)** Abundant macrophages in the interstitium and lumen of the alveoli (immunohistochemical staining with CD68 antibody) and **(F)** scattered T-lymphocytes (staining with CD3 antibody). *Microphotographs were taken using the Leica DM4000 B LED light microscope (Leica, Wetzlar, Germany) and Leica DFC295 digital camera (Leica, Heerbrugg, Switzerland).

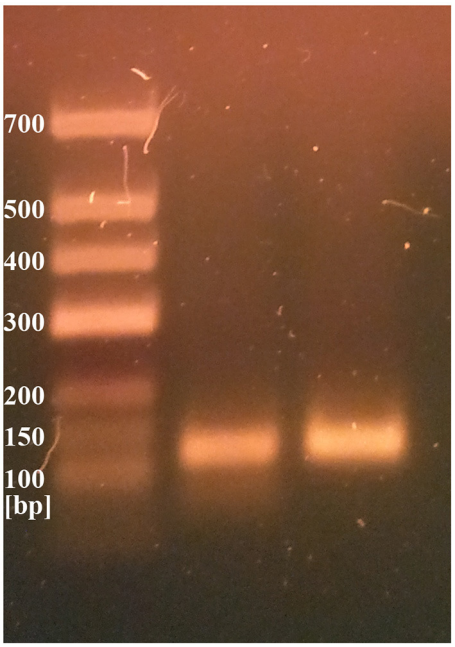

FIGURE 2 | IP2 and IP4 PCR products on 2% agarose gel.

the standard procedure. Gross autopsy findings revealed heavy, grossly firm, and rubbery lungs with severe bilateral edema. On the cut section, the lungs were dark red without purulent discharge. The hilar lymph nodes were slightly enlarged. The findings in other organs were unremarkable.

Initially, the death was attributed to pneumonia of unknown origin but after additional diagnostic procedures conducted 3 months later, it was proven that death was caused by COVID-19.

Histopathological Analysis

Histopathological analysis (HP) of the lungs revealed exudative and early organizing phases of diffuse alveolar damage (DAD) in all sections (**Figure 1**). The findings of intraalveolar protein-rich edema, capillary congestion, and formation of hyaline membranes corresponded to the exudative phase of DAD. DAD was focally associated with fibrinous pneumonia with a mixed inflammatory infiltrate in the lumen of the alveoli and interstitium, consisting of abundant monocytes/macrophages (CD68 staining), rare T-cells (CD3 staining), and scattered granulocytes. Megakaryocytes were observed in the microvasculature. The focal proliferation of type 2 pneumocytes, thickening of the alveolar septae, fibroblast proliferation, and loose interstitial fibrosis corresponded to the early organizing phase of DAD. Few hyaline microthrombi were present in sections. HP findings of other organs were unremarkable (**Figure 1**).

Molecular Testing

Two different genetic analyses of VH samples (RNA isolation/reverse transcription and Real-Time PCR/amplicon sequencing) were performed with the aim of confirming the validity of results and eliminating the possibility of false-positive results. Two regions of SARS-CoV-2 orf1ab were amplified using the Institute Pasteur primers (IP2 and IP4)[1]. To confirm the specificity, agarose gel extraction of IP4 amplicon and DNA sequencing were performed (**Figure 2**). The obtained sequence was aligned against reference genomes in the NCBI database using MEGABLAST. IP2 and IP4 regions were amplified with CT values 35 and 34, respectively. PCR products matched the expected lengths for IP2 and IP4 segments, with a minor non-specific band in the P2 lane. MEGABLAST results showed that the sequence without primer binding regions matched SARS-CoV-2 with an E value of 2e−15, as well as 97.92% of percentage identity with NC_045512.2 (**Figure 3**).

Confirmatory Assay

Since this was presumably the first confirmed case of COVID-19 in our country, we performed a second molecular analysis of different gene targets of SARS-CoV-2, as suggested by the Center for Disease Control and Prevention. Total RNA was separately isolated from an additional sample of 200 μl of VH. The sequences from the SARS-CoV-2-specific RdRp gene, as well as from the human RNase P gene as internal control, were detected.

DISCUSSION

The case represents the very first fatal outcome of COVID-19 in Europe and the first post-mortem-confirmed COVID-19 case after retrospective VH analysis. The patient died on February 5th, after 5 days of intensive respiratory symptoms, indicating that he was probably infected in January. The fact that the patient had not traveled abroad and the fact that there were no known epidemiological links with other countries with an active COVID-19 epidemic at that moment indicate that SARS-CoV-2 had probably already been spreading among the population in the Balkans at the beginning of 2020. The study of Deslandes et al. (7) confirmed the suspicion that the virus had come to Europe earlier than it was officially registered. Based on the retrospective testing of respiratory swabs, this group of researchers detected one SARS-CoV-2-positive case in France in late December 2019 (7).

An important conclusion is that VH is a very useful post-mortem sample for SARS-CoV-2 preservation, as it allowed us to detect the virus 3 months after the death, especially in cases when the virus has not been isolated from the blood (8–11). We isolated the virus from VH, although VH underwent freezing at −20°C and subsequent thawing and the confirmatory analysis was done on that thawed sample a few weeks after the first one. This suggests that SARS-CoV-2 is more resistant to temperature changes. Recently published manuscripts showed different results regarding the presence of SARS-CoV-2 in the ocular structures (12–15). In the study of Sawant et al., vitreous swabs were positive for SARS-CoV-2 RNA in 2/10 patients with COVID-19 (14), while one other study did not find SARS-CoV-2 RNA in the vitreous fluid of COVID-19-positive patients (13). On the other hand, Casagrande et al. showed that the prevalence rate of SARS-CoV-2 RNA in the retinal biopsies

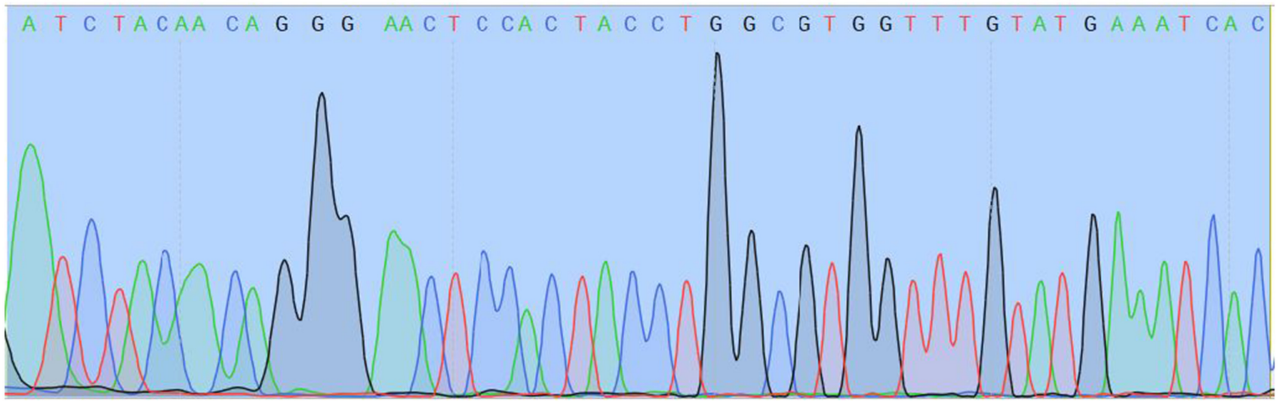

FIGURE 3 | Readable sequence of IP4 amplicon without primer binding sites.

of patients with COVID-19 is 21% (3/14) (15). Therefore, the possibility of infiltration of VH via retinal vasculature could not be eliminated. VH could be used as a valuable specimen for post-mortem virological analyses, as it stays stable and preserved for a long time. The sequencing of the viral RNA is undoubtedly confirmation that this was in fact SARS-CoV-2, while the possibility of a falsely positive result is excluded.

In the presented case, clinical, radiological, and biochemical findings are in accordance with the severe form of COVID-19. Macroscopically, it is typical of COVID-19 pneumonia to be presented with large and heavy lungs due to retained fluid, as in the presented case. Edler et al. noticed that the lung surface sometimes showed signs of pleurisy and mosaic-like pattern of pale fields and slightly protruding dark purple sections with prominent capillary drawing (16). Also, cut sections of the lungs revealed either ubiquitously dark red or, alternately, faded appearance. In some COVID-19 deaths, purulent respiratory tract infection with abscessed bronchopneumonia was observed (16–18), which is macroscopically different from our findings. Here, the main HP finding was DAD in exudative and early organizing phases, in accordance with the recently published studies (19, 20). Capillary congestion and multifocal microthrombotic disease in capillaries and small vessels were noticed and described in some papers (20). In comparison to other published data, we only detected scattered hyaline microthrombi in the lung capillaries (21, 22). In addition, an increased number of megakaryocytes in the lungs (and other organs) is described in COVID patients, along with some COVID-19-unrelated conditions, like intravascular coagulation, acute infection, shock,and fever (21, 23).

Based on this case, it appears that SARS-CoV-2 has been spreading much earlier than it was considered and that COVID-19 was probably the cause of much-reported pneumonia of unknown origin in early 2020. Important insights into this novel disease and its course can be obtained by post-mortem HP and molecular analyses of the VH, as an excellent sample for SARS-CoV-2 detection, in patients with pneumonia of unknown origin who died in the last one year.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/nuccore/MW471658.1/.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MB, IS, TA, and AB: drafted manuscript. IS, OS, VP, SS, ZM, and BR: autopsy and pathohistological and molecular analyses. TA, ID, and SD: review of literature. All authors: revised and prepared together the final version of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.612758/full#supplementary-material

Supplementary Figure 1 | SYBR green RT PCR amplification using Pasteur primers. Linear scale representation. Order of Ct values: 34 for IP2, 33 for IP4, 40 for internal (human) control.

Supplementary Figure 2 | SYBR green RT PCR amplification using Pasteur primers. Log scale representation. Order of Ct values: 34 for IP2, 33 for IP4, 40 for internal (human) control.

Supplementary Figure 3 | Result of RT-PCR assay.

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How Cooperative Engagement Programs Strengthen Sequencing Capabilities for Biosurveillance and Outbreak Response

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The threat of emerging and re-emerging infectious diseases continues to be a challenge to public and global health security. Cooperative biological engagement programs act to build partnerships and collaborations between scientists and health professionals to strengthen capabilities in biosurveillance. Biosurveillance is the systematic process of detecting, reporting, and responding to especially dangerous pathogens and pathogens of pandemic potential before they become outbreaks, epidemics, and pandemics. One important tool in biosurveillance is next generation sequencing. Expensive sequencing machines, reagents, and supplies make it difficult for countries to adopt this technology. Cooperative engagement programs help by providing funding for technical assistance to strengthen sequencing capabilities. Through workshops and training, countries are able to learn sequencing and bioinformatics, and implement these tools in their biosurveillance programs. Cooperative programs have an important role in building and sustaining collaborations among institutions and countries. One of the most important pieces in fostering these collaborations is trust. Trust provides the confidence that a successful collaboration will benefit all parties involved. With sequencing, this enables the sharing of pathogen samples and sequences. Obtaining global sequencing data helps to identify unknown etiological agents, track pathogen evolution and infer transmission networks throughout the duration of a pandemic. Having sequencing technology in place for biosurveillance generates the capacity to provide real-time data to understand and respond to pandemics. We highlight the need for these programs to continue to strengthen sequencing in biosurveillance. By working together to strengthen sequencing capabilities, trust can be formed, benefitting global health in the face of biological threats.

Keywords: cooperative threat reduction, emerging diseases, next generation sequencing, pandemic preparedness, One Health

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INTRODUCTION

Emerging and re-emerging infectious diseases are a major challenge for public health and economic security worldwide (1). Zoonotic diseases, those pathogens that can infect both animals and humans, pose the greatest risk to humans. Changes in environmental conditions (e.g., climate change and habitat degradation), biodiversity loss, habitat encroachment, and increased globalization all increase the risk that novel pathogens will spill over into humans

(1–4). Preventing and dealing with the consequences of these diseases requires a global effort. Cooperative engagement programs are designed to build and strengthen capabilities and capacities in biosurveillance, biosecurity, and biosafety around the world. These programs aim to create, foster, and sustain international collaborations among health professionals, disease diagnostic laboratories, and infectious disease scientists (5, 6). They primarily focus on especially dangerous pathogens and pathogens of pandemic potential, and include those that may be used in bioterrorism. These programs have been implemented in the United States, Canada, Germany, and other countries with the goal of reducing the threat of infectious diseases by promoting global health security.

One aspect of cooperative engagement programs is to build, promote, and strengthen biosurveillance capabilities. Biosurveillance is "the ongoing systematic collection, analysis, and interpretation of health data, essential to the planning, implementation, and evaluation of public health practice, closely integrated with the timely dissemination of these data to those who need to know and linked to prevention and control" (7, 8). Early detection of pathogens is crucial for mitigation efforts and to limit the spread of a pathogen before it grows from a small outbreak to a larger epidemic or pandemic. One critical tool in biosurveillance and early detection has now become sequencing. Sequencing is not just a critical tool in biosurveillance, it can also play a major role in pandemic response. Here, we discuss the importance of cooperative engagement programs in strengthening sequencing capabilities in countries around the world.

COOPERATIVE ENGAGEMENT PROGRAMS STRENGTHEN BIOSURVEILLANCE AND RESEARCH CAPABILITIES

Cooperative engagement programs bring together researchers, scientists, and health professionals from all over the world with the shared goal of reducing the threat of infectious diseases (5, 6). These programs help to establish and strengthen both biosurveillance efforts and research capabilities to answer important questions regarding infectious diseases (**Figure 1**). A vital consequence of these programs is trust. Trust is formed in the initial capacity building for sequencing and biosurveillance through reachback efforts. Reachback is the technical training and support from partner institutions. Participating countries and institutions can also help each other to perform sequencing and to analyze sequence data, which further enhances relationships. Once the sequencing capability is built, sustaining this capability is challenging; sequencing technology and bioinformatics tools are constantly changing and labs need to keep purchasing supplies and reagents. Collaborations, research networks and projects, and reachback support are important pieces to sustainability. These allow participating countries to continue to actively use sequencing and bioinformatics tools to answer important questions regarding, for example, pathogen biology and disease distribution. Cooperative engagement programs help build the initial capacity and continue to play a role in sustaining these capabilities.

Every country is at a different level of readiness for sequencing and genomics, from laboratory expertise to computational and bioinformatics proficiency. Various funding agencies assess if sequencing is appropriate and is able to be sustained in a given country. Difficulties in building a genomics capability include cost of equipment, obtaining service contracts for instruments, obtaining kits and reagents in country, sustaining a trained workforce, and having the proper infrastructure. For low-income institutions, funds from sponsors help with the initial capacity building and then through collaborative research projects. Once partnerships are formed, cooperative engagement programs foster trust by encouraging participants to work together side-by-side to solve problems and challenges. Other trust-building activities include trainings, mentoring and advising in professional development, and developing personal relationships through in-person meetings. All of these activities increase the chances that a genomics capacity will be developed and used in biosurveillance.

Collaborations among researchers and institutions can lead to the creation of research networks (**Figure 1**). These research networks are typically centered around a common pathogen, disease, or host of interest, such as a research network on bat-borne viruses or Rickettsial pathogens (9). Often called "threat reduction research networks," they increase sustainability and maintain relationships. Trust is key to creating and sustaining networks, which allows for data sharing among collaborators within the network. Threat reduction research networks encourage collaborations on specific research projects, thereby creating new connections and maintaining those already established. The expertise of each institution can be leveraged; and therefore, they can provide support to each other throughout the duration of a funded research project. With increased travel and globalization, the world is more connected than ever, highlighting the importance of the saying "a threat anywhere is a threat everywhere" (6). Having threat reduction research networks in place with the trust that has been formed benefits the broader scientific community, such as in the early stages of an epidemic or pandemic through data sharing (**Figure 1**).

Cooperative engagement programs support and promote the One Health initiative (**Figure 1**). One Health is the integrative and multidisciplinary approach to reducing disease risk by focusing on the health of people, animals, and the environment. A One Health approach to biosurveillance and threat reduction research networks is an important framework for understanding the whole ecology of disease systems, from wildlife reservoir hosts to the role of environmental conditions and habitat disturbances (10). We need to be able to reliably identify pathogens of importance in humans and domestic animals. Wildlife and changes in biodiversity need to be understood and addressed as well, which is an often-overlooked component of the global health security agenda (11, 12). The whole ecology of a disease system leads to a better understanding of the disease and provides information to enhance the effectiveness of biosurveillance efforts. This knowledge maximizes limited resources by allowing

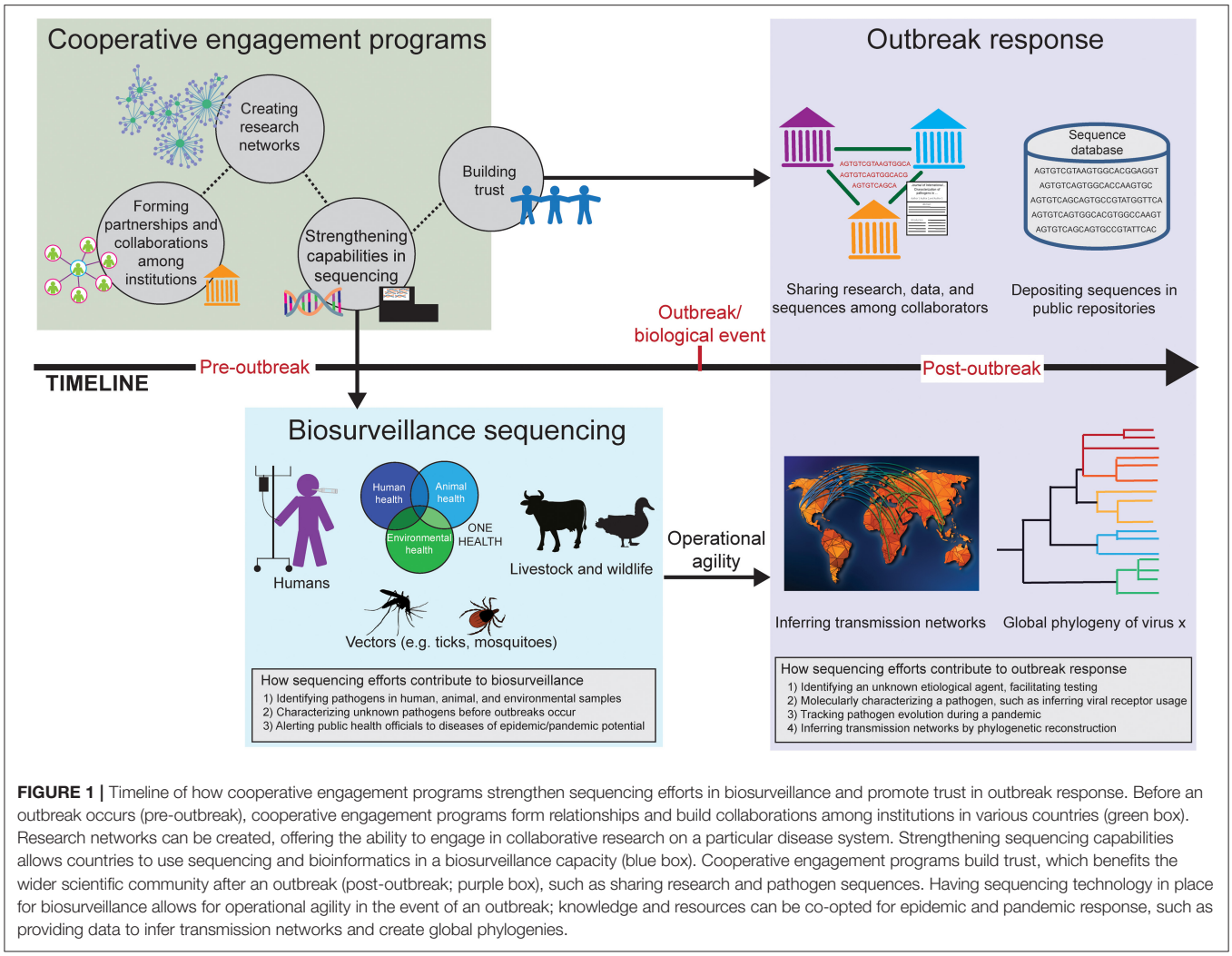

FIGURE 1 | Timeline of how cooperative engagement programs strengthen sequencing efforts in biosurveillance and promote trust in outbreak response. Before an outbreak occurs (pre-outbreak), cooperative engagement programs form relationships and build collaborations among institutions in various countries (green box). Research networks can be created, offering the ability to engage in collaborative research on a particular disease system. Strengthening sequencing capabilities allows countries to use sequencing and bioinformatics in a biosurveillance capacity (blue box). Cooperative engagement programs build trust, which benefits the wider scientific community after an outbreak (post-outbreak; purple box), such as sharing research and pathogen sequences. Having sequencing technology in place for biosurveillance allows for operational agility in the event of an outbreak; knowledge and resources can be co-opted for epidemic and pandemic response, such as providing data to infer transmission networks and create global phylogenies.

researchers to target specific hosts and environments important to the pathogen in question (13).

SEQUENCING IN BIOSURVEILLANCE

Sequencing is an essential and critical tool in biosurveillance (14). In biosurveillance, early detection is crucial. Identifying pathogens in human, animal, and environmental samples cannot be completed without next generation sequencing. Sequencing is being introduced to and adopted by countries all over the world. Democratization of sequencing is a crucial aspect of threat reduction by making sure all countries can afford to adopt this technology and by making the process of sequencing and the analysis of genomic data more feasible (15). Next generation sequencing is still challenging to adopt because of the high learning curve associated with understanding the theory and techniques and gaining practical experience.

In an effort to build sequencing capabilities in partner countries, trainings and workshops led by partner institutions are needed to teach both "wetlab" techniques and bioinformatics. Wetlab topics include sample collection, storage, and preparation; DNA and/or RNA extraction; and performing a sequencing run. Along with trainings in these wetlab techniques, cooperative engagement programs promote training in applying bioinformatics tools to genomic data. Bioinformatics tools are essential to analyzing sequence data. Quality control, taxonomy identification tools (e.g., Kraken2, GOTTCHA2, MetaPhlAn2), phylogenetics, and reference-based analysis are all needed to identify and characterize pathogens in a particular sample and to get the most information out of genomic data as possible. Software such as Los Alamos National Laboratory's EDGE (Empowering the Development of Genomics Expertise) Bioinformatics platform (www.edgebioinformatics.org) are designed to make the analysis of genomic data relatively straightforward (16).

An essential component of sequencing is the collection and inclusion of metadata. Metadata are the information that describe a given sample. Metadata are important throughout the entire life of a sample; from sample collection through sequencing

and bioinformatic analyses. When a sample is first collected, important metadata include, for example, time of collection, what kind of sample it is (e.g., throat swab, fecal sample, blood sample), where the sample was taken, from what host species it came, and location and environmental data (e.g., GPS coordinates and habitat type). Extraction methods, library preparation, and sequencing platform are also important to include as the sample is processed. Furthermore, recording bioinformatic analyses used to process data helps researchers use data that are deposited in public repositories. Integration of metadata with genomic data is key when adding it to a repository, especially linking raw data and processed data that may end up in different databases. These data are extremely important for research related to biosurveillance and sequencing pathogens during a pandemic, and help with contextualizing sequencing data and decision making. For example, without the proper metadata, conclusions regarding pathogen origin and evolution would not be possible. Metadata are especially vital for samples that are in long-term storage that may be processed at a later date.

Applying sequencing and bioinformatics to biosurveillance and threat reduction research networks is key to understanding the whole ecology of disease systems in a One Health context. Knowledge of potential reservoir hosts and/or vectors, the circumstances of transmission events, and the effects of environmental conditions helps to reduce the risk of infectious disease threats and provides information to enhance biosurveillance efforts and effectiveness (17). For example, shotgun metagenomics and metatranscriptomics, and associated taxonomic ID tools, can be used to identify known and novel pathogens in human, animal, and environmental samples. By surveying the types of pathogens present in different regions, public health officials can be alerted about a potential threat and limit the severity and cost of an epidemic or pandemic (18) (**Figure 1**).

Putting the sequencing systems in place and conducting trainings before an epidemic or pandemic occurs gives operational agility to respond to an outbreak and help with mitigation efforts (**Figure 1**). The technology and training need to be put in place prior to public health crises. Once in place, the technology can be co-opted to pandemic response; only the goals and questions being addressed change. Cooperative engagement programs begin establishing relationships and developing collaborations before an outbreak or a biological event occurs. Setting up sequencing machines and training local staff members how to go from sample to sequence to analysis is the first step. By investing time and effort into strengthening sequencing and bioinformatics capabilities through partnerships, trust is built, which can be leveraged both for biosurveillance (pre-outbreak) and outbreak response (post-outbreak; **Figure 1**).

BENEFITS OF SEQUENCING THROUGHOUT A PANDEMIC

A major tool in the fight against a pandemic is pathogen sequencing. Whereas, sequencing in a biosurveillance capacity deals with questions related to identifying and characterizing pathogens before outbreaks occur, sequencing throughout a pandemic provides real-time data on pathogen transmission and evolution (**Figure 1**). The trust gained from and promoted by cooperative engagement programs plays a major role in the sharing of sequence data among institutions and countries, as well as depositing sequences in public repositories (e.g., GISAID [https://www.gisaid.org]) (**Figure 1**). Having the technology and tools allows for operational agility; if the tools for biosurveillance are in place, it allows institutions to shift to pandemic response (**Figure 1**). In general, global sequencing efforts during an epidemic or pandemic can (1) identify unknown etiological agents, facilitating testing; (2) molecularly characterize pathogens such as inferring viral receptor usage; (3) track pathogen evolution throughout the duration of a pandemic; and 4) infer transmission networks by phylogenetic reconstruction. This information can be used to guide effective intervention strategies and track response efforts over time.

Early in pathogen emergence, quickly identifying the etiological agent is critical for developing diagnostic tools, slowing transmission, guiding patient care, and designing therapeutics. For some agents, microbiological techniques such as microscopy and differential growth media can identify pathogens. These techniques likely lead to a broad understanding of the pathogen identity (i.e., bacterial genus, virus family). If genetic data are available for the pathogen group, PCR can be used to identify/confirm the pathogen identity. Using next-generation shotgun sequencing is a much faster and specific way to identify a pathogen requiring little *a priori* knowledge. Thus, sequencing patient samples with instruments like Oxford Nanopore's MinION or Illumina's MiSeq can identify pathogens, including those that are well-known, emerging, or completely novel, days earlier than conventional microbiological and sanger sequencing methods. For COVID-19, the pathogen genome was sequenced and compared to available sequences on public databases (19, 20). This revealed it was related to previously sequenced human pathogenic coronaviruses, such as the viruses that caused the 2003 SARS and 2012 MERS epidemics (21). However, it showed greater similarity to a coronavirus sequence obtained from bats (19). Interestingly, the spike protein, responsible for viral entry into cells, was more closely related to a separate published coronavirus sequence identified in pangolins (22). This paved the way for a more nuanced understanding of the evolutionary origins of the novel coronavirus responsible for the pandemic. Having a large amount of cataloged sequence data allowed for these conclusions to be made quickly, informing decision-making regarding mitigation efforts.

The number of microorganisms in sequence databases is staggering, and the more broadly researchers sample, the more prepared the scientific community will be to respond to novel emerging pathogens. Collaborative engagement programs and threat reduction networks have the ability to generate sequencing data from understudied regions, closing a knowledge gap in sequence databases. Leveraging molecular studies of related pathogens to understand a current epidemic or pandemic pathogen is predicated on knowing its genome sequence. The genome sequence of SARS-CoV-2 allowed the inference of host receptor usage.

Based on similarity to related coronaviruses, especially SARS-CoV-1, it was likely the virus used the angiotensin-converting enzyme 2 (ACE2) of human lung cells to gain entry (23, 24). This initial understanding, plus other homology-based insights, led to a list possible therapeutics (25), with current therapeutic options and promising candidates [reviewed in (26)].

With each additional country that participates in sequencing a pandemic pathogen, the power to identify loci contributing to adaptation to humans increases. Every transmission cluster that has sequences available is an independent evolution experiment. Variants emerging during a pandemic are of great concern because they can affect viral virulence, immunogenicity, and transmission, but also can impact diagnostic tests and vaccine effectiveness. For instance, 10,000 sequences deposited in GISAID (27) were used to assess that SARS-CoV-2 appears not to be responding to human T-cell immune pressure, but is responding to B-cell epitopes, indicating humoral immunity imparts a significant selective pressure on the virus (28). Genome sequences from all over the world being deposited in GISAID allowed Korber et al. (29) to show that the emergent variant leading to the amino acid change D614G of the coronavirus spike protein, which came to dominate SARS-CoV-2 sequences, is more transmissible. They went on to link clinical data to viral genome sequences and show that the increased transmissibility is likely due to increased viral shedding. Having many publicly available pathogen sequences can also help researchers predict the robustness of diagnostic tools to pathogen evolution (30). All of these research directions are critical for a real-time, robust response to an ongoing pandemic, and are greatly strengthened by sequence contributions across all geographic regions, including those engaged in cooperative biological engagement programs.

During a pandemic, one of the most vital contributions of collaborative sequencing efforts is to expand the number and geographic distribution of sequenced samples. Sequencing labs originally set up to address local outbreaks of endemic pathogens, can rapidly pivot to sequencing samples of a pandemic pathogen. For many countries, cross border movement of goods and workers is essential to the economy, individual livelihoods, and more importantly food distribution (31). Therefore, evidence-based decision making is critical. Identifying sources of new infections across country borders can be very difficult with traditional tracking and tracing methods, especially where infrastructure is limited. Thus, it is hard to know the efficacy of shutting down international travel for preventing pathogen spread. Phylogenetic analysis of sequences from an ongoing pandemic can identify if cases are from local transmission events or imported from other countries through human travel (32–34). These analyses are predicated on robust sequencing operations across regions including all countries with travel ties; the more sequencing across a region, the more everyone benefits. This makes collaborative efforts to stand up sequencing infrastructure an important component in decision making during an ongoing pandemic.

DISCUSSION

Emerging and re-emerging diseases will continue to spread to new areas and affect millions of people around the world. Even without including viruses with pandemic potential (e.g., coronaviruses and influenza viruses), zoonotic infectious diseases affect tens of millions of people per year and cause substantial economic and human health impacts (1, 4, 18, 35). These diseases often emerge with no warning, and as a result, health officials in charge of mitigation efforts begin at a disadvantage. In response to the threats of unforeseen pathogens, effective biosurveillance needs to be implemented. The goal of cooperative engagement programs is to increase the ability and capacity to detect and respond to infectious disease outbreaks, specifically those involving especially dangerous pathogens and pathogens of pandemic potential.

Cooperative engagement programs help to strengthen both biosurveillance and research capabilities to answer important questions regarding infectious diseases. An important component of these programs is building trust among institutions and countries by strengthening sequencing capabilities together. Sustaining the collaborations and capabilities through threat reduction research networks and collaborative research projects further builds trust among researchers, institutions, and countries. This enables sample and data sharing, which is critical throughout the duration of an epidemic or pandemic. We urge the continued effort of cooperative engagement programs to strengthen sequencing capabilities for biosurveillance and research regarding emerging and re-emerging diseases. We need global unity in the fight against emerging diseases that pose significant risk to global health security, and cooperative engagement programs are leading the way.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JF and AB conceived the idea of the manuscript. AB and EM wrote the first draft of the manuscript. JF and AR provided critical comments and edits. All authors contributed to manuscript revision, read, and approved the submitted version.

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Assessment of Quarantine Understanding and Adherence to Lockdown Measures During the COVID-19 Pandemic in Palestine: Community Experience and Evidence for Action

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Background: Containment of the coronavirus pandemic relied extensively on the combination of early implementation of quarantine and massive behavioral changes to ensure effectiveness. Decision-makers need to constantly monitor the outbreak situation and the impact of the measures implemented. Yet little is known about the factors influencing adherence and understanding of lockdown measures among the Palestinian community. This study aimed to assess the impact and factors affecting these early public health interventions.

Materials and Methods: A cross-sectional web-based questionnaire was distributed throughout social media (Facebook and Instagram). We used a snowball recruiting technique to target Palestinian adult citizens during the coronavirus pandemic quarantine between 6 and 16 April 2020, which corresponded to almost the middle interval of the strict massive lockdown measures in Palestine that lasted from 22 March to 5 May 2020. Multivariate logistic regression models were developed for the outcome variables (staying home adherence, in-home precautions adherence, and quarantine understanding).

Results: Our questionnaire was completed by 2,819 participants. The mean (range) age was 29.47 (18–71) years. Of them, 1,144 (40.6%), 1,261 (44.7%), and 1,283 (45.5%) reported low levels of staying home adherence, in-home precautions adherence, and quarantine understanding, respectively. Females, city residents, those with higher educational levels, and those informed by official government sources were associated significantly with higher levels of both staying home adherence and quarantine understanding. Adequate food supply was associated with a higher level of staying home adherence. Higher levels of in-home precautions adherence were noticed in the elderly and those with a high-risk group living at home. Higher monthly income was inversely associated with higher levels of in-home precautions adherence and lower levels of quarantine understanding ($P < 0.05$).

Conclusions: The socio-economic and financial status of the general population and coordination between the major information resources (official government), social media,

and the press were the major factors affecting the community in regard to quarantine adherence. For maximum effectiveness and commitment levels amongst the people to decrease the spread of infection, policymakers need to address all those factors. In addition, clear communication between policymakers and the population is essential for reassuring the people and minimizing their fears regarding the unknown future.

Keywords: COVID-19, lockdown, Palestine, pandemic, perceptions, quarantine, adherence

BACKGROUND

On 11 March 2020, the WHO declared COVID-19 to be a pandemic (1). As the treatment was mainly symptomatic and supportive, protection, and prevention of infection transmission were the best choices worldwide (2). Quarantine announcements were asserted simultaneously almost all over the world. In Palestine, quarantine took place on 22 March 2020 as the first cases of COVID-19 were confirmed (3).

According to the Center for Disease Control and Prevention (CDC), quarantine was adopted as an obligatory means to separate and restrict the movement of people who had potentially been exposed to a contagious disease. People also had to follow appropriate infection control measures which included bans on large social gatherings, school closures, the ban of weddings, parties, and funerals, closures of entertainment venues, various restrictions on restaurant dining areas and gyms, such as increasing the distance between tables and gym machines and improving ventilation to prevent the virus droplet transmission. Adding to this, travel restrictions and social distancing measures were introduced during quarantine (4–7). These measures were implemented to limit disease spread, morbidities, mortalities, and decrease the burden on the health care system, as witnessed before in history with cholera, plague, and influenza (8).

The utility of quarantine is undetermined, and whether or not overusing it can be of any benefit lacks any scientific basis. However, one thing is certain according to a rapid review on how to improve adherence with quarantine: quarantine does not work if people do not adhere to it (9, 10).

Previous surveys on factors that affect adherence to quarantine in outbreaks were reviewed. Multiple factors were studied to assess their effect on the adherence to quarantine and protective health behaviors such as hand washing, avoiding crowds, and maintaining social distance between individuals. Some of these factors were of direct influence and reflected higher adherence actions, such as knowledge about the infectious disease outbreak and quarantine protocol, the perceived benefits of quarantine, the grasped risks of the disease, and the social norms that pressured others to comply with the quarantine. Other factors were of alternative effects, such as where people got their knowledge of quarantine protocols from, with no difference in adherence rates between those who sourced information from official vs. non-official sources. In addition, practical issues such as financial consequences or employees in insecure jobs who lacked leave entitlement would result in individuals being less likely to comply with social distance measures (11). Trust in the government's public health interventions, pre-existing positive appraisal of the health care system, and trust in the national response predicts more adherence to the quarantine (8, 9, 12).

A study in Norway found that adherence to quarantine has been low, especially after the initial surge of infections faded nationwide, which suggests that people are influenced by the perceived infection risk or that the population experiences quarantine fatigue and a wish to return to normality (12).

Recent studies on the topic of the associated predictors with quarantine and health measure compliance showed that gender, age, geographic area, and employment status, as well as the person's fear for themselves and others to contract COVID-19, were significantly predictive (13).

There is very limited data that evaluates the possible predictors which could influence the general population's staying home adherence and the understanding of quarantine and lockdown measures during the COVID-19 pandemic in Palestine. This study is dedicated to providing a clear vision regarding the situation by expanding on the limited knowledge about the possible implicated factors in quarantine compliance. Overall, this could allow the decision-makers to constantly monitor and maintain the balance between the implementation of quarantine and public health measures.

MATERIALS AND METHODS

Study Population, Sample, and Setting

The target population comprised every Palestinian who lived in the West Bank, Gaza, or Jerusalem during coronavirus-2 quarantine and who was equal to or more than 18 years old. We adopted a cross-sectional web-based survey design to assess the public's adherence to quarantine and infection control instructions during the lockdown of coronavirus-2 pandemic by using an anonymous online questionnaire. Every person had a number that reflected their order by the time they finished the questionnaire. A snowball sampling technique was used and focused on recruiting any Palestinian who lived in Palestine during the pandemic. The online survey was disseminated on Facebook and Instagram to friends and local pages and they were encouraged to pass it on to others. A mandatory question was added on the first page of the questionnaire regarding current residency. Those who reported living outside Palestine were automatically excluded from the study. We were able to recruit 2,819 participants in this study who completely filled and returned the questionnaire, with an age range between 18 and 71 years old.

Questionnaire Development

After reviewing related factors that affect adherence to quarantine in outbreaks (9, 14, 15), we included additional questions related to the COVID-19 pandemic. The structured questionnaire consisted of questions that covered several areas: (1) informed consent, (2) demographic data, (3) knowledge and concerns about quarantine, and (4) compliance to precautionary measures against coronavirus inside and outside the home. The data collection tool was revised by two experts in the field. Then, a pilot study was performed on 56 volunteers of the author's Facebook friends and relatives and their friends (nine of them were older than 30) for feedback to identify ambiguities, questionnaire structure errors, difficult questions, and to record the time taken to complete the questionnaire. Then we took into consideration their notes and edited them as needed; after that, they reviewed the second version and accepted it.

Procedure and Ethical Consideration

As the Palestinian Government recommended the public to minimize face-to-face interaction and isolate themselves at home, the questionnaire was distributed electronically. Participants completed it in Arabic through an online survey. Expedited ethical approval was obtained from the Institutional Review Board (IRB) at An-Najah National University. Privacy was strictly protected during the procedure as we avoided any questions that could expose the identity of respondents. Information and the purpose of the study were posted on the first page of the questionnaire. All respondents provided electronic informed consent before starting the questionnaire. Data collection took place over 10 days (6–16 April 2020) which corresponded to almost the middle interval of the massive quarantine in Palestine where restriction measures were at their highest (22 March to 5 May 2020).

Statistical Analysis

Quarantine understanding outcome reflects the knowledge and information the person has about the pandemic and quarantine regardless of the source. It was initially evaluated through five statements: (1) quarantine is needed where I live, (2) not committing to quarantine will raise the number of cases, (3) measures taken by the government are necessary, (4) quarantine should not only be limited to infected people and those who are in contact with them, and (5) hygiene measures in the house are part of quarantine. A 5-point Likert scale [strongly agree (4), agree (3), neutral (2), disagree (1), and totally disagree (0)] was used to respond to each statement. By summing the points of each statement, a scale from 0 to 20 was created for each respondent. We then used the median as a cutoff point to categorize this outcome into a low level (0–17) and a high level (18–20).

Staying home adherence outcome reflects the compliance of the individual to the main instruction given by the government: "Do not leave the house if it is not necessary." It was initially evaluated through five statements: (1) going grocery shopping or to the bakery, (2) going out meeting friends or family, (3) going out to spend time and have fun, (4) attending social events, and (5) going to the pharmacy. The answer to each statement is composed of [never going out (3), some days (2), more than half of days (1), and every day (0)].

In-home precautions adherence outcome reflects the compliance to infection control measures while staying inside the home to decrease the spread of infection between family members. It was initially evaluated through five statements: (1) washing your hands for 20 seconds or more, (2) decrease the time of interaction with other family members, (3) washing hands after returning from outside, (4) sneezing appropriately according to guidelines (using a tissue or using elbow), and (5) not sharing towels and items between family members. The answer to each statement is composed of [never do them (0), do them sometimes (1), do them most of the time (2), and always do them (3)].

For these last two outcomes separately, we summed up the points of each statement. A scale from 0 to 15 was created for each respondent. Then the median was used as the cutoff point to categorize staying home adherence outcome to a low level (0–12) and a high level (13–15) while categorizing in-home precautions adherence outcome to a low level (0–10) and a high level (11–15).

The 27th version of IBM SPSS (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp) was used for data coding, entry, and analysis. All parts of the analysis were performed by the authors themselves. Descriptive statistics (median, mean, standard deviation, and independent student *t*-test) were calculated for continuous variables while frequencies/percentages and Chi-square test were used for categorical variables. $P < 0.05$ was always considered significant.

Statistically significant variables in bivariate analysis were included in the multivariate logistic regression model developed for each of the study outcomes.

RESULTS

Socio-Demographic Characteristics of the Study Population

In this study, the questionnaire was introduced to 2,819 individuals, all of whom completed and returned the questionnaire electronically (**Table 1**). The mean (range) age of respondents was 29.47 (18–71) years. We divided the population into three groups according to age: young (18–35), middle (36–53), and elderly (>53). 73.9% were young and only 4% of participants were elderly. More than two-thirds (72.6%) of respondents were female and nearly half (51.4%) were single. The majority live in the West Bank (83.5%). Most of the participants (78.4%) currently study in college or had graduated recently. Almost one-quarter (24.6%) were smokers. Only 11.8% were health care workers and 45.5% admitted that they had a high-risk group living with them currently.

It was found that 1,144 (40.6%), 1,261 (44.7%), and 1,283 (45.5%) of respondents had low levels of staying home adherence, in-home precautions adherence, and quarantine understanding, respectively.

Quarantine Characteristics of the Population

As shown in **Table 2** 98% of respondents believed that quarantine is important, and 2,173 (77.1%) expressed fear of getting COVID-19 or transmitting it to others. Only 14.9% of respondents

TABLE 1 | Bivariate analysis of socio-demographic characteristics with dependent variables (Staying home adherence; In-home precautions adherence; Quarantine understanding; *P*-value presented was Chi-square significance; *N* = 2,819).

Variables	*N* (%)	Staying home adherence		*P*-value	In-home precautions adherence		*P*-value	Quarantine understanding		*P*-value
Age		**Low level** ***n* = 1,144**	**High level** ***n* = 1,675**		**Low level** ***n* = 1,261**	**High level** ***n* = 1,558**		**Low level** ***n* = 1,283**	**High level** ***n* = 1,536**	
18–35	2,083(73.9)	825(39.6)	1,258(60.4)	0.106	979(47)	1,104(53)	<0.001*	897(43.1)	1,186(56.9)	<0.001*
36–53	624(22.1)	276(44.2)	348(55.8)		247(39.6)	377(60.4)		329(52.7)	295(47.3)	
>53	112(4)	43(38.4)	69(61.6)		35(31.3)	77(68.7)		57(50.9)	55(49.1)	
Sex										
Male	768(27.2)	468(60.9)	300(39.1)	<0.001*	377(49.1)	391(50.9)	0.04*	409(53.3)	359(46.7)	<0.001*
Female	2,051(72.8)	676(33)	1,375(67)		884(43.1)	1,167(56.9)		874(42.6)	1,177(57.4)	
Social status										
Single	1,449(51.4)	539(37.2)	910(62.8)	<0.001*	669(46.2)	780(53.8)	0.114	593(40.9)	856(59.1)	<0.001*
Relationship	1,370(48.6)	605(44.2)	765(55.8)		592(43.2)	778(56.8)		690(50.4)	680(49.6)	
Residency										
Village	1,380(49)	618(44.8)	762(55.2)	<0.001*	631(45.7)	749(54.3)	0.113	657(47.6)	723(52.4)	0.01*
City	1,292(45.8)	463(35.8)	829(64.2)		576(44.6)	716(55.4)		550(42.6)	742(57.4)	
Camp	147(5.2)	63(42.9)	84(57.1)		54(36.7)	93(63.3)		76(51.7)	71(48.3)	
Geographic area										
West bank	2,354(83.5)	969(41.6)	1,385(58.4)	0.03*	1,060(45)	1,294(55)	0.768	1,059(45)	1,295(55)	0.014*
Gaza strip	270(9.6)	118(43.7)	152(56.3)		116(43)	154(57)		144(53.3)	126(46.7)	
Jerusalem	195(6.9)	57(29.2)	138(71.8)		85(43.6)	110(56.4)		80(41)	115(59)	
Educational level										
Secondary or less	326(11.6)	166(50.9)	160(49.1)	<0.001*	151(46.3)	175(53.7)	0.068	207(63.5)	119(36.5)	<0.001*
Collage	2,211(78.4)	865(39.1)	1,346(60.9)		1,002(45.3)	1,209(54.7)		964(43.6)	1,247(56.4)	
Master or doctorate	282(10)	113(40.1)	169(59.9)		108(38.3)	174(61.7)		112(39.7)	170(60.3)	
Health care worker										
Yes	332(11.8)	131(39.5)	201(60.5)	0.657	139(41.9)	193(58.1)	0.264	141(42.5)	191(57.5)	0.236
No	2,487(88.2)	1,013(40.7)	1,474(59.3)		1,122(45.1)	1,365(54.9)		1,142(45.9)	1,345(54.1)	
Monthly income (Shekel)										
<2,000	568(20.1)	240(42.3)	328(57.7)	0.512	232(40.9)	336(59.)	0.032*	297(52.3)	271(47.7)	<0.001*
2,000–5,000	1,552(55.1)	631(40.7)	921(59.3)		692(44.6)	860(55.4)		706(45.5)	846(54.5)	
>5,000	699(24.8)	273(39.1)	426(60.9)		337(48.2)	362(51.8)		280(40.1)	419(59.9)	
Smoking/Shisha										
Yes	693(24.6)	350(50.5)	343(49.5)	<0.001*	328(47.3)	365(52.7)	0.113	363(52.4)	330(47.6)	<0.001*
No	2,126(75.4)	794(37.4)	1,332(62.6)		933(43.9)	1,193(56.1)		920(43.3)	1,266(56.7)	
High risk group in home										
Yes	1,283(45.5)	539(42)	744(58)	0.158	536(41.8)	747(58.2)	0.004*	705(55)	831(45)	0.653
No	1,536(54.5)	605(39.4)	931(60.6)		725(47.2)	811(52.8)		578(37.6)	705(62.4)	

**P-value is statistically significant.*

had jobs that required them to leave home during quarantine, and only 85 (3%) had at least one of their relatives infected with COVID-19. The two most common sources of information about quarantine and precautions were social media and television/radio (59.5% and 18.6%, respectively). Nearly 80.2% admitted that they were properly informed about the quarantine, and 29.3% documented inadequate food supplies to withstand the quarantine period.

However, most people (38.2%) used to spend between 6 and 10 h outside the home before the quarantine. Most respondents (94.1%) correctly identified that quarantine aimed to protect society. Only 52.6% understood that quarantine restrictions aimed to protect members of their household. Nearly 59.4% correctly reported that quarantine would not protect them.

Bivariate Analysis of the Study Main Outcomes

Staying home adherence outcome was found to have statistically significant associations with the following socio-demographic variables [(sex, social status, residency, geographic area, educational level, and smoking); $P < 0.05$, **Table 1**] and the following quarantine characteristic variables [(quarantine type, fear of getting COVID-19 or transmitting it, being properly

TABLE 2 | Bivariate analysis of quarantine characteristics with dependent variables (Staying home adherence; In-home precautions adherence; Quarantine understanding; *P*-value presented was Chi-square significance; *N* = 2,819).

Variables	*N* (%)	Staying home adherence			In-home precautions adherence			Quarantine understanding		
		Low level *n* = 1,144	High level *n* = 1,675	*P*-value	Low level *n* = 1,261	High level *n* = 1,558	*P*-value	Low level *n* = 1,283	High level *n* = 1,536	*P*-value
Do you think quarantine is important?										
Yes	2,763(98)	1,116(40.4)	1,647(59.6)	0.147	1,232(44.6)	1,531(55.4)	0.248	1,238(44.8)	1,525(55.2)	<0.001*
No	56(2)	28(50)	28(50)		29(51.8)	27(48.2)		45(80.4)	11(19.6)	
Type of quarantine										
Obliged to stay at home	2,398(85.1)	902(37.6)	1,496(62.4)	<0.001*	1,046(43.6)	1,334(56.4)	0.356	1,060(44.2)	1,338(55.8)	0.001*
I have to work outside home	421(14.9)	242(57.5)	179(42.5)		197(46.8)	224(35.2)		223(53)	198(47)	
Any of relatives or acquainted infected?										
Yes	85(3)	34(40)	51(60)	0.912	40(47.1)	45(52.9)	0.661	39(45.9)	46(54.1)	0.945
No	2,734(97)	1,110(40.6)	1,624(59.4)		1,221(44.7)	1,513(55.3)		1,244(45.5)	1,490(54.5)	
Afraid of getting COVID-19 or transmit it?										
Yes	2,173(77.1)	852(39.2)	1,321(60.8)	0.006*	950(43.7)	1,223(56.3)	0.047*	897(41.3)	1,276(58.7)	<0.001*
No	646(22.9)	292(45.2)	354(54.8)		311(48.1)	335(51.9)		386(59.8)	260(40.2)	
Properly informed about quarantine										
Yes	2,262(80.2)	884(39.1)	1,378(60.9)	0.001*	976(43.2)	1,286(56.8)	0.001*	984(43.5)	1,278(56.5)	<0.001*
No	557(19.8)	260(46.7)	279(53.3)		285(51.2)	272(48.8)		299(53.7)	258(46.3)	
Source of information										
Television or radio	525(18.6)	219(41.7)	306(58.3)	0.027*	221(42.1)	304(57.9)	<0.001*	259(49.3)	266(50.7)	<0.001*
Official government agencies	359(12.7)	134(37.3)	225(62.7)		120(33.4)	239(66.6)		132(36.8)	227(63.2)	
A health care worker	159(5.6)	67(42.1)	92(57.9)		58(36.5)	101(63.5)		63(39.6)	96(60.4)	
Social media	1,676(59.5)	669(39.9)	1,007(60.1)		806(48.1)	870(51.9)		770(45.9)	906(54.1)	
Conversation with other people	100(3.6)	55(55)	45(45)		56(56)	44(44)		59(59)	41(41)	
Enough food supply to withstand quarantine period?										
Yes	1,994(70.7)	750(37.6)	1,244(62.4)	<0.001*	876(43.9)	1,118(56.1)	0.184	855(42.9)	1,139(57.1)	<0.001*
No	825(29.3)	394(47.8)	431(52.2)		385(46.7)	440(53.3)		428(51.9)	397(48.1)	
Quarantine duration										
1–2 weeks	187(6.6)	98(52.4)	89(47.6)	<0.001*	86(46)	101(54)	0.103	102(54.5)	85(45.5)	0.023*
2–3 weeks	847(30.1)	355(41.9)	847(58.1)		357(42.2)	490(57.8)		396(46.8)	847(53.2)	
3–4 weeks	786(27.9)	331(42.1)	786(57.9)		378(48.1)	408(51.9)		357(45.4)	786(54.6)	
>4 weeks	999(35.4)	360(36)	639(67)		440(44)	559(56)		428(42.8)	571(57.2)	
Average hours out home before quarantine										
<2 h	584(20.7)	206(35.3)	378(64.7)	<0.001*	261(44.7)	323(55.3)	0.851	289(49.5)	295(50.5)	0.020*
2–6 h	776(27.5)	337(43.4)	439(56.6)		356(45.9)	776(54.1)		344(44.3)	432(55.7)	
6–10 h	1,075(38.2)	415(38.6)	660(61.4)		478(44.5)	1,075(55.5)		460(42.8)	615(57.2)	
>10 h	384(13.6)	186(48.4)	198(51.6)		166(43.2)	384(56.8)		190(49.5)	194(50.5)	

**P-value is statistically significant.*

informed about quarantine, source of information, having an adequate food supply, quarantine duration, and average hours outside the home before quarantine); $P < 0.05$, **Table 2**].

Regarding in-home precautions adherence outcome, statistically significant associations were found with the following socio-demographics [(age, sex, monthly income, and high-risk group in the home); $P < 0.05$, **Table 1**] and the following quarantine characteristics [(fear of getting COVID-19 or transmitting it, being properly informed about quarantine, and source of information); $P < 0.05$, **Table 2**].

On the other side, quarantine understanding outcome was found to be significantly associated with these socio-demographics [(age, sex, social status, residency, geographic area, educational level, monthly income, and smoking); $P < 0.05$, **Table 1**] and the following quarantine characteristic variables [(belief in the importance of quarantine, quarantine type, fear of getting COVID-19 or transmitting it, being properly informed about quarantine, source of information, having enough food supply, quarantine duration, and average hours outside the home before quarantine); $P < 0.05$, **Table 2**].

As shown in **Figure 1**, those with higher mean scores of the self-reported rating of adherence to quarantine were significantly more likely to have a high-level of staying home adherence compared to those who reported lower mean scores (mean ± SD = 9.11 ± 1.26 and 7.56 ± 2.15; $P < 0.001$; respectively).

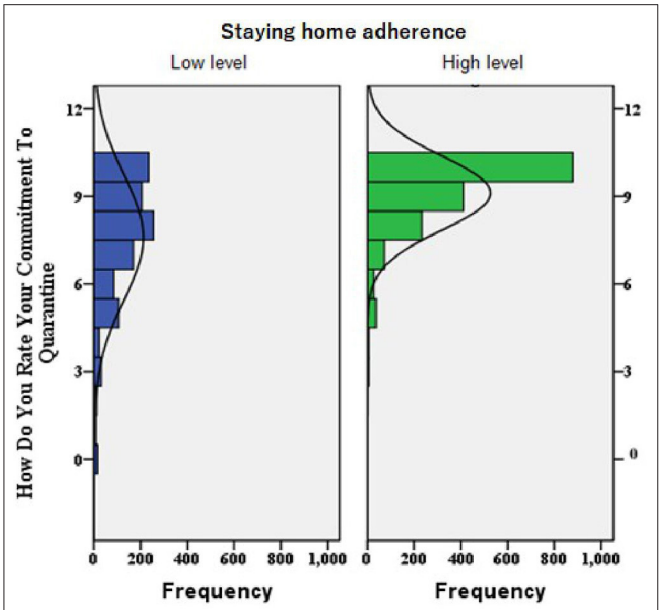

FIGURE 1 | A histogram chart built for the distribution of participants' self-rating of adherence to quarantine among the high and low levels of staying home adherence (*P*-value of *t*-test analysis for the mean difference between the two groups was <0.001; i.e., High-level group showed significantly higher Mean ± SD self-rating of adherence to quarantine compared to low-level, 9.11 ± 1.26 and 7.56 ± 2.15, respectively).

Multivariate Logistic Regression for Staying Home Adherence

The multivariate logistic regression model for staying home adherence outcome predictors is shown in **Table 3**. As shown, female sex, city residents, and higher educational levels (master and doctorate) were associated with a higher level of staying home adherence [ORs (95%CIs) = 2.72 (2.27–3.37); 1.37 (1.16–1.64); and 1.51 (1.06–2.16); respectively]. Furthermore, being informed through official government sources and having adequate food supply were more likely to result in a higher level of staying home adherence [ORs (95%CIs) = 1.38 (1.03–1.86) and 1.23 (1.03–1.47); respectively). Being obliged to stay at home was also a significant positive predictor of a higher level of staying home adherence. On the contrary, being in a relationship (engaged or married) was inversely related to staying home adherence [OR (95%CI) = 0.7 (0.59–0.83)]. Other variables did not remain significant after adjusting for other variables in the model.

Multivariate Logistic Regression for In-Home Precautions Adherence

In-home precautions adherence model shown in **Table 4**; older age groups (36–53 and 54–71 years) showed strong positive associations with a higher level of in-home precautions adherence compared to younger adults [OR (95%CI) = 1.37 (1.14–1.66) and 2.17 (1.42–3.30); respectively].

Furthermore, female sex, having a high-risk group in the home, and considering official government agencies as a source of information were significantly associated with a higher level of in-home precautions adherence [OR (95%CI) = 1.35 (1.14–1.61), 1.23 (1.06–1.43), and 1.58 (1.19–2.10); respectively]. Being properly informed about quarantine was also a significant positive predictor. On the other hand, higher monthly income (>5,000 Shekels) was inversely related to in-home precautions adherence [OR (95%CI) = 0.72 (0.57–0.90)].

Multivariate Logistic Regression for Quarantine Understanding

The multivariate logistic regression model for quarantine understanding outcome predictors is shown in **Table 5**. Female sex, city residents, and a higher educational level (master and doctorate) were associated with a higher level of quarantine understanding [ORs (95%CIs) = 1.29 (1.06–1.58); 1.21 (1.02–1.44); and 2.29 (1.60–3.27); respectively]. Furthermore, being informed through official government sources, being properly informed, and fear of catching COVID-19 were significant predictors of a higher level of quarantine understanding [ORs (95%CIs) = 1.64 (1.23–2.20); 1.32 (1.08–1.62); and 2.03 (1.68–2.45); respectively]. Moreover, higher monthly income (>5,000 shekels), being obligated to stay at home, and those who believe that quarantine is important were associated with a higher level of quarantine understanding. On the contrary, being in a relationship (engaged or married) and smokers (cigarette or shisha) were inversely related to quarantine understanding [OR (95%CI) = 0.71 (0.59–0.85) and 0.80 (0.66–0.97); respectively]. Other variables did not remain significant after adjusting for other variables in the model.

DISCUSSION

The present study aimed to assess staying home adherence, in-home precautions adherence, and quarantine understanding among Palestinian society during the COVID-19 pandemic lockdown.

Females, city residents, those with a higher level of education, those obliged to stay at home as a type of quarantine, and those considering official government agencies as a source of information were associated with a higher level of staying home adherence and quarantine understanding. This could be explained by the cultural background of Palestinian society where males usually spend more time working outside the home. In our study, 47% of females and 64.4% of males reported more than 6 h on average outside the home before the quarantine. Police forces are usually more distributed in city centers compared to villages, and cities are usually more crowded; therefore the risk of COVID-19 is higher. On one hand, higher educated-people understand the risk of transmission and infection more which could affect their understanding and adherence compared to less-educated individuals. On the other hand, higher-educated people usually have jobs that can be performed from the home through online applications, whereas less-educated people usually have craft jobs that require them to leave the home. In a study in Israel during the same pandemic, it was noted that the compliance rate to self-isolation was affected by loss of income, as the compliance rate dropped from 94 to 57% when income was not compensated through the government (16). A study

TABLE 3 | Multivariate logistic regression model for factors associated with staying home adherence ($N = 2,819$).

Explanatory variable	Beta coefficient	Standard error	AOR[!]	95% CI[~]	*P*-value
Sex					
Female	1.02	0.10	2.77	2.27–3.37	<0.001
Male*	–	–	–	–	–
Social status					
Relationship	−0.36	0.09	0.70	0.59–0.83	<0.001
Single*	–	–	–	–	–
Residency					
City	0.32	0.09	1.37	1.16–1.64	<0.001
Camp	0.27	0.19	1.31	0.90–1.92	0.162
Village*	–	–	–	–	–
Geographic area					
West bank	−0.34	0.17	0.71	0.51–1.0	0.053
Gaza	−0.63	0.22	0.54	0.35–0.82	0.004
Jerusalem*	–	–	–	–	–
Educational level					
Collage	0.24	0.13	1.27	0.98–1.64	0.070
Master or doctorate	0.41	0.18	1.51	1.06–2.16	0.023
Secondary or less*	–	–	–	–	–
Smoking/Shisha					
Yes	−0.12	0.10	0.89	0.73–1.09	0.246
No*	–	–	–	–	–
Type of quarantine					
I am obliged to stay at home	0.63	0.12	1.87	1.49–2.34	<0.001
My work requires that I stay outdoors*	–	–	–	–	–
Afraid of getting COVID-19 or transmit it?					
Yes	0.18	0.10	1.2	0.99–1.45	0.063
No*	–	–	–	–	–
Do you think that you have been properly informed about quarantine?					
Yes	0.18	0.10	1.19	0.98–1.46	0.087
No*	–	–	–	–	–
Source of information					
Official government agencies	0.32	0.15	1.38	1.03–1.86	0.031
A health care worker	0.02	0.20	0.02	0.70–1.51	0.903
Social media	0.09	0.11	1.09	0.89–1.35	0.405
Conversations with other people	−0.31	0.24	0.74	0.46–1.17	0.193
Television or radio*	–	–	–	–	–
Enough food supply to withstand quarantine period					
Yes	0.21	0.09	1.23	1.03–1.47	0.023
No*	–	–	–	–	–
Quarantine duration					
2–3 Weeks	0.23	0.17	1.26	0.90–1.77	0.186
3–4 Weeks	0.15	0.18	1.16	0.82–1.63	0.407
>4 Weeks	0.30	0.17	1.35	0.97–1.90	0.080
1–2 Weeks*	–	–	–	–	–
Average hours out of home before quarantine					
<2 h	0.27	0.15	1.32	0.99–1.76	0.063
2–6 h	−0.13	0.14	0.88	0.67–1.15	0.338
6–10 h	0.11	0.13	1.11	0.86–1.44	0.421
>10 h*	–	–	–	–	–

**Reference category. [~]CI, Confidence interval; [!]AOR, Adjusted odds ratio (AOR for high level as compared with low level). Enter method was used.*

TABLE 4 | Multivariate logistic regression model for factors associated with in-home precautions adherence ($N = 2,819$).

Explanatory variable	Beta coefficient	Standard error	AOR[!]	95% CI[~]	*P*-value
Sex					
Female	0.30	0.09	1.35	1.14–1.61	0.001
Male*	–	–	–	–	–
Age					
36–53	0.32	0.10	1.37	1.14–1.66	0.001
54–71	0.77	0.22	2.17	1.42–3.30	<0.001
18–35*	–	–	–	–	–
Monthly income (shekel)					
2,000–5,000	−0.19	0.10	0.83	0.68–1.01	0.066
>5,000	−0.33	0.12	0.72	0.57–0.90	0.005
<2,000*	–	–	–	–	–
High risk group in home					
Yes	0.21	0.08	1.23	1.06–1.43	0.008
No*	–	–	–	–	–
Afraid of getting COVID-19 or transmit it?					
Yes	0.14	0.09	1.15	0.96–1.38	0.120
No*	–	–	–	–	–
Do you think that you have been properly informed about quarantine?					
Yes	0.21	0.10	1.23	1.01–1.49	0.036
No*	–	–	–	–	–
Source of information					
Official government agencies	0.46	0.15	1.58	1.19–2.10	0.002
A health care worker	0.33	0.19	1.40	0.96–2.03	0.080
Social media	−0.19	0.10	0.83	0.68–1.02	0.070
Conversations with other people	−0.36	0.23	0.70	0.45–1.09	0.113
Television or radio*	–	–	–	–	–

**Reference category. ~CI, Confidence interval; [!]AOR, Adjusted odds ratio (AOR for high level as compared with low level). Enter method was used.*

during the H1N1 pandemic quarantine in Victoria found that people who used official sources of information only compared to those who used both official and unofficial sources showed no differences in the odds of compliance (Odds Ratio 1.00, 95% CI = 0.69–1.44) (17). Official sources of information are usually trusted and considered as a clear source that people commit to and understand more clearly. However, in Australia, a study during the H1N1 pandemic found no differences in adherence rates between those who took the information from official vs. nonofficial sources (9), whereas in a Canadian study, the source of information was found to be significantly associated with quarantine understanding (18).

Having an adequate food supply in the home was associated with a higher level of staying home adherence. It is reasonable that those who secure their food resources before the quarantine can avoid leaving the home easily in contrast to others who will be worried about protect their family from starving. However, monthly income (>5,000 Shekels), fear of getting COVID-19 or transmitting it, and being properly informed about quarantine were associated with a high-level of quarantine understanding. These again reinforce the importance of proper delivery of information to the public and the underlying fears from COVID-19 transmission and infection rate. An Australian study during the H1N1 pandemic reported that people who understand quarantine were more compliant with it compared to people who reported inadequacy of information (17). However, in the UK, a study during the COVID-19 pandemic (16) reported that functional fear rather than sociopolitical factors increased compliance rates, which highlights the effect of fear on public response. Those with higher monthly incomes might not have resisted the quarantine and they understood it more as they had enough currency and didn't worry about financial shortages. It is noteworthy that loss of income was found to be the most frequently cited problem in compliance with quarantine (19).

However, being in a relationship (engaged or married) was inversely related to a higher level of both staying home adherence and quarantine understanding. This may be in part due to more responsibilities toward household members to supply the home with what is needed during the quarantine. On the other side, anxiety and stress might play a role in this due to over-stress between family members during the quarantine. Therefore, going out could be an opportunity to relax and to avoid more stress. Smoking (cigarette or Shisha) was inversely associated with quarantine understanding. Cigarette/Shisha smokers usually seek meeting friends more than nonsmokers. They are usually stressed and might not be able to handle and understand quarantine intentionally due to their carelessness and under-estimation of the risk. Moreover, the effect of financial status on their ability of smoking due to job loss may make them more stressed.

The elderly and those with a high-risk group living with them were more likely to have higher in-home precautions adherence. It is worth mentioning that the elderly are usually considered a high-risk group if infected with COVID-19, and

TABLE 5 | Multivariate logistic regression model for factors associated with quarantine understanding ($N = 2,819$).

Explanatory variable	Beta coefficient	Standard error	AOR[!]	95% CI[~]	*P*-value
Sex					
Female	0.26	0.10	1.29	1.06–1.58	0.012
Male*	–	–	–	–	–
Age					
36–53	−0.30	0.11	0.74	0.60–0.93	0.008
54–71	−0.05	0.22	0.95	0.62–1.45	0.817
18–35*	–	–	–	–	–
Social status					
Relationship	−0.35	0.10	0.71	0.59–0.85	<0.001
Single*	–	–	–	–	–
Residency					
City	0.19	0.09	1.21	1.02–1.44	0.025
Camp	−0.00	0.19	0.10	0.69–1.45	0.984
Village*	–	–	–	–	–
Geographic area					
West bank	0.03	0.16	1.03	0.75–1.42	0.858
Gaza	−0.45	0.21	0.64	0.42–0.97	0.035
Jerusalem*	–	–	–	–	–
Educational level					
Collage	0.53	0.13	1.69	1.30–2.19	<0.001
Master or doctorate	0.83	0.18	2.29	1.60–3.27	<0.001
Secondary or less*	–	–	–	–	–
Monthly income (shekel)					
2,000–5,000	0.16	0.11	1.17	0.95–1.45	0.146
>5,000	0.27	0.13	1.31	1.01–1.69	0.041
<2,000*	–	–	–	–	–
Smoking/Shisha					
Yes	−0.23	0.10	0.80	0.66–0.97	0.025
No*	–	–	–	–	–
Do you think quarantine is important?					
Yes	1.28	0.36	3.61	1.79–7.25	<0.001
No*	–	–	–	–	–
Type of quarantine					
I am obliged to stay at home	0.28	0.11	1.33	1.06–1.66	0.012
My work requires that I stay outdoors*	–	–	–	–	–
Afraid of getting COVID-19 or transmit it?					
Yes	0.71	0.10	2.03	1.68–2.45	<0.001
No*	–	–	–	–	–
Do you think that you have been properly informed about quarantine?					
Yes	0.28	0.10	1.32	1.08–1.62	0.007
No*	–	–	–	–	–
Source of information					
Official government agencies	0.50	0.15	1.64	1.23–2.20	0.001
A health care worker	0.36	0.20	1.44	0.98–2.11	0.061
Social media	0.12	0.11	1.12	0.91–1.38	0.276
Conversations with other people	−0.30	0.24	0.74	0.46–1.17	0.200
Television or radio*	–	–	–	–	–
Enough food supply to withstand quarantine period					
Yes	0.09	0.09	1.10	0.92–1.32	0.309
No*	–	–	–	–	–

(Continued)

TABLE 5 | Continued

Explanatory variable	Beta coefficient	Standard error	AOR[I]	95% CI~	*P*-value
Quarantine duration					
2–3 Weeks	0.19	0.17	1.20	0.86–1.68	0.279
3–4 Weeks	0.20	0.17	1.22	0.87–1.71	0.240
>4 Weeks	0.23	0.17	1.26	0.91–1.76	0.169
1–2 Weeks*	–	–	–	–	–
Average hours out of home before quarantine					
<2 h	−0.04	0.14	0.96	0.73–1.28	0.785
2–6 h	0.10	0.14	1.10	0.84–1.44	0.488
6–10 h	0.11	0.13	1.12	0.87–1.44	0.378
>10 h*	–	–	–	–	–

**Reference category. ~CI, Confidence interval; [I]AOR, Adjusted odds ratio (AOR for high level as compared with low level). Enter method was used.*

by the time of the study, the only two deaths from COVID-19 in Palestine were two elderly patients with co-morbidities (20). In-home precautions adherence might be considered crucial to protect those with a high-risk group in the home. Conversely, during a mumps outbreak at an American University, isolation compliance didn't significantly differ by gender, age, location of residence, or employment status (21). Surprisingly, high monthly income (>5,000 Shekels) was inversely associated with in-home precautions adherence. This is in opposition to our expectations as those with a higher monthly income can usually afford to buy sanitizers and protective equipment. But it seems that the ability to buy differs from adherence. Their feelings of being able to be treated if infected might affect their adherence as they thought they have more currency for better affordable treatment. Furthermore, those people might have a higher nutritional status and were not afraid of COVID-19 infection and thought that they were strong enough not to catch the infection, mainly due to false information during COVID-19. A more likely explanation is that people with a monthly income of more than 5,000 Shekels have a healthier family, and are less likely to have a high-risk group in the home. In our study, 42.2% of people who had >5,000 Shekels as monthly income reported having a high-risk group in the home, while 57.8% of people with monthly income <5,000 Shekels reported having a high-risk group in the home.

It should be noted that only two factors (females and those who consider official government agencies as a source of information) were significantly associated with a higher level of the three study outcomes. Average hours spent outside of the home before quarantine and duration of quarantine did not affect any of the study outcomes. This is in accordance with other studies during the H1N1 pandemic in Australia (17). People might appreciate more factors and the severity of the disease and its transmission for adherence and quarantine understanding than the length of quarantine and the hours they usually spent outside of the home before the quarantine. Forcing the quarantine through the declaration of emergency bylaws might leave people to concentrate more on staying at home rather than the length of quarantine itself. As the study focused on factors affecting adherence to quarantine measures and one of the inclusion criteria was Palestinian individuals in Palestine under quarantine, the West Bank had the highest number of responders because both Gaza and Jerusalem were not under lockdown until the last few days of the study.

This study could have some limitations. Selection bias could have occurred due to the sampling technique. Due to social distancing during quarantine, we disseminated the survey on social media, and this might in part exclude people who do not have access to the internet and social media, and also limit access to children and the elderly. Any participant who was younger than 18-years-old was excluded. Furthermore, only 4% of the participants were older than 53 years old. However, according to Index Mundi, only 8% of the Palestinian population were older than 55 years old, and around 36% of the population were younger than 15 years in 2020 (22), We believe that the elderly use the internet less frequently than other age groups, and for this reason, although the elderly group had been represented to some extent in our sample (4%), our study could not represent all age groups. However, this was the only possible procedure to perform during the lockdown measures and it was useful in collecting the required information as fast and safely as possible. Systematic bias where over- or under-estimation of some measures due to self-reporting might also have been encountered. This study has several strengths, including a large sample size and the sampling timeframe that corresponded to the peak surge of COVID-19 cases in Palestine, which has had 613 cases and five deaths as per writing this paper (20). From an epidemiological point of view, our study might not represent the national level; however, taking into account the worldwide nature of the risk in this pandemic, we strongly believe that these data could provide useful information to be generalized to other countries and future pandemics.

CONCLUSIONS

It was seen that major effects depend mainly on the socio-economic and financial status of the general population and the coordination between the major information resources (official government), social media, and the press. Hence, addressing such factors could enable the country to achieve higher adherence rates that can effectively decrease the spread of infection. It is important for policymakers to reach out to the community by every possible means during the lockdown to prevent the spread of false news, enhance their understanding, and update them

with new measures. Policymakers' clear communication with the people is crucial for their reassurance, as such communication minimizes their fears of the unknown future. As financial status has a great role in the level of adherence, compensation of income loss and giving access to online jobs may decrease the burden of these lockdown measures on the population and ensure higher compliance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB of An-Najah National University. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

HA, NY, TA, and MH-Y designed study protocol and drafting the manuscript. HA coordinated the study protocol and conducted the statistical analysis. NY, TA, and MH-Y collected the data. All authors read and approved the final manuscript.

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REVIEW
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International Rickettsia Disease Surveillance: An Example of Cooperative Research to Increase Laboratory Capability and Capacity for Risk Assessment of Rickettsial Outbreaks Worldwide

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Cooperative research that addresses infectious disease surveillance and outbreak investigations relies heavily on availability and effective use of appropriate diagnostic tools, including serological and molecular assays, as exemplified by the current COVID-19 pandemic. In this paper, we stress the importance of using these assays to support collaborative epidemiological studies to assess risk of rickettsial disease outbreaks among international partner countries. Workforce development, mentorship, and training are important components in building laboratory capability and capacity to assess risk of and mitigate emerging disease outbreaks. International partnerships that fund cooperative research through mentoring and on-the-job training are successful examples for enhancing infectious disease surveillance. Cooperative research studies between the Naval Medical Research Center's Rickettsial Diseases Research Program (RDRP) and 17 institutes from nine countries among five continents were conducted to address the presence of and the risk for endemic rickettsial diseases. To establish serological and molecular assays in the collaborative institutes, initial training and continued material, and technical support were provided by RDRP. The laboratory methods used in the research studies to detect and identify the rickettsial infections included (1) group-specific IgM and IgG serological assays and (2) molecular assays. Twenty-six cooperative research projects performed between 2008 and 2020 enhanced the capability and capacity of 17 research institutes to estimate risk of rickettsial diseases. These international collaborative studies have led to the recognition and/or confirmation of rickettsial diseases within each of the partner countries. In addition, with the identification of specific pathogen and non-pathogen *Rickettsia* species, a more accurate risk assessment could be made in surveillance studies using environmental samples. The discoveries from these projects reinforced international cooperation benefiting not only the partner countries but also the scientific community at large through presentations (n = 40) at international scientific meetings and peer-reviewed publications (n = 18).

The cooperative research studies conducted in multiple international institutes led to the incorporation of new SOPs and trainings for laboratory procedures; biosafety, biosurety, and biosecurity methods; performance of rickettsia-specific assays; and the identification of known and unknown rickettsial agents through the introduction of new serologic and molecular assays that complemented traditional microbiology methods.

Keywords: rickettsioses, scrub typhus, cooperative international research, surveillance, orientia

INTRODUCTION

Rickettsial diseases are vector-borne diseases caused by agents of the genus *Rickettsia* (1, 2). However, the definition of rickettsial diseases can also be more inclusive to include diseases caused by agents that are genetically related to *Rickettsia*, such as *Orientia* species of the family Rickettsiaceae, and *Anaplasma, Ehrlichia*, and *Neorickettsia* species of the family Anaplasmataceae (3). Both families, Rickettsiaceae and Anaplasmataceae, are members of the order Rickettsiales within the class Alphaproteobacteria and phylum Proteobacteria. Lastly, there are some diseases such as Q fever and trench fever that are often associated with rickettsial diseases because the causative agents at one time were considered *Rickettsia* species (i.e., *Coxiella burnetii–Rickettsia burnetii* and *Bartonella quintana–Rickettsia quintana,* respectively) (3, 4). For the purposes of this report, rickettsial diseases will be limited to those diseases caused by *Rickettsia* and *Orientia* species.

Rickettsial diseases (and their causative agents) have been traditionally separated into three major groups based on their disease presentation, antigenicity, and vectors (**Table 1**). Those groups include the **typhus group** (epidemic typhus [*Rickettsia prowazekii*] and murine typhus [*Rickettsia typhi*]); **spotted fever group** (Rocky Mountain spotted fever (RMSF) [*Rickettsia rickettsii*], Mediterranean spotted fever (MSF) [*Rickettsia conorii*], African tick-bite fever [*Rickettsia africae*], flea-borne spotted fever [*Rickettsia felis*], Queensland tick typhus [*Rickettsia australis*], Japanese spotted fever [*Rickettsia japonica*], etc.), and **scrub typhus group** (scrub typhus [*Orientia tsutsugamushi, Candidatus* Orientia chuto, and *Candidatus* Orientia chiloensis]) (4–7) (**Table 1**). Genotyping of pathogenic and non-pathogenic rickettsial agents have led to over a dozen genogroups (8). These genogroups are not addressed herein.

Rickettsial diseases are military and public health concerns because they are distributed widely throughout the world (9–14). Though many rickettsial diseases are mild and self-limiting, there are several of them such as epidemic typhus, RMSF, scrub typhus, murine typhus, and MSF that can be quite severe and life threatening (6, 14). Such rickettsial agents have the potential for use as biological weapon (BW) agents (15). Since the early 2000s, the United States (US) Department of Defense (DoD) has funded and implemented a Biological Threat Reduction Program (BTRP) through the Defense Threat Reduction Agency (DTRA). DTRA BTRP funded multiple cooperative biological research (CBR) multi-year and Threat Agent Detection and Response Activity Project (TAP) single-year biosurveillance studies in countries throughout the world (16). Moreover, the Global Emerging Infections Surveillance (GEIS) Branch of the Armed Forces Health Surveillance Division, responsible for identifying military health relevant threats to inform force health protection decision making, has supported infectious disease surveillance globally (17, 18). These two agencies are major sources of funding supporting the development of and provision of rickettsial assays and methodology by the Rickettsial Diseases Research Program (RDRP) at the Naval Medical Research Center (NMRC). With this support, RDRP participates in international cooperative research and herein describes collaborations with nine countries resulting in the development/support of rickettsial disease research that provided partner countries with the capacity and capability to conduct rickettsial diseases surveillance that informed medical and scientific leaders as to the risk of rickettsial outbreaks in their area of responsibility.

The distribution of rickettsial diseases is varied throughout all continents except Antarctica (5–7). The specific knowledge of the presence, identity, prevalence, and distribution of the rickettsioses and their causative rickettsial agents are only partially known and varies significantly from country to country. This lack of knowledge is often directly tied to limited laboratory diagnostic capability and access to rickettsial assays and therefore places many countries and regions at risk of underestimating the impact and risk of rickettsial diseases, both sporadic occurrences and outbreaks (6). To overcome the shortfall of rickettsiology in underserved countries/regions, we have conducted cooperative research to determine the risk of various rickettsial diseases. Our team provided the rickettsial reagents and assays needed to initiate this work and increased local laboratory capability and capacity through general and specific laboratory training, access to and training on rickettsial assays and reagents, assistance with the evaluation of results, and drawing proper conclusions to be shared with local public health leaders and the international scientific community. The cooperative research among participating institutions has led to enhanced laboratory capability and reinforced knowledge on the presence, identity, distribution, and prevalence of rickettsial agents and diseases within their sphere of responsibility. This enhanced capability has led to partner country scientists' capacity to determine the risk of rickettsial diseases, identify outbreaks, publish results for general observation, and submission of grant applications to further rickettsial disease research (16, 19).

The goal of this paper is to describe the particular components utilized and outcomes obtained during the development of international cooperative rickettsial diseases research to determine the risk of rickettsial disease outbreaks in nine countries from 2008 to 2020: Azerbaijan, Chile, Georgia, India, Kazakhstan, Madagascar, Thailand, Ukraine, and Vietnam. The

TABLE 1 | Three major groups of rickettsial diseases exist based on their causative agents, host seroreactivity to group-specific antigens, arthropod vectors, and their distribution.

Diseases	Etiologic agents	Serologic reactivity to antigens from***	Vectors	Distribution
Typhus Group (TG)				
Epidemic Typhus	*Rickettsia prowazekii*	TGR	*Pediculus humanus corporis*—human body louse; *Glaucomys volans*—flying squirrel ectoparasites	Worldwide
Murine Typhus	*Rickettsial typhi*	TGR	*Xenopsylla cheopis* Oriental rat flea	Worldwide
**Spotted Fever Group (SFG)*, ** **				
Rocky Mountain spotted fever (RMSF)	*Rickettsia rickettsii*	SFGR	*Dermacentor variabilis* *Dermacentor andersoni* *Rhipicephalus sanguineus* *Amblyomma cajennense* *Amblyomma aureolatum*	Western Hemisphere
Mediterranean spotted fever (MSF)	*Rickettsia conorii* subsp. *conorii*	SFGR	*Rhipicehpalus sanguineus* Brown dog tick	Europe, northern Africa, western and southern Asia
Scalp eschar and neck lymphadenopathy after tick bite (SENLAT)	*Rickettsia slovaca* *Rickettsia raoultii* *Candidatus* Rickettsia rioja	SFGR	*Dermacentor marginatus* *Dermacentor reticulatus*	Europe and Central Asia
African tick-bite fever (ATBF)	*Rickettsia africae*	SFGR	*Amblyomma variegatum* *Amblyomma hebraeum*	Sub-Saharan Africa and Caribbean Islands
Japanese spotted fever (JSF)	*Rickettsia japonica*	SFGR	*Haemaphysalis longicornis, Haemaphysalis flava, Dermancentor taiwanensis, Ixodes ovatus*	Japan and Asia
Queensland tick typhus (QTT)	*Rickettsia australis*	SFGR	*Ixodes holocyclus, Ixodes tasmania*	Australia
Flea-borne spotted fever (FBSF)	*Rickettsia felis*	SFGR	*Ctenocephalides felis* Cat flea	Worldwide
Rickettsialpox	*Rickettsia akari*	SFGR	*Liponyssoides sanguineus* House mouse mite	Eastern Europe and Northeastern USA
Scrub Typhus Group (STG)				
Scrub typhus	*Orientia tsutsugamushi*	STGO	*Leptrombidum* species Trombiculid mites	Asia, Australia, and Islands of Indian and Pacific Oceans
Scrub typhus	*Candidatus* Orientia chuto	STGO	*Microtrombicula natalensis* Trombiculid mites	United Arab Emirates, Africa
Scrub typhus	*Candidatus* Orientia chiloensis	STGO	*Herpetacarus* species Trombiculid mites	Chile

*Other SFG diseases and causative agent(s) include Israeli spotted fever (R. conorii subsp. israelensis), Astrakhan spotted fever (R. conorii subsp. caspia), Indian tick typhus (R. conorii subsp. Indica), Tidewater spotted fever (R. parkeri), Siberian tick typhus (R. sibirica), Lymphangitis-associated rickettsiosis (R. sibirica mongolitimonae), Aneruptive fever (R. helvetica), Far eastern tick-borne rickettsiosis (R. heilongjiangensis), and Flinders Island spotted fever (R. honei).

**Potential SFG rickettsial pathogens include R. monacensis, R. aeschlimannii, R. massiliae, and R. asembonensis.

***TGR, typhus group rickettsiae; SFGR, spotted fever group rickettsiae; STGO, scrub typhus group orientiae.

narrative is divided into specific areas that address the (1) development of collaborations; (2) general description of 17 research institutes from nine countries; (3) overall goals of the research projects for each institute or combination of institutions; (4) serologic and molecular assays utilized to assess the presence, identity, distribution, and prevalence of rickettsial agents; (5) risk assessments made for rickettsial diseases; (6) training provided; and (7) results obtained by partner countries and the important knowledge gain from the studies making great contribution to rickettsiology, and enhancement of capacity and capability of the institutes. Lastly, there is a discussion of the importance of continuing these collaborations, especially in regard to the results obtained. The overall benefit of international collaborations is to improve partner countries assessment of infectious diseases by enhancing their ability to accurately assess the risk of endemic diseases and the potential of outbreaks of infectious diseases such as the recent COVID-19 pandemic.

DEVELOPMENT OF COLLABORATIONS

Discussions with potential collaborators were initiated in-person at conferences or correspondence by email, teleconference,

through colleague referrals, and funding agencies' annual meetings. The Research Topics addressed included specific rickettsial disease research ($n = 15$ projects) as well as rickettsial disease research included in febrile disease projects ($n = 2$) and arthropod-borne and/or zoonotic disease research projects ($n = 9$). The proposals for these projects were initiated by collaborators with mutual interests but were often augmented by additional collaborators and institutions to broaden the scope. The final proposals were subsequently submitted to institutions for approval prior to submission to funding agencies. The institutes received approval for research grants from one or more funding organizations. The projects that were performed and their funding sources are shown in **Table 2**. The funding organizations included DTRA ($n = 16$), GEIS ($n = 9$), Nacional de Desarrollo Cientifico y Technologico (FONDECYT) ($n = 7$), National Foundation for Science and Technology Development of Vietnam (NAFOSTED) ($n = 3$), Indian Council for Medical Research (ICMR) ($n = 3$), Institut Pasteur de Madagascar (IPM) ($n = 1$), and Khon Kaen University (KKU) ($n = 1$).

RESEARCH INSTITUTES BY COUNTRY

Seventeen research institutes from nine countries collaborated with the Rickettsial Diseases Research Program (RDRP) of the Naval Medical Research Center (NMRC), Silver Spring, Maryland, USA, including (1) **Azerbaijan**: Republican Antiplague Station, Baku; Republican Hygiene and Epidemiology Center, Baku; and Ministry of Defense (MoD) Laboratory, Baku; (2) **Chile**: School of Medicine, Pontifi via Universidad Católica de Chile, Santiago; Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia; Clínica Alemana de Santiago, Facultad de Medicina Clínica Alemana, Universidad del Desarrollo, Santiago; (3) **Georgia**: the National Center for Disease Control and Public Health (NCDC); and Laboratory of the Ministry of Agriculture, Tbilisi, Georgia; (4) **India**: Northeast Regional Medical Research Centre (NRMR), Dibrugarh, India; (5) **Kazakhstan**: M. Aikimbayev's National Scientific Center for Especially Dangerous Infections (NSCEDI), formerly named Kazakh Scientific Center of Quarantine and Zoonotic Diseases (KSCQZD) and the Scientific Practical Center for Sanitary Epidemiological Expertise and Monitoring (SPC-SEEM), Almaty, and Uralsk Anti-Plague Station (UAPS), Uralsk; (6) **Madagascar**: Institut Pasteur de Madagascar (IPM), Antananarivo; (7) **Thailand**: Khon Kaen University (KKU), Khon Kaen, Thailand; (8) **Ukraine**: Lviv Scientific Research Institute of Epidemiology and Hygiene (LSRIEH), Lviv; and 9) **Vietnam**: Hanoi Medical University (HMU) and the National Hospital for Tropical Diseases (NHTD), Hanoi, Vietnam.

OVERALL GOALS OF THE RESEARCH PROJECTS FOR EACH INSTITUTE OR COMBINATION OF INSTITUTIONS

The research goals of a single institute was often to investigate a newly described or recently rediscovered rickettsial disease(s) and/or agent(s) in a particular region in these countries: (1) **India:** "Identify previously recognized scrub typhus as well as determine the presence of other rickettsial diseases and/or agents in Northeast India" by RMRC, Dibrugarh, India; (2) **Madagascar:** "Identify flea-borne rickettsial agents near the capital city" by Institut Pasteur, Antananarivo, Madagascar; (3) **Thailand:** "Assess the role of cats and cat fleas in presence of spotted fever group rickettsiae in Northeast Thailand" by Khon Kaen University, Khon Kaen, Thailand; and (4) **Ukraine:** "Ascertain whether typhus group rickettsiae are still present and whether spotted fever group rickettsiae are present by assessing the seroprevalence of the agents infecting humans residing in Lviv Oblast," by the LSRIEH, Lviv, Ukraine.

Unlike the above single-institute studies, many of the projects discussed below involved multiple institutes within a country, because investigating the presence and distribution of rickettsial diseases and/or agents was often a similar goal of multiple institutes due to the collaborative nature of the projects performed within the countries (e.g., Azerbaijan, Chile, Georgia, Kazakhstan, and Vietnam). These institutes worked together or independently on the following research goals within the countries: (1) **Azerbaijan:** Initially, there were three goals to assess the risk of rickettsial diseases in Azerbaijan, which involved multiple institutes: (a) "Determine presence of TGR and SFGR infections by seroprevalence study of rural populations in 3 regions of Azerbaijan"; (b) "Determine incidence and prevalence of rickettsial infections among a cohort of military individuals"; and (c) "Ascertain whether arthropods from rodents contained rickettsial agents." These studies conducted independently were conducted by the Republican Antiplague Station, Baku; Republican Hygiene and Epidemiology Center, Baku; and Ministry of Defense (MoD) Laboratory, Baku, Azerbaijan. (2) **Chile:** Scrub typhus for hundreds of years was thought to be only found in the Asia–Australia region. So, when an individual presented to a clinic in 2006 in Chile with signs and symptoms of rickettsial disease, it was quite unexpected and even more unexpected that it was subsequently determined to be scrub typhus. This led to clinicians and researchers searching for further evidence of scrub typhus in Chile. The clinical and scientific investigations utilize various expertise of clinicians, scientists, and institutions. Thus, the overall goal in this country was to determine the clinical presentation, distribution, prevalence, incidence, vectors, reservoirs, and genetic characteristics of the disease and its agents. Thus, the multiple institutions worked well together on various aspects of the overall goal. The institutes included the following: School of Medicine, Pontifi via Universidad Católica de Chile, Santiago; Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia; Clínica Alemana de Santiago, Facultad de Medicina Clínica Alemana, Universidad del Desarrollo, Santiago, Chile. (3) **Georgia:** For the country of Georgia, a current assessment of rickettsial diseases/agents was needed as only limited knowledge of rickettsial diseases existed. Thus, the goal was to ascertain the presence of rickettsial infections and rickettsial agents in Georgia by (a) determining the role of rickettsial agents among febrile patients and (b) assessing ticks for the presence of rickettsial agents and specifically identifying them with new molecular assays. The institutes involved in these studies included the

TABLE 2 | Collaborative rickettsial research projects by country.

Host countries and institutes	Project nomenclature*	Projects
Azerbaijan - Republican Antiplague Station, Baku; - Ministry of Defense, Head Medical Office, Baku; - Republican Hygiene and Epidemiology Center, Baku	DTRA AJ-TAP-2	A seroprevalence study of prior exposure to select arthropod-borne and zoonotic infections among rural populations in three regions of Azerbaijan.
	DTRA AJ-TAP-4	A prospective cohort study of the incidence and prevalence of select arthropod-borne and zoonotic infections among Azerbaijani military personnel.
	DTRA-RDRP	Analysis of tick samples from Georgia and Azerbaijan.
Chile - School of Medicine, Pontificia Universidad Católica de Chile, Santiago; - Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia; - Clínica Alemana de Santiago Facultad de Medicina Clínica Alemana, Universidad del Desarrollo, Santiago	FONDECYT, OSU, and GEIS-RDRP	Potential scrub typhus case in Chile.
	FONDECYT and GEIS-RDRP	Assessment of domestic dogs for evidence of rickettsial infection. Case report of Korean traveler with scrub typhus. Distribution of scrub typhus cases in Chile. Identify potential scrub typhus vectors. Genetically characterize orientiae from scrub typhus cases. Conduct serosurvey of rickettsial disease in Chile.
Georgia - National Center for Diseases Control and Prevention, Tbilisi; - Laboratory of the Ministry of Agriculture, Tbilisi	GEIS-RDRP and DTRA:GG-21	Human disease epidemiology and surveillance of especially dangerous pathogens in Georgia.
	DTRA: GG-TAP-4 and DTRA: GG-TAP-12	Prevalence of *Rickettsia*, *Ehrlichia,* and *Borrelia* species pathogens in ticks from Georgia. Analysis of previously identified *Rickettsia*-positive Georgia ticks by multi-locus sequence typing.
India - Regional Medical Research Centre, Dibrugarh	DTRA-RDRP and ICMR	Assess individuals from Northeast India for seroprevalence of rickettsioses. Determine genetic characterization of *Orientia tsutsugamushi* in NE India Determine the risk of spotted fever in Northeast India. Determine the seroprevalence of typhus group rickettsia in Northeast India.
Kazakhstan - Uralsk Anti-plague Station, Uralsk; - Scientific Center of Quarantine and Zoonotic Diseases, Almaty - Scientific and Practical Center of Sanitary and Epidemiological Expertise and Monitoring, Almaty	DTRA: KZ-TAP-2	Species identification of tick vectors associated with infectious disease in Kazakhstan.
	DTRA: KZ-29	The epidemiology of Crimean-Congo hemorrhagic fever, hantavirus (hemorrhagic fever with renal syndrome), and tick-borne viral and rickettsial diseases in the Republic of Kazakhstan.
	DTRA: CAP-1 and DTRA: KZ-31	Flea-borne disease surveillance and Effect of *Rickettsia* spp. upon fitness of *Yersinia pestis* in fleas that vector plague in the Republic of Kazakhstan.
Madagascar - Institut Pasteur de Madagascar, Antananarivo	IPM and GEIS-RDRP	Flea-borne rickettsial diseases in Madagascar.
Thailand - Khon Kaen University, Khon Kaen	GEIS-RDRP and KKU	Role of companion cats and cat fleas play in rickettsial diseases in Northeast Thailand.
Ukraine - Lviv Scientific Research Institute of Epidemiology and Hygiene, Lviv	DTRA PDG for UP-1	Evaluation of arthropod-borne infections in Ukraine.
Vietnam - Hanoi Medical University, Hanoi - National Hospital for Tropical Diseases, Hanoi	NAFOSTED	The presence and prevalence of rickettsial infections among humans in northern Vietnam.
	DTRA-RDRP and NAFOSTED	Characterize clinical manifestations of rickettsial diseases and determine the applicability of molecular assays in rickettsial diagnosis.
	DTRA-RDRP and NAFOSTED	Determine the genetic makeup of *O. tsutsugamushi* causing scrub typhus in Vietnam.

**Project Nomenclature: RDRP, Rickettsial Diseases Research Program of the Naval Medical Research Center; DTRA, Defense Threat Reduction Agency; TADR, Threat Agent Detection and Response of DTRA; TAP, TADR Activity Project of DTRA; GEIS, Global Emerging Infections Surveillance and Response Research Support; DTRA-RDPR, Support in conducting rickettsial diseases research in CBR and TADR projects, and providing reagents and training; GEIS-RDRP, Development, production, and supply of assays/reagents to support rickettsial disease surveillance by GEIS partners worldwide; OSU, Ohio State University; FONDECYT, Nacional de Desarrollo Científico y Tecnológico; ICMR, Indian Council for Medical Research; IPM, Institut Pasteur de Madagascar; KKU, Khon Kaen University; NAFOSTED, National Foundation for Science and Technology Development of Vietnam.*

National Center for Disease Control and Public Health (NCDC); and Laboratory of the Ministry of Agriculture, Tbilisi, Georgia. (4) **Kazakhstan:** Tick-borne (Crimean-Congo hemorrhagic fever; tick-borne encephalitis) and flea-borne (plague) diseases are endemic to Kazakhstan. However, there was only limited knowledge of the presence of rickettsial diseases in Kazakhstan. Thus, the overall goal of the rickettsial disease studies was to augment the minimal knowledge of the presence of rickettsiae in Kazakhstan. Thus, various studies of rickettsial disease agents were investigated, especially those associated with ticks and fleas, including (a) "Identify Tick-borne Rickettsial Agents in Kazakhstan" and (b) "Determine the Presence and Distribution

of Flea-borne Rickettsiae in Kazakhstan" conducted by NSCEDI and SPC-SEEM Almaty, and UAPS Uralsk, Kazakhstan; and (5) **Vietnam:** Similar to other countries, Vietnam had historical evidence of rickettsial diseases; however, for several decades, there was limited investigation into the presence, prevalence, and distribution of rickettsial diseases and agents. Thus, the overall goal for Vietnam in the past decade has been to rectify the deficiency by conducting seroprevalence, clinical, and environmental studies in various number of locations. Three studies addressed the limited knowledge of rickettsial infections in northern Vietnam: (a) "The presence and prevalence of rickettsial infections among humans in northern Vietnam"; (b) "Characterization of the clinical manifestations of rickettsial diseases and determine the applicability of molecular assays in rickettsial diagnosis"; and (c) "Ascertain the genetic makeup of *Orientia tsutsugamushi* causing scrub typhus in northern Vietnam." The institutes involved in these studies included the National Hospital for Tropical Diseases and the Hanoi Medical University, Hanoi, Vietnam.

LABORATORY ASSAYS

Serological Assays

Commercially available and NMRC's serological assays that they developed in-house and not for commercial use were utilized. NMRC's serological assays with standard operating procedures (SOPs) included typhus group rickettsiae (TGR)-specific enzyme-linked immunosorbent assay (ELISA)-immunoglobulin gamma (IgG), spotted fever group rickettsiae (SFGR)-specific ELISA-IgG, and scrub typhus group orientiae-specific (STGO) ELISA-IgG (13, 20, 21). Positive controls ($n = 1$) and negative controls ($n = 3$) for each assay were provided to confirm that the assays were performing correctly (4). Subsequently, the institutions identified positive and negative control sera from their studies to use in these assays.

Commercial serological assays with instructions for ELISA and indirect immune fluorescence assay (IFA) were used according to manufacturers' instructions. These assays included InBios Scrub Typhus IgM ELISA (InBios International Inc., Seattle, WA) and Scrub Typhus IFA-IgG (Fuller Laboratories, Fullerton, CA).

Molecular Assays

PCR

Four types of polymerase chain reaction (PCR) were used: (1) standard PCR (sPCR), (2) nested PCR (nPCR) or hemi-nested PCR (hnPCR), and (3) quantitative real-time PCR (qPCR) assays.

qPCR

The qPCR assays (genus-, group-, or species-specific) were either developed at NMRC or found in peer-reviewed publications, and were used to screen for and identify rickettsial and oriental agents (4). The primers, probes, and controls [positive controls included either plasmids containing the target gene fragment(s) or linear target gene fragment(s), and molecular-grade water served as the negative controls] were used as described (4). Either reagents for the qPCR assays were supplied; their product numbers were provided; the primers, probes, and linear positive control oligonucleotide sequences were provided; or a combination of reagents, oligonucleotide sequences, and product numbers were provided. With the provided information for the reagents, the institutes could subsequently obtain the reagents independently of NMRC.

sPCR/nPCR

The sPCR and nPCR/hnPCR were used to produce amplicons for specific gene fragment sequencing either for a single gene or multiple genes in multilocus sequence typing (MLST) to identify and characterize rickettsial agents (4). Multiple gene fragment sequences were used as described in the MLST scheme initially described by Fournier et al. (22) to identify known *Rickettsia* species, incompletely characterized *Candidatus* Rickettsia species, and not previously described rickettsial agents (4). The genes most commonly used in the described studies for the identification of rickettsiae included 17-kDa antigen gene, *rrs*, *gltA*, *ompB*, *ompA*, and *sca4* (4). Identification of *Orientia* species by MLST utilized the following genes: *rrs*; 47-kDa antigen high-temperature requirement A protease gene (*htrA*); and the 56-kDa type-specific antigen gene (*tsa56*) (23).

Sequencing and Phylogenetic Analysis

The products from sPCR/nPCR for a single gene or for multiple genes to conduct MLST were sequenced in-house or using commercial companies as previously described (24). The sequencing data were assembled by Lasergene version 15.0 software (DNASTAR, Inc. Madison, WI, USA) or similar software, and sequences were compared with sequences available in GenBank (NCBI) using the BLAST search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Gene sequences, excluding the primer regions, were aligned by the ClustalW and phylogenetic analysis performed using MEGA X software (or similar software). The phylogenetic trees were constructed based on the alignment of the various gene fragment sequences (described above) obtained using the maximum likelihood method and Tamura-Nei model (25), and bootstrap analysis (1,000 reiterations) was carried out according to the Kimura 2-parameter method. All positions containing alignment gaps and missing data were eliminated.

RISK ASSESSMENTS

Assessing the risk of endemic rickettsial diseases and the potential for outbreaks requires measuring epidemiological metrics such as determining the presence, spatial and temporal distribution, prevalence for individual samples or minimal infection rate for pooled samples, and incidence of rickettsial infections and their causative agents. The evidence of rickettsial infections (e.g., antibodies against group-specific rickettsial antigens and/or detection of rickettsial agents in clinical samples) clearly indicates the presence of rickettsial pathogens in a location/region/country. Subsequent studies to determine their prevalence, incidence, and distribution are required to better localize the risk of rickettsioses. Molecular studies utilizing assays such as qPCR provide the specificity required to identify

Rickettsia pathogens; however, if only genus- or group-specific assays are used, non-pathogens within the genus or group may be detected and confused with pathogens. Therefore, more species-specific assays are needed in accurately determining rickettsial disease risk assessments.

TRAINING

NMRC staff scientists trained and mentored partner country scientists involved in the rickettsial diseases research projects prior to and during performance of laboratory work (**Table 3**). Training provided was project driven and included the following subjects: (1) conducting BSL-2 general laboratory procedures as they pertained to rickettsial studies; and (2) developing, updating, and/or augmenting SOPs to include rickettsial specific assays and general procedures for sequencing and MLST. New assays/procedures utilized in the laboratories were conducted with appropriate controls and standards to evaluate performance. Once partner country scientists were confident with the new procedures, they used the assays to assess environmental and/or clinical samples. Performance of laboratory assays was routinely assessed by comparing results of controls with the same controls performed at NMRC or described by manufacturers. The commercial and in-house assays and their sources that were utilized by the various laboratories are shown by country in **Table 4**.

SPECIFIC FINDINGS BY COUNTRY

The results of the various rickettsial disease research projects ($n = 26$) were completed at 17 institutions from nine countries and are described in detail below by country. The findings are summarized and appropriate references provided in **Table 2** and **Supplementary Table 1**.

Azerbaijan

Serological data showed the prevalence of IgG against SFGR (3.7–15.9%) and TGR (0–0.6%) among individuals in Azerbaijan and indicated a low to moderate exposure to SFGR and a very low risk of TGR infections (**Supplementary Table 1**). Antibodies against SFGR are lifelong and therefore the point prevalence studies do not allow for a strong evaluation of outbreak risk. Additional serosurveys throughout Azerbaijan need to be performed, as well as tick and flea field studies in locations positive and negative for evidence of rickettsia agents following results of serosurveys. Moreover, clinical studies should be carried out to assess incidence over time as well as during outbreaks to determine the risk levels of endemic rickettsioses spatially and temporally. Molecular evidence of *R. felis* group was found among a small number of ixodid ticks collected from rodents in the Lankaran district located in the southeast of Azerbaijan near the border with Iran (**Supplementary Table 1**). However, the total number of arthropods collected was low, and the assay used to identify *R. felis* in this study has subsequently been found to have low specificity, both of which decrease our ability to determine risk of human infection with *R. felis* group of

TABLE 3 | Training utilized by research institutes to identify rickettsiae and rickettsial infections.

Training	Development of a protocol(s) to assess risk or rickettsial diseases utilizing rickettsial laboratory procedures utilizing pre-existing and/or collection of environmental and/or clinical samples	Rickettsiology and introduction to laboratory assays for obtaining evidence of rickettsial diseases and agents	SOPs: review/updated for general procedures of BSL-2 laboratories	Laboratory assays: general, serological, and /or molecular	Use of commercial and non-commercial serologic assays to detect evidence of previous rickettsial infection—group-specific	Use of commercial and non-commercial molecular assays to detect and identify rickettsiae	Determine identity of rickettsiae by sPCR, nPCR, and MLST using GFS and phylogeny
Azerbaijan	AJ-TAP-2; AJ-TAP-4	Yes	BSL-2	Yes, yes, yes	Non-commercial ELISA TGR and SFGR	Non-commercial Rick17b and RfelG qPCR assays	n/a
Chile	FONDECYT-need title and GEIS-RDRP	Yes	BSL-2	Not needed	Commercial and non-commercial: TGR, SFGR, STGO	Non-commercial: Otsu47; Orien16S	Yes for *rrs*, *htrA*, and *tsa56*
Georgia	GG-TAP-4; GG- TAP-12; GG-21	Yes	BSL-2	Not needed	Non-commercial ELISA TGR, SFGR and STGO	Non-commercial: Rick17b, Raesch, Rraoul, Rslov, Rmon, Rconor, and Rmass9666 qPCR assays	Yes for *gltA*, *ompA*, *ompB*, and *sca4*
India	ICMR-need title and DTRA-RDRP	Yes	BSL-2	Yes, yes, no	Commercial and non-commercial STGO, TGR and SFGR ELISAs	n/a	n/a
Kazakhstan	KZ-29; KZ-31; KZ-TAP-2; KZ-CAP-1	Yes	BSL-2	Yes, yes, yes	Non-commercial ELISAs for TGR and SFGR	Non-commercial Rick17, RfelB, Rasemb	n/a
Madagascar	IP-need title and GEIS-RDRP	Yes		Yes, yes, yes	Non-commercial STGO, TGR and SFGR ELISAs	Non-commercial Rtyph and RfelB	n/a
Thailand	KKU-need title; GEIS-RDRP	Yes		Yes, yes, yes	Non-commercial ELISAs for SFGR	Non-commercial Rick17b.	n/a
Ukraine	UP1-UDP	Yes	BSL-2	Yes, yes, no	Non-commercial ELISAs for TGR and SFGR	n/a	n/a
Vietnam	DTRA-RDRP and NAFOSTED	Yes	BSL-2	Yes for new ELISAs and qPCR assays	Commercial and non-commercial: TGR, SFGR, STGO	Non-commercial: Rick17b, Rtyph and Otsu47 qPCR assays	Yes for *tsa56*

TABLE 4 | Serological and molecular assays utilized in rickettsial investigations by country.

Countries	Source of assays*	Serologic evidence of rickettsial infections (group-specific assays) Molecular evidence of rickettsiae (genus-, group-, and/or species-specific assays)		
		Type of assays**	Specific assays***	Host samples****
Azerbaijan	NMRC	ELISAs	TGR- and SFGR-ELISAs-IgG	Human sera
		qPCR	Rick17, Trick, RfelG, Raesch, Rraoul, Rslov	Tick
Chile	NMRC	ELISAs	STGO	Human serum
	OSU	Sequencing	*rrs*	Human eschar
	NMRC	ELISA	STGO	Dog sera
	InBios Fuller Laboratories	ELISA-IgM IFA-IgG	*O. tsutsugamushi* antigens	Human serum
	NMRC	qPCR	Otsu47	Human eschar
		MLST	*rrs, htrA*, and *tsa56*	Human eschar
	InBios Fuller Laboratories	ELISA-IgM and IgG IFA-IgG	*O. tsutsugamushi* antigens	Human sera
	NMRC	qPCR	Orien16S	Human eschas
		MLST	*rrs* and *htrA*	
	NMRC	qPCR	Orien16S	Mites
	NMRC	qPCR	Orien16S	Human eschars and buffy coat
	PUCC	MLST	*rrs, htrA*, and *tsa56*	
	NMRC	ELISAs	TGR-, SFGR-, and STGO-ELISAs-IgG	Human sera
Georgia	NMRC	qPCR assays	Rick17, Trick, Raesch, Rraoul, Rslov	Ticks
		MLST	*gltA, ompA, ompB, sca4*	
		ELISAs	TGR- and SFGR-ELISAs-IgG	Human sera
		qPCR assays	Rick17b, Rraoul, Rslov, Raesch, Rmona, Rconor, Rmass9666	Ticks
		MLST	*gltA, ompA, ompB, sca4*	
India	NMRC	ELISAs	TGR-, SFGR-, and STGO-ELISAs-IgG	Human sera
	InBios	ELISA	InBios Scrub typhus ELISA-IgM	
	NMRC	Sequencing	*tsa56*	Human blood
		ELISAs	TGR-, SFGR-, and STGO-ELISAs-IgG	Human sera
		MLST	17kDa, *gltA, ompA, ompB, sca4*	Human blood
		ELISA	TGR-ELISA-IgG	Human sera
Kazakhstan	USAMRIID	qPCR assays	*Dermacentor, Hyalommma, Rhipicephalus*, and *Ixodes* genus-specific assays	Ticks
	NMRC	qPCR	Rick17b	Ticks
		qPCR	Rick17b	Ticks
		qPCR assays	Rick17b, Raesch, Rraoul, Rslov	Ticks
		qPCR assays	Rick17b, Rtyph, RfelG, RfelB, Rasemb	Rodent fleas
Madagascar	NMRC	ELISAs	TGR- and SFGR-ELISAs-IgG	Human sera Small mammal sera
	NMRC and IPM	qPCR assays	RKND, RfelG, Rasemb	Small mammal fles
	NMRC and IPM	Sequencing	*ompB*	
Thailand	NMRC	ELISA	SFGR-ELISA-IgG	Cats
		qPCR assays	Rick17b, Rtyph, RfelG, RfelB, Rasemb	Cat fleas
		Sequencing	*ompB*	
Ukraine	NMRC	ELISA	TGR-ELISA-IgG	Human
			SFGR-ELISA-IgG	
Vietnam	NMRC	ELISAs	TGR-, SFGR-, and STGO-ELISAs-IgG	Human sera
		ELISAs	TGR-, SFGR-, and STGO-ELISAs-IgG	Human sera
		qPCR assays	Otsu47, Rick17b, Rtyph	Human buffy coats
		qPCR	Otsu47	Human PBMCs
				Human eschars
	HMU and NHTD	Sequencing	*tsa56*	Human PBMCs
				Human eschars

**Source of assays: NMRC, Naval Medical Research Center; OSU, Ohio State University; InBios, InBios International, Inc; USAMRIID, United States Army Medical Research Institute of Infectious Diseases; IPM, Institut Pasteur de Madagascar; HMU, Hanoi Medical University; NHTD, National Hospital for Tropical Diseases.*

***Type of assays: ELISAs, enzyme-linked immunosorbent assays; qPCR, quantitative real-time PCR; MLST, multilocus sequence typing.*

****Specific assays: TGR, typhus group rickettsiae; SFGR, spotted fever group rickettsiae; STGO, scrub typhus group orientiae; IgG, immunoglobulin gamma; Rick17, a genus-specific qPCR assay for Rickettsia species (1st generation); Rick17b, a genus-specific qPCR assay for Rickettsia species (2nd generation); RKND, a genus-specific qPCR assay for Rickettsia species; Trick, a group-specific qPCR assay for tick-borne spotted fever group Rickettsia species; Rtyph, a species-specific qPCR assay for Rickettsia typhi; Rasemb, a species-specific qPCR assay for Rickettsia asembonensis; RfelG, a group-specific qPCR assay for flea-borne spotted fever group Rickettsia species; Orien16S, a genus-specific qPCR assay for Orientia species; Raesch, a species-specific qPCR assay for Rickettsia aeschlimannii; Rraoul, a species-specific qPCR assay for Rickettsia raoultii; Rslov, a species-specific qPCR assay for Rickettsia slovaca; Rmona, a species-specific qPCR assay for Rickettsia monacensis; Rconor, a subspecies-specific qPCR assay for Rickettsia conorii subsp. conorii; Rmass, a species-specific qPCR assay for Rickettsia massiliae; rrs, 16S rRNA gene; htrA, 47-kDa antigen gene; tsa56, 56-kDa type-specific antigen gene; 17 kDa, 17-kDa antigen gene; gltA, citrate synthase gene; ompA, outer membrane protein A gene; ompB, outer membrane protein B gene; sca4, surface cell antigen 4 gene.*

*****Host samples: PBMC, peripheral blood mononuclear cell.*

rickettsiae in this district or Azerbaijan. More arthropod surveys, both ticks and fleas, are needed.

Chile

The first recognized human case of scrub typhus in 2006 in South America was confirmed by molecular and serological studies and reported in 2011 (26) (**Supplementary Table 1**). An additional three cases were subsequently identified and reported in 2016 (27). Since then, more than 40 cases have occurred throughout Chile (**Supplementary Table 1**). The disease has been characterized clinically, and serological studies of dogs (sentinel animals) and humans have shown the distribution both locally and nationally (**Supplementary Table 1**). In addition, human serological studies have identified spotted fever and typhus group rickettsial infections in Chile. Molecular characterization of the causative agent, *Candidatus* Orientia chiloensis, from human, rodent, and mite samples has shown that it is distinct from *O. tsutsugamushi* and *Candidatus* O. chuto (**Supplementary Table 1**). Additional studies are needed to show the transmission of the agent by mites to confirm the vector and hospital studies to better characterize the disease, incidence, and potential for scrub typhus outbreaks in Chile.

Georgia

Initially, only a single rickettsial agent, *Rickettsia conorii*, was known to be endemic to Georgia (28). More recently, ixodid ticks acquired from livestock, trapped rodents, and tick drags in May 2008 ($n = 653$) and 2009 ($n = 264$) from areas in the districts of Akhaltsikhe, Aspindza, Gori, and Kaspi of the country of Georgia were found to contain three additional rickettsial pathogens *R. aeschlimannii*, *R. roultii*, and *R. slovaca* (**Supplementary Table 1**). Additional studies followed utilizing new molecular assays and MLST to characterize the rickettsiae that were unable to be identified by qPCR. In the end, a total of nine tick-borne rickettsiae, six pathogens, and three rickettsial agents of unknown pathogenicity were identified. A hospital-based fever study determined that among 655 fever patients, 10 (1.5%), 2 (0.3%), and 2 (0.3%) patients had evidence of a previous infection with SFGR, STGO, and TGR, respectively (**Supplementary Table 1**). These results suggest the presence of rickettsial diseases among Georgians but at a very low prevalence, suggesting that the risk of a rickettsial outbreak is low. These results need to be confirmed with additional nationwide hospital-based fever studies.

Northeast India

Four epidemiological studies of rickettsial infections were initiated after it was discovered that scrub typhus had returned to Northeast India after a gap of 67 years (29). In the first study, of 1,264 human serum samples assessed by ELISA-IgG 390 (30.8%), 175 (13.8%), and 53 (4.2%) individuals had antibodies against STGO, SFGR, and TGR, respectively. Molecular studies of the positive serum samples identified two individuals had *O. tsutsugamushi* DNA. Investigation of arthropods (ticks, fleas, and mites) from domestic animals and rodents of the study area found only fleas that were positive for rickettsiae by the Rick17b genus-specific qPCR assay (4 of 16 individuals) and sequencing determined that the agent was *Candidatus* R. senegalensis (**Supplementary Table 1**). This study showed conclusively the presence of rickettsial diseases in this underserved area of India. In the second study, to determine the genotype of *O. tsutsugamushi* causing scrub typhus in NE India, patients screened positive by InBios Scrub Typhus ELISA-IgM blood samples were assessed for the presence of *tsa56*. Those positive amplicons were sequenced and three distinct genotypes were identified (**Supplementary Table 1**). This knowledge can be used in planning control strategies and prophylactic measures and identifying outbreak sources. The third study, involving the investigation of serum samples ($n = 317$) from individuals in another scrub typhus endemic region of NE India, found seroprevalence against scrub typhus, typhus, and spotted fever of 35.6, 2.2, and 0%, respectively. DNA extraction of seven SFGR positive blood samples only obtained a single amplicon for the 17-kDa gene. Sequence typing identified *R. felis*, a flea-borne rickettsia associated worldwide with flea-borne spotted fever (**Supplementary Table 1**). Future studies are needed to look for *R. felis* infection in patients with fever of unidentified origin (FUO) to determine the epidemiology and to understand the complex paradigm of *R. felis* transmission in India. Lastly, a study involving 2,199 clinical patients (762 with acute encephalitis syndrome and 1,437 with FUO) and 40 (1.8%) samples were found positive for IgG against *R. typhi* (**Supplementary Table 1**). This prevalence among patients suggests that a low risk of murine typhus exists in NE India and thus additional epidemiological studies throughout NE India should be performed to assess the risk of murine typhus with special attention to urban settings.

Kazakhstan

To assess arthropod-borne rickettsial diseases in Kazakhstan, new molecular methods were introduced to identify tick and *Rickettsia* species via United States Army Medical Research Institute of Infectious Diseases and NMRC in collaboration with NSCEDI-KSCQZD, SPC-SEEM, and UAPS. In Kazakhstan, North Asian tick typhus, also known as Siberian tick typhus, caused by *Rickettsia sibirica*, is endemic. However, it was unclear if other tick-borne diseases due to rickettsial infections occurred. To address this issue, *Rickettsia* species were identified initially only at the genus level with the Rick17b qPCR assay. The preliminary results showed the presence of tick-borne rickettsiae in Kazakhstan and the utility of molecular assays in arthropod-borne rickettsial surveillance. The addition of three species-specific qPCR assays identified never before known endemic regions for *R. aeschlimannii*, *R. raoultii*, and *R. slovaca*. In addition, flea-borne rickettsiae were identified in southwestern Kazakhstan that included *R. felis/Ca.* R. senegalensis and *R. asembonensis* (**Supplementary Table 1**). These new rickettsial pathogens identified in Northern, Western, Southern, and Southwestern Kazakhstan were significant in identifying health issues and stimulation of additional surveillance studies and brought awareness of the potential for rickettsial outbreaks in addition to the recognition of potential endemic rickettsioses.

Madagascar

Due to the presence of flea-borne diseases such as plague in Madagascar, this study was conducted to determine if a risk to flea-borne rickettsial diseases also exists in Madagascar. Notably,

a vector for both plague and murine typhus is the Oriental rat flea (*Xenopsylla cheopis*). Thus, a survey among humans and rodents was conducted to assess the risk of flea-borne rickettsial diseases. A seroprevalence study was performed among humans and peri-domestic small mammals. The seroprevalence of SFGR and TGR among humans was 34 and 39%, respectively. However, among the small mammals collected, only 4.4% were IgG positive against TGR *R. typhi* antigens and none of the animals had evidence of IgG against SFGR antigens. Interestingly, among the peri-domestic small mammals' fleas collected and assessed for rickettsiae, 24.3 and 1.9% of Oriental rat fleas (*X. cheopis*) were positive for *R. typhi* (a TGR) and *R. felis* (a SFGR), respectively, and 30.8% of *P. irritans* were positive for *R. felis* (**Supplementary Table 1**). These results showed that at least the two rickettsial agents identified in the rodent fleas assessed have possible roles in the TGR and SFGR infections among people in Madagascar. Additional studies to determine the spatial and temporal distribution of flea-borne rickettsiae are needed, in addition to hospital-based studies to determine incidence of these rickettsial diseases.

Northeast Thailand

A study of the role of domestic cats in the presence of rickettsial disease in Northeast Thailand investigated cat sera (42 serum samples) for the presence of antibodies against SFGR and cat fleas ($n = 23$) for molecular evidence of rickettsiae. Two cats (4.8%) had antibodies against SFGR, and 21 cat fleas (91.3%) were positive for *R. asembonensis* DNA (**Supplementary Table 1**). Thus, for Northeast Thailand, cats and cat fleas show evidence of spotted fever group rickettsiae, and physicians and veterinarians should be aware of the risk for rickettsial disease. These preliminary results should be followed up with additional surveillance and hospital studies to more clearly determine the risk of rickettsial diseases among the animal and human populations in Thailand.

Ukraine

Seroprevalence of 1,000 non-febrile hospital patients in western Ukraine for TGR was determined to be 1.5%, and for SFGR, it was 5.1%, indicating a low prevalence among non-febrile patients from two hospitals for previous exposure to rickettsiae (**Supplementary Table 1**). Remarkably, seropositivity to TGR was only 1.5%, and the study population included people who lived in an area where epidemic typhus was previously endemic. The seroprevalence studies should be increased to take in all of Ukraine. Moreover, a hospital-based fever study should be conducted to assess the identities, incidence, and distribution of rickettsial diseases for Ukraine.

Vietnam

Historically, rickettsial disease has been understudied in Vietnam. The following three studies conducted in northern Vietnam have added significantly to the knowledge of the presence of various rickettsial diseases, including scrub typhus and murine typhus and potentially spotted fever. In a serological study, the seroprevalence was determined to be 6.5, 1.1, and 1.7% for TGR, SFGR, and STGO infections of healthy humans, indicating the presence of all three groups infecting people in northern Vietnam, but at low levels. A subsequent study of hospitalized patients at two referral hospitals in Hanoi found that 34.1 and 3.3% of patients suspected of rickettsial disease were confirmed by serological and molecular assays to have scrub typhus and murine typhus, respectively. To follow up on these results, a second hospital-based study was conducted to characterize the *O. tsutsuagmushi* causing scrub typhus in northern Vietnam. It was determined that three geno-groups (Karp, Kato, and Gilliam) predominated. This information is important in the development of laboratory assays and vaccine candidates. Studies on rickettsial diseases with an emphasis on scrub typhus and murine typhus, but also including spotted fever, should continue to precisely determine the risk of endemic disease and the possibility of outbreaks for all of Vietnam.

In addition to providing leaders of the partner countries, research institutions, funding organizations, scientists, and clinicians with important rickettsial disease surveillance information based on these findings, the research was also made available to the international scientific community by providing abstracts to presentations ($n = 40$) given at national and international conferences and the publication of peer-reviewed articles ($n = 18$) in international scientific journals (**Table 5**). The important contributions of the rickettsia surveillance conducted due to the collaborations described herein to the scientific community for each country is exemplified when comparing the number of publications of rickettsial research before and after the collaborations. By searching PubMed for rickettsia, and rickettsia associated with arthropod-borne diseases and zoonosis, we found the number of publications varied significantly by country, with the most articles (without counting those described herein) published by India (281), Thailand (165), Chile (18), Vietnam (12), Ukraine (5), Madagascar (5), and Kazakhstan (3). Azerbaijan and Georgia had no other rickettsia publications by the PubMed search within the last 10 years other than the two described herein (**Table 5**). Thus, with the exception of India and Thailand, the percentage of publications due to those reported herein from seven countries (mean, 47.6%; range, 0–100%) shows the important knowledge that collaborative research provides to the partner institutes and global health.

DISCUSSION

During the implementation of our cooperative research, each country and institute(s) faced similar and unique challenges. Some laboratories were able to quickly incorporate rickettsial disease research or augment what they had into a self-sustaining capability. The primary challenge shared by all of the laboratories was recognizing the limited knowledge on rickettsial disease presence, identity, distribution, and prevalence, within their area of responsibility. Moreover, clinical presentations of rickettsial diseases are not distinct and are easily confused with multiple other infectious diseases such as dengue, leptospirosis, flu, malaria, etc. (6, 14). This issue is compounded by the lack of access to reliable diagnostic tests, especially in resource-limited and middle-income countries (2). Certain issues could not be

TABLE 5 | Rickettsia abstracts and publications by country.

Countries	Number of rickettsia abstracts due to collaborations (reference numbers)	Number of rickettsia publications due to collaborations (reference numbers)	Number of rickettsia publications from PubMed Search*** (references numbers)	Number of rickettsia publications by collaboration/ total publications in past 10 years
Azerbaijan	4 (30–33)	1 (34)	0	1/1 (100%)
Chile	8 (35–42)	5 (43–47)	19 (27, 48–65)	5/24 (20.8%)
Georgia	12 (66–77)	2 (34, 78)	0	2/2 (100%)
Northeast India*	2 (79, 80)	2 (81, 82)	4 (29, 83–85)	2/6 (33.3%)
Kazakhstan	8 (86–93)	3 (16, 19, 94)	1 (95)	3/4 (75%)
Madagascar	0	1 (96)	5 (97–101)	1/6 (16.7%)
Northeast Thailand**	1 (102)	1 (103)	2 (104, 105)	1/3 (33.3%)
Ukraine	2 (106, 107)	0	5 (108–112)	0/5 (0%)
Vietnam	3 (113–115)	3 (116–118)	12 (119–130)	3/15 (20%)

Search results for only Northeast India. Search results for all of India were 281 items.

**Search results for only Northeast Thailand. Search results for all of Thailand were 165 items.*

***Search words: rickettsia, arthropod borne, and zoonosis; search time period: past 10 years—does not include those listed for collaborations.*

overcome, especially those political in nature that arose between collaborating countries.

NMRC staff scientists provided training as needed and scheduled to conduct serological and molecular assays utilized to identify rickettsia and rickettsial infections (4). Performance of general laboratory procedures was discussed early on to ensure that all involved had the same knowledge and perceptions of working in BSL-2 laboratories. Trainings for rickettsial assays were accomplished in a mentoring fashion that included didactic lectures, hands-on laboratory instruction, and overseeing supervised instruction. Positive and negative controls, initially provided by NMRC, were utilized to ensure that laboratory results were obtained similarly in the partner laboratory and NMRC laboratory. Follow-up training was conducted as new procedures were added to the institutes' portfolios. This additional training was performed in the partner countries during visits and at NMRC where scientists from Chile, Georgia, India, Kazakhstan, and Vietnam visited and worked side by side with members of RDRP.

NMRC staff scientists also provided guidance and training on data analysis especially in the proper evaluation of laboratory results, most importantly, data quality: when to accept and when not to accept the results. This required the evaluation of standard controls (commercial and non-commercial) as acceptable ranges established and could be used for reference and quality control. Understanding how to analyze results relative to the controls and to know when there is an issue with a control are vital for interpreting results. This allowed for the development of appropriate conclusions from data obtained (4, 131–133). Examples of these discussions often occurred during partner country visits, side meetings of conferences, and video conference calls.

When detection of rickettsial disease is limited to serological data, it is difficult to determine the causative agent responsible for antibody response in a patient. Thus, the knowledge necessary to assess the specific rickettsial risk(s) is relegated to the general assumptions as to the cause of the infection, being limited to group specificity. Therefore, it is important to obtain specific information associated with proposed agents such as the host(s), the hosts' natural settings, prevalence of infected hosts, and their distribution (4, 14, 133).

Limited molecular identification of rickettsiae to the genus or group level in the environment does not allow one to determine accurately the risk of particular rickettsial diseases. Detection of pathogenic rickettsiae must be accomplished by *Rickettsia* species identification, as sympatric non-pathogenic rickettsiae can be found among the same arthropod hosts (134). For example, one can commonly find sympatric pathogens with non-pathogenic *Rickettsia* species among the same arthropod species [e.g., *Rickettsia rickettsii* and *Rickettsia montanensis* in *Dermacentor variabilis* (135); *Rickettsia parkeri* and *Rickettsia andeanae* in *Amblyomma maculatum* (136, 137); *Rickettsia felis* and *Rickettsia asembonensis* in *Ctenocephalides felis* (24, 138)].

The commonly used method of preparing arthropod vectors is to employ pooling, which groups the same arthropod species from one location, the same host, or drag/flag sheet into one pool sample (94). The pool samples can be restricted to individuals of the same arthropod species and life stage. As indicated above, since more than one rickettsial agent may be found within a single species of tick or flea, then pooled samples may contain more than one *Rickettsia* species. In this situation, one of the *Rickettsia* species might be missed and therefore one cannot determine the prevalence of each rickettsia accurately. The minimum infection rate (MIR) equals the total number of positive pools divided by the total number of individual arthropods in all the pools assessed multiplied by 100. A positive pool with 5 individuals in it is not counted the same as a pool with 50 individuals; i.e., the prevalence of each pool would be considered 100%, whereas the MIR would be considered to be 20 and 5%, respectively. If a pool has more than one *Rickettsia* species in it, then it will be assumed that only one agent of each species is among the pool sample (78). For example, if a pool of 10 *A. maculatum* individuals had both qPCR assays for *R. parkeri* and *R. andeanae* as positive, then the MIR for both agents would be 10%. Therefore, the most

accurate way, though more time-consuming and costly, is to determine the prevalence of rickettsiae and the agents' identity within their arthropod hosts based on individual arthropods, not pools. Lastly, if you have both individual and pooled samples, you will have to determine both prevalence and MIR for the respective sample types (78). By definition, a pool sample cannot contain a single arthropod. To address the issues above, a strategy of processing arthropod hosts individually, screening by pools, and testing each individual arthropod in a positive pool can be used.

Unlike the genus *Rickettsia* where there is much more information on the pathogenicity of the individual species, significantly less is known about *Orientia* species. Moreover, the rodent animal models for *O. tsutsugamushi* strains do not necessarily mimic the virulence of the same agents in humans and non-human primates, thus confounding the matter of identifying pathogens (139, 140). However, it has been known for a very long time that differences in pathogenicity exist among the large diversity of *O. tsutsugamushi* strains (141). The scrub typhus story becomes more compelling now that we know there are additional *Orientia* species found outside the endemic region of *O. tsutsugamushi* (known as the Tsutsugamushi Triangle incorporating lands in Asia, Australia, and islands in the Indian and Pacific oceans). Scrub typhus is now considered endemic for multiple areas throughout the world (7). With the recent added complexity of orientiae, there is need for more research to be performed to determine presence of pathogen and non-pathogens within *Orientia*. For the present, detection of *Orientia* species is considered evidence of a causative agent for and therefore used in the risk assessment of scrub typhus.

The projects described herein clearly demonstrate that the partner country laboratories benefited from enhanced training, capacity/capability, education (science, laboratory procedures, language, and risk assessment), and appropriate funding support. The ultimate consequences were not only the data collected, which, following analysis, allowed the institutes and partner countries to determine risk of rickettsial diseases for districts, regions, and/or countries and which can subsequently be used by medical leaders and policy makers to institute further education and projects and augment preventive medicine, diagnostic, and treatment modalities to enhance public health. The true success of these projects is best measured by the ability of the partner laboratories to continue research on their own in this area of research as well as research in other infectious diseases. Fortunately, as can be seen by this paper and others (16, 19), the collaborating governments, funding partners, and research institution involvements were extremely positive, resulting in host country institutions and personnel enhancement in conducting rickettsial disease research, which also enhanced scientific and medical knowledge worldwide. Ultimately, collaborative researchers are able to provide information to public health decision makers with advanced awareness on emerging infectious disease threats such as rickettsial diseases, and thereby promote the timely, science-based disease outbreak prevention, preparedness, and control-and-response actions necessary to improve global public health.

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SUPPLEMENTARY MATERIAL

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REVIEW
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An Update on Advances in COVID-19 Laboratory Diagnosis and Testing Guidelines in India

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The declaration of COVID-19 as a global pandemic has warranted the urgent need for technologies and tools to be deployed for confirming diagnosis of suspected cases. Diagnostic testing for COVID-19 is critical for understanding epidemiology, contract-tracing, case management, and to repress the transmission of the SARS-CoV-2. Currently, the Nucleic Acid Amplification Test (NAAT)-based RT-PCR technique is a gold standard test used for routine diagnosis of COVID-19 infection. While there are many commercially available RT-PCR assay kits available in the market, selection of highly sensitive, specific, and validated assays is most crucial for the accurate diagnosis of COVID-19 infection. Laboratory diagnosis of SARS-CoV-2 is extremely important in the disease and outbreak management. Development of rapid point of care tests with better sensitivity and specificity is the critical need of the hour as this will help accurate diagnosis and aid in containing the spread of SARS-CoV-2 infection. Early detection of viral infection greatly enhances implementation of specific public health intervention, such as infection control, environmental decontamination, and the closure of specific high-risk zones. Large-scale sequencing of SARS-CoV-2 genome isolated from affected populations across the world needs to be carried to monitor mutations that might affect performance of molecular tests. Creation of genome repositories and open-source genetic databases for use by global researchers is clearly the way forward to manage COVID-19 outbreak and accelerate vaccine development. This review summarizes various molecular diagnostics methods, technical guidelines, and advanced testing strategies adopted in India for laboratory diagnosis of COVID-19.

Keywords: COVID-19, RT-PCR, guidelines, laboratory diagnosis, molecular testing

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INTRODUCTION

On March 12, 2020, the World Health Organization (WHO) declared the novel coronavirus disease 2019 (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) as global pandemic. According to the WHO data on May 14, 2020, there have been 4,218,212 confirmed cases of COVID-19, including 290,242 deaths reported globally and 74,281 confirmed cases reported from India (1). The declaration of COVID-19 as a global pandemic has warranted the urgent need for technologies and tools to be deployed for confirming diagnosis of suspected cases.

Diagnostic testing for COVID-19 is critical for understanding epidemiology, contract-tracing, case management, and to repress the transmission of the SARS-CoV-2. Hence, there is an urgent need for deployment of rapid, highly specific and ultra-sensitive molecular diagnostic tests. Most importantly, accurate and rapid diagnosis of SARS-CoV-2 infection will help to identify, isolate, and treat the patients in order to minimize risk of public contamination and drastically reduce the mortality rates. As per the existing guidelines, the clinicians should work with their local and state health departments to coordinate laboratory diagnosis of COVID-19 through government laboratories or work with commercial clinical laboratories (2).

Currently, WHO recommends for laboratory diagnosis COVID-19 based on detection of unique sequences of virus RNA by Nucleic Acid Amplification Test (NAAT) such as real-time reverse-transcriptase polymerase chain reaction (rRT-PCR). Similarly, the Indian Council of Medical Research (ICMR) also recommended use of rRT-PCR-based tests approved by the US FDA EUA/CE-IVD approved kits, under intimation to Drug Controller General of India (DCGI) and Ministry of Health and Family Welfare (MoH&FW). The FDA EUA/CE-IVD approved RT-PCR kits are highly specific and detects the presence or absence of SARS-CoV-2 viral nucleic acid and thus directly confirms viral infection in a human sample. The RT-PCR test is the current gold standard diagnostic method for the diagnosis of COVID-19. Other molecular methods such as virus antigen or serological antibody testing are currently recommended for use only in research settings and not in clinical decision-making (3).

Structure of SARS-CoV-2

SARS-CoV-2 is a large positive-sense single-stranded ribonucleic acid (RNA) virus belonging to the family Coronaviridae.

The SARS-CoV-2 genome is approximately 30,000 nucleotides in length and encodes several proteins including an RNA-dependent RNA polymerase (RdRP) and four structural proteins viz., nucleocapsid protein (N), spike protein (S), envelope protein (E), and membrane protein (M) (**Figure 1**). RdRP helps in maintaining fidelity of viral genome by acting in conjunction with nonstructural proteins, and the spike protein (S) plays an important role in transmission of the virus by functioning in receptor binding and membrane fusion in the host (5). The S gene of the SARS-CoV-2-encoding spike protein is found to have <75% nucleotide sequence similarity when compared to other SARS-related coronaviruses. The E, M, and N structural proteins are more conserved than the spike protein and are essential for general function of coronavirus (6, 7).

Viral Replication

Coronaviruses have complex machinery comprising of multisubunits for replication and transcription. Around 8–10 open reading frames (ORFs) are found in most of the coronaviruses, among them ORF1a and ORF1b are important in SARS-CoV-2. ORF1a and ORF1b are translated into polyprotein 1a (pp1a) and pp1ab. RNA-dependent RNA polymerase enzyme (RdRp) is produced as a result of viral protease cleavage of pp1a and pp1ab polyproteins. RdRp, also known as nsp12, plays a predominant role in the replication and transcription cycle of COVID-19 virus and hence is being considered as primary target for antiviral inhibitors like remdesivir (8, 9). Coronaviruses generate 6–9 subgenomic mRNAs (sgmRNAs) during replication, and structural and accessory proteins are translated from this sgmRNAs (10).

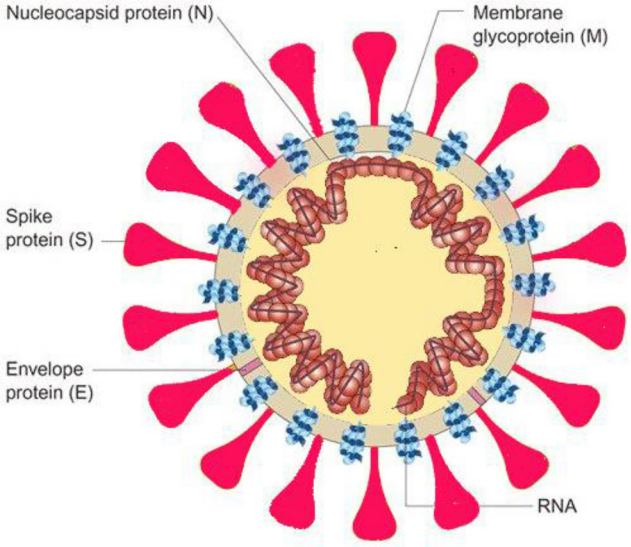

FIGURE 1 | Illustration of viral structure with its structural viral proteins (4).

SAMPLE REQUIREMENT FOR COVID-19 TESTING

Appropriate sample collection is the most important step in the laboratory diagnosis of any infectious disease. Improper specimen collection may contribute to false negative test results. **Table 1** summarizes different types of samples used for COVID-19 testing as recommended by ICMR (11). The guideline mandates clinical sample collection by trained laboratory personnel/health-care workers in the presence of a clinician. Generally, samples for COVID-19 diagnosis are collected from two major sources: upper respiratory tract and lower respiratory tract. Upper respiratory tract specimens are collected by nasopharyngeal (NP) swab or the oropharyngeal (OP) swab, whereas the bronchoalveolar lavage, tracheal aspirate, or sputum will be collected from the lower respiratory tract. ICMR has created a comprehensive Specimen Referral Form for COVID-19 for use by all specimen collection centers and testing labs (12).

Nasopharyngeal Swabs

The NP specimen is a vital and sensitive sample to test the SARS-CoV-2 virus. As suggested by the Centers for Disease Control and Prevention (CDC), it is highly recommended to collect only the NP swab, although OP swabs remain an acceptable specimen type. In the case that both NP and OP swabs are collected, they should be combined in a single tube to maximize sample load and test sensitivity. Synthetic fiber swabs with plastic shafts

TABLE 1 | Description of samples used for COVID-19 detection.

Specimen type	Collection materials	Transport to laboratory	Storage till testing	Suitable for Tests
Nasopharyngeal and oropharyngeal swab	Flocked swabs	4°C	≤5 days: 4°C > 5 days: −70°C	RT-PCR, Rapid Diagnostic Test
Bronchoalveolar lavage	Sterile container	4°C	≤48 h: 4°C > 48 h: −70°C	RT-PCR, Rapid Diagnostic Test
Tracheal aspirate, nasopharyngeal aspirate, or nasal wash	Sterile container	4°C	≤48 h: 4°C >48 h: −70°C	RT-PCR, Rapid Diagnostic Test
Sputum	Sterile container	4°C	≤48 h: 4°C > 48 h: −70°C	RT-PCR, Rapid Diagnostic Test
Whole blood (5 ml)	EDTA Vial	4°C	≤5 days: 4°C	ELISA, Immunodiagnostics

are recommended for NP and OP samples collection. Calcium alginate or wooden-shaft swabs are not recommended because they might inactivate the virus and could provide a negative result. NP and OP samples should be placed immediately into an appropriate sterile medium or saline for proper transportation.

Oropharyngeal Swabs

The OP swab is another important specimen recommended by the WHO and CDC to detect SARS-CoV-2 infection. The OP swab is collected from the posterior pharynx region, avoiding contact with the tongue.

Bronchoalveolar Lavage and Tracheal Aspirate

Collection of the bronchoalveolar lavage (BAL) and tracheal aspirate is recommended only in severely ill and hospitalized patients.

Sputum

Sputum is collected from patients who have severe coughing symptoms. Sputum is collected by asking patients to expectorate deep-cough sputum directly straight into a sterile, leak-proof, screw-cap sputum collection cup or sterile dry container.

Other Samples

Blood and stool samples are also used in diagnosis of infection since SARS-CoV-2 is known to present in blood and stool. A small study reported the presence of SARS-CoV-2 in anal or oral swabs of patients in the Hubei Province (13). However, the utility of these samples remains unclear, because the data on viral shedding post-infection is still preliminary.

Nucleic Acid Amplification Test (NAAT)

NAAT-based RT-PCR technique is currently a gold standard test used for routine confirmation of cases of COVID-19. The viral genes targeted in the RT-PCR test so far include the N, E, S, and RdRP genes. According to the WHO guidelines, in order to confirm the positive diagnosis, a validated RT-PCR assay targeting a minimum of two regions on the SARS-CoV-2 genome must be chosen, with one being specific for SARS-CoV-2. Alternatively, the test can include a primer specific for betacoronavirus and presence of SARS-CoV-2 must be confirmed by sequencing partial or whole genome of the virus (14).

ICMR recommends use of commercial RT-PCR-based tests approved by the US FDA EUA/CE-IVD approved kits, under intimation to DCGI and MoH&FW under Biosafety 2 level (BSL-2) conditions and with appropriate biosafety precautions. The commercial testing kits also need to be validated by ICMR before by mass testing. These tests are validated at National Institute of Virology (NIV), Pune, and 14 other validation centers approved by ICMR. As of May 25, 2020, 83 commercial RT-PCR kits have been validated by ICMR validation centers, and 33 kits were found to be satisfactory for testing (15). Conventional PCR assays, in-house developed RT-PCR assays, and antigen/antibody testing are not recommended for clinical diagnosis of COVID-19 (16). ICMR NIV has issued technical guidelines for testing of suspected human sample by RT-PCR assay. According to this guideline, samples should be first tested in the E gene screening assay and later tested in the RdRp, ORF1b, and N gene confirmatory assays. These assays are highly sensitive and specific and do not cross-react with other coronavirus or human clinical samples that contain respiratory viruses. **Table 2** summarizes examples of primer and probe details used in screening and confirmatory assay (17, 18).

ICMR also recommends use of FDA approved Cartridge-Based Nucleic Acid Amplification Test (CBNAAT) using Cepheid Xpert Xpress SARS-CoV2 for use under an emergency use authorization (EUA) only. CBNAAT test should be run under biosafety 2 level (BSL-2) conditions and with appropriate biosafety precautions. This test detects E gene and also the SARS-CoV-2 specific N2 region of the N gene.

RT-PCR tests involve fairly complex steps and take nearly 24–48 h for generating the results. RT-PCR tests also require trained personnel and well-equipped modern laboratories with BSL-2 set up for their use. Advent of rapid nucleic-acid-detection-based tests appear to accelerate the COVID-19 diagnosis in India. One such test is TruNat, an indigenous testing developed originally for tuberculosis, has been explored and is now being used for COVID-19 testing in India. TruNat beta CoV test, a

TABLE 2 | Details of primers and probes for screening and confirmatory assay.

	Assay	Oligonucleotide ID	Sequence (5′-3′)
Primers and probes screening assay (E gene assay)	E gene	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT
		E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA
		E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG -BHQ
	RNaseP gene (Internal control)	RNaseP Forward	AGATTTGGACCTGCGAGCG
		RNaseP Reverse	GAGCGGCTGTCTCCACAAGT
		RNaseP Probe	FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ
Primers and probes confirmatory assay (RdRp and ORF gene assay)	RdRp	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG
		RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA
		RdRP_SARSr-P2 Specific for Wuhan-CoV	FAM-CAGGTGGAACCTCATCAGGAGATGC-QSY
	HKU ORF gene	HKU-ORF1b-nsp14F	TGGGGYTTTACRGGTAACCT′
		HKU-ORF1b-nsp14 R	AACRCGCTTAACAAAGCACTC
		HKU-ORF1b-nsp14 P	FAM-TAGTTGTGATGCWATCATGACTAG-QSY

microchip-based real-time PCR assay, runs on TruNat machines with a very short test duration of 1 h. The Truelab workstation (Molbio Diagnostics, India) includes sample preparation, an RNA extraction system, an RT-PCR machine, and disposable kit components. It is a chip-based, real-time quantitative PCR system that is portable, battery-operated, fully automated, and weighs around 3 kg. This system can be used in remote areas and has network data transfer ability and an automated reporting system. The advantage of TruNAT is that the virus is lysed during the testing process, minimizing the risk of infection and contamination by the virus. The TruNat test is a semi-quantitative real-time PCR assay that has two steps. Step 1 is and E gene screening assay. All negatives are to be considered as true negatives. All positive samples should be subjected to confirmation by Step 2 RdRp gene confirmatory assay. All samples that test positive by this assay are considered as true positive. Further RT-PCR-based confirmation is not required for samples that are positive after Step 2 of the TruNat assay (19).

On May 19, 2020, ICMR declared TruNat system as a comprehensive assay for screening and confirmation of COVID-19 cases in India. State health departments have been working with the National Tuberculosis Elimination Programme (NTEP) to establish TruNAT test for COVID-19 diagnosis. TruNAT test is promising especially in areas/districts where modern laboratories are not available. ICMR has scaled up COVID-19 testing laboratories in partnership with DST, DBT, ICAR, CSIR, DRDO, MHRD, medical colleges, and private laboratories. As of May 30, 2020, there has been a total of 669 COVID-19 testing labs in India, including 466 government laboratories and 203 private laboratories. Of these, 480 labs are using RT-PCR-based tests, 134 labs are using TruNat tests, and 55 labs are using CBNAAT-based COVID-19 tests (20).

Pooling of Samples for Surveillance Purposes

COVID-19 cases are increasing exponentially in India. Surveillance of migrant workers and international passengers in institutional quarantine facilities is of utmost importance to understand the disease status and ultimately containing the disease spread. Hence, it becomes crucially important to scale-up and increase the numbers of tests conducted by laboratories. Because positivity rates in suspected cases are still low, pooling of samples for screening seem to be a viable option with substantial time and cost saving benefits. A pooled testing algorithm involves the PCR screening of a specimen pool comprising multiple identified individual specimens, followed by individual specimen testing only if a pooled sample tests positive. All individual samples in a negative pool are regarded as negative, and the test result requires it to be conveyed to the concerned quarantine facility within 24 h (21).

Limitations of NAAT

Various factors contribute to a false negative result in NAAT. Factors such as poor quality of the specimen, specimen with little patient material, specimen collected late or very early in the infection, improperly handled and shipped specimens, and inherent technical reasons such as virus mutation will hamper the results of RT-PCR-based testing. WHO recommends that each NAAT run should include both external and internal controls, and they encourage laboratories to participate in external quality assessment schemes (14).

Serology-Based Antibody Testing

Antibodies in the blood are detected when the body is responding to a specific infection, like COVID-19. IgM is one of the first types of antibodies to be produced and is the most useful for determining recent infection. Antibodies may not be detected in the early days of an infection when the body's adaptive immune response is still building. In a study, the presence of IgM antibodies for SARS-CoV-2 has been observed to range from 7 to 10 days after the onset of symptoms (22). Therefore, serology-based antibody testing is not used as the sole basis to diagnose or exclude SARS-CoV-2 infections. However,

antibody testing could play a role in the fight against COVID-19 by helping health-care professionals identify individuals have developed an adaptive immune response to SARS-CoV-2. In addition, these test results can help in identifying individuals who can donate convalescent plasma, which may serve as a possible treatment for those who are seriously ill from COVID-19.

ICMR recommends using a rapid antibody test as a surveillance tool for epidemiological purposes in hot spot areas and in such areas where cases have not emerged so far. ICMR-NIV has successfully developed and validated anti-SARS-CoV-2 human IgG ELISA test kit for antibody detection of COVID-19. In an external validation, the sensitivity and specificity IgG ELISA kit was found to be of 98.7 and 100%, respectively. The ELISA test has the advantage of processing 90 samples together in a single run of two-and-a-half hours and also has minimal biosafety and biosecurity requirements as compared to the real-time RT-PCR test. The IgG ELISA test has been proposed to be used for surveillance of the population exposed to SARS-CoV-2 coronavirus infection (23).

ANTIGEN TESTING

Antigen-based rapid tests detects the presence of SARS-CoV-2 antigens such as the nucleocapsid (N) protein and the S1 or S2 domains of the spike (S) protein. The antigen(s) detected are expressed only when the virus is actively replicating and when antigen is present in sufficient concentrations and run a higher risk of not being able to detect viral material from a swab, and are prone to produce false negative diagnosis. Therefore, antigen tests are best used to identify acute or early infection, especially in settings where a rapid test turnaround time is required. The sensitivity of antigen tests reportedly varies from 34 to 80%, which means there could be nearly 50% of false negative results, depending on the group of patients tested (3, 24). Hence, it is important for clinicians and laboratory personnel to understand the analytic performance characteristics of antigen test assays, including sensitivity, specificity, and positive and negative predictive values. As of January 6, 2021, ICMR has validated 63 antigen-based Rapid Test Kits approved for use in India. Details of these kits can be accessed on https://www.icmr.gov.in/pdf/covid/kits/List_of_rapid_antigen_kits_17022021.pdf. Antigen-based rapid tests that are US-FDA approved can be used directly after due marketing approval from DCGI.

In September 2020, ICMR issued advisory recommending the use of a rapid antigen test (RAT) as an initial test for surveillance in containment zones and for screening at points of entry. Negative RAT result should always to be confirmed by RT-PCR/TruNat/CBNAAT. Whereas in noncontainment zones and in hospital settings, RT-PCR/TruNat/CBNAAT is recommended for initial screening and followed by RAT (25). The clinical performance of RATs largely depends on the circumstances in which they are used and perform best when the viral load of the sample is generally highest. In December 2020, the CDC issued interim guidelines on the use of antigen tests for screening in high-risk congregate settings in which repeat testing could quickly identify persons with a SARS-CoV-2 infection to inform infection prevention and control measures. Laboratory professionals who perform antigen tests need to understand the factors that affect the accuracy of antigen testing, as described in this guidance (26).

CONCLUSION

COVID-19 is a global pandemic and currently is the most dreadful viral disease faced by the global community. In many countries including India, the government is making enormous efforts to contain the spread of virus by implementing measures like countrywide shutdown of public places, isolating infected individuals, treatment, primary, and secondary contact tracing, decontaminating and sealing down the infected zones, etc. The ICMR, the apex body in India for the biomedical research, is at the forefront of the battle against COVID-19. ICMR is issuing advisories and appropriate guidelines regularly for tackling operational challenges, including logistics of sampling materials, testing kits, validation of kits, developing protocols, etc.

Laboratory diagnosis of SARS-CoV-2 is extremely important in the disease and outbreak management. Development of rapid point-of-care tests with better sensitivity and specificity is the critical need of the hour because this will help accurately diagnose and aid in containing the spread of SARS-CoV-2 infection. Early detection of infection greatly enhances implementation of specific public health intervention, such as infection control, environmental decontamination, and the closure of specific high-risk zones. Many aspects of the COVID-19 virus and disease are still being explored. A better understanding of viral dynamics, immunological response, duration of viral shedding etc. will help decide optimal type and timing of clinical material to be sampled for molecular testing and most importantly help decide on treatment modalities. Large-scale sequencing of the SARS-CoV-2 genome isolated from affected population across the world needs to be carried to monitor mutations that might affect performance of molecular tests. Creation of genome repositories and open-source genetic databases for use by global researchers is clearly the way forward to manage the COVID-19 outbreak and accelerate vaccine development.

AUTHOR CONTRIBUTIONS

KK: literature review, concept outline development, and drafting of manuscript. RN: critical review. DH: supervision. VS: literature review. SM: concept development and guidance. All authors contributed to the article and approved the submitted version.

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frontiers in Medicine

CASE REPORT
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Case Report: A Severe SARS-CoV-2 Infection in a Teenager With Angelman Syndrome

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Teenagers generally present mild to no symptoms of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. In the present report, we present the case of a 14-year-old boy with Angelman syndrome (AS) who presented with severe COVID-19 symptoms. He spent 20 days in the ICU with elevated inflammatory biomarkers (C-reactive protein and D-dimer) and increased peaks of neutrophil-to-lymphocyte ratio, which is uncommon for teenagers diagnosed with COVID-19. Although he showed physiological instability, he was able to produce neutralizing antibodies, suggesting a functional immune response. The literature concerning the immune response to infections in patients with AS is still poor, and to our knowledge, this was the first report of a patient with AS diagnosed with COVID-19. As such, the present study may alert other patients with AS or other rare diseases that they lack a competent immune response and could suffer severe consequences of SARS-CoV-2 infection.

Keywords: COVID19, SARS-CoV-2, Angelman syndrome, neutralizing antibodies, intensive care unit, inflammation, immune response

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INTRODUCTION

Coronaviruses are enveloped, non-segmented, positive-sense RNA viruses belonging to the family Coronaviridae. The latest human pathogenic coronavirus to be identified was SARS-CoV-2, which causes COVID-19 (1). It was first reported in December 2019 in Wuhan, China, and declared a pandemic by the World Health Organization (WHO) in March 2020. It continued to spread across the globe, alternating hotspots during the last months (2). The number of confirmed pediatric cases is much lower than that of adult cases, and the severity and mortality rates are even lower (3). However, at all ages, patients with hypertension, diabetes, obesity, or chronic lung disease are more prone to severe disease (2). In the present report, we describe the case of a 14-year-old boy with asthma and Angelman syndrome (AS) who developed a life-threatening manifestation of COVID-19 due to severe respiratory distress syndrome. Although allergy does not appear to

be a predisposing factor for COVID-19 (4), it is well-documented that children with neurological disorders are more prone to develop severe clinical course in respiratory tract infections (5).

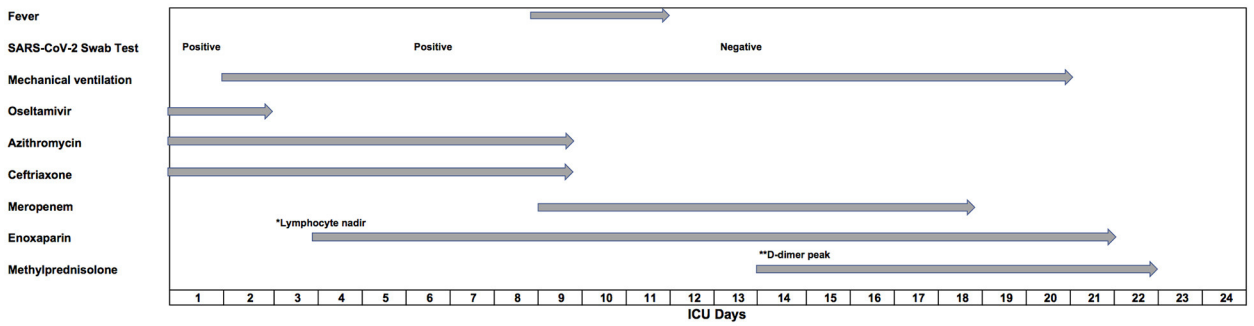
CASE DESCRIPTION

A 14-year-old boy with asthma and AS visited the emergency department of Hospital Municipal Infantil Menino Jesus, São Paulo, Brazil in April 2020; he had a history of fever episodes of 38°C, respiratory distress, and perioral cyanosis. At home, he had performed inhalation with salbutamol and ipratropium, but developed worsening wheezing. When he arrived at our center, he was hypothermic (34.8°C), tachycardic, tachydyspneic, and had an O_2 saturation of 77% on peripheral oximetry. His mother also had flu-like symptoms and complained of anosmia. At that time, Brazil was in quarantine and hospitals were implementing extensive measures to prevent COVID-19, providing personal protective equipment to hospital care team members, patients, and their families. In addition, our center was executing a specific COVID-19 protocol to isolate patients who met the following criteria: fever, cough, runny nose, shortness of breath, difficulty breathing with hospital admission needed, or underlying uncontrolled medical condition. With the hypothesis of severe acute respiratory syndrome due to infection by SARS-CoV-2, the patient was admitted to the hospital and subjected to a viral panel (6), which included adenovirus (AdV), respiratory syncytial virus (RSV-A, -B), parainfluenza virus (PIV-1,−2,−3,−4), influenza virus (Flu-A, -B), human metapneumovirus (hMPV), seasonal coronavirus (CoV-OC43, -HKU1, -NL63,−229E), enterovirus (EV), rhinovirus (RV), and SARS-CoV-2 (7).

Initial chest X-ray showed multiple bilateral consolidation foci with a cottony aspect. The patient received supplemental oxygen therapy, volume expansion with saline solution, oseltamivir, and antibiotic therapy with azithromycin and ceftriaxone. He evolved on his second day of hospitalization, with worsening of his breathing pattern, and was transferred to the intensive care unit (ICU). Mechanical ventilation was initiated on the following day (**Figure 1A**). At this time, the patient had mild anemia (hemoglobin: 11.6 g/dL, normal reference [NR]: 13.0–17.0 g/dL) and thrombocytopenia (platelet count: 125 × 10^9/L, NR: 150–400 × 10^9/L), with normal leukocytes (5.83 × 10^9/L, NR: 4.5–11 × 10^9/L), and monocytes (3.27 × 10^9/L, NR: 1–4 × 109/L). Although lymphopenia is commonly seen in patients with severe COVID-19, lymphocyte count was normal in the patient (2.45 × 10^9/L, NR 1–3 × 10^9/L), as were platelet to lymphocyte ratio and neutrophil count. However, the neutrophil-to-lymphocyte (N/L) ratio was elevated during the patient's stay in ICU (**Figure 2**). Although renal function was not affected, as indicated by creatinine and urea levels, increased liver enzymes were observed [aspartate aminotransferase (AST): 184 U/L, alanine aminotransferase (ALT): 253 U/L; NR 15–40 U/L], as were hypoalbuminemia (2.8 g/dL, NR: 3.5–5 g/dL), and hypernatremia (151 mmol/L, NR: 137–145 mmol/L). The patient's serum chloride levels were also elevated (119 mmol/L, NR: 98–107 mmol/L), while his ionized calcium levels were decreased (0.86 mmol/L, NR: 1.12–1.32 mmol/L). Other electrolytes presented normal levels in the serum, as follows: lactate (1.3 mmol/L, NR: 0.5–1.6 mmol/L), potassium (4.4 mmol/L), phosphate (4.1 mg/dL), and magnesium (1.8 mg/dL). Elevated ferritin levels (483 ng/mL, NR: 7–140 ng/mL) were found in the blood on the following day, indicating liver damage. D-dimer and C-reactive protein (CRP) were somewhat elevated (D-dimer: 858.1 ng/mL, NR < 500 ng/mL; CRP: 7.3 mg/dL, NR < 1 mg/dL), and lactate dehydrogenase (LDH) showed increased levels (636 U/L, NR: 120–246 U/L) (**Figure 3**). These three biomarkers are positively correlated with the severity of COVID-19 (8).

Blood culture was negative, confirming no bacterial infection. On day 2, the result of the viral panel confirmed infection with SARS-CoV-2, with a swab qPCR viral load of 5.9 × 10^6 RNA copies/mL and a cycle threshold (Ct) 24.72 (9). The patient was negative for other viruses (**Figure 1A**). Thus, oseltamivir was suspended and a viral panel from the patient's mother was collected, which later identified the same virus. The patient remained on mechanical ventilation for 19 days.

On day 4 of hospitalization, a chest CT scan was performed (**Figure 1B**), demonstrating extensive pulmonary consolidation in the left lower lobe and in a limited part of the right lobe. Diffuse ground-glass densification was observed in the unconsolidated pulmonary portions. The scan also showed moderate pleural effusion on the left side. On day 5 of hospitalization, echocardiography showed a hyper-refringent image in the inferior vena cava, suggestive of a thrombus measuring 15.5 × 8.7 mm. Enoxaparin was started at an anticoagulant dose until the lesion resolved 18 days later. A progressive increase in D-dimer was observed, with the initial level of 858.1 ng/mL reaching a peak of 2023.5 ng/mL on day 15 of hospitalization (**Figure 3**). On day 6 of hospitalization, the SARS-CoV-2 test was repeated and demonstrated a decrease in viral load to 3.5 × 10^4 copies/mL, with a Ct value of 32.20. On day 10 of ceftriaxone and azithromycin, the patient started to show feverish peaks and worsening respiratory parameters. Laboratory tests showed leukocytosis (22.2 × 10^9/L), with a left shift. Antibiotic therapy was staggered with meropenem because the patient was suspected to have pneumonia associated with mechanical ventilation. He evolved with improvement of the fever on day 3 of meropenem and completed a total of 10 days of antibiotic therapy. On day 14 of hospitalization, the viral panel was repeated, with a negative result. On day 15 of hospitalization, corticosteroid therapy with methylprednisolone was started, prompted by his pulmonary condition and personal history of asthma. We intended to carry out 5 days of this treatment, but it was extended for another 4 days as the patient started to show urticaria. He subsequently showed progressive improvement in respiratory parameters and was extubated on day 21 of hospitalization. The criteria for hospital discharge were clinical resolution of symptoms, stabilization of underlying diseases, and negative PCR test for SARS-CoV-2. The boy was discharged from hospital negative for SARS-CoV-2 and in good general condition and controlled asthma, without the need for home oxygen therapy. In total, he had been hospitalized for 36 days, with 20 days in intensive care.



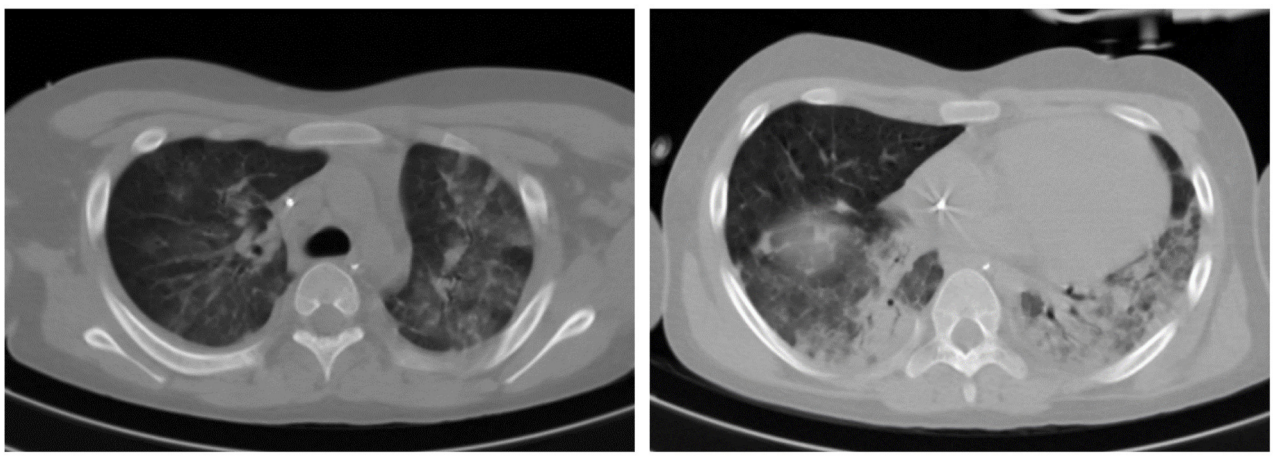


FIGURE 1 | Clinical parameters evolution. **(A)** Timeline of symptoms and treatment during ICU stay. **(B)** Chest tomography of the fourth day of hospitalization with considerable pulmonary consolidation in the left lower lobe and in a smaller part of the right, with diffuse ground-glass densification in the unconsolidated pulmonary portions. ICU, intensive care unit. *Lymphocyte = 1.21 × 10^9/L; **D-dimer = 2,023.5 (ng/mL).

After 80 days, neutralizing antibodies (nAbs) was measured in the patient's plasma; the neutralization end point titer was 640 (high titer), while the expected negative control titer was < 20. The methodology for quantification of nAbs was previously described by Wendel et al. (10). Cytokines were also measured at this time using an 8-Plex Human Cytokine Kit (BioRad); lower levels of inflammatory cytokines (IL-8 and TNFa) were detected in the patient's plasma (**Table 1**) than control (six children without AS that had COVID-19).

DISCUSSION

AS is a rare neurogenetic disorder characterized by microcephaly, severe intellectual deficit, speech impairment, epilepsy, EEG abnormalities, ataxic movements, tongue protrusion, paroxysms of laughter, abnormal sleep patterns, and hyperactivity (11). It has a prevalence of between 1/10,000 and 1/20,000 individuals (12) and is considered a syndromic form of autism spectrum disorder (13). AS results from loss of function of the imprinted ubiquitin-protein ligase E3A (*UBE3A*) gene on chromosome 15q11.2-q13 (11, 14). Although life expectancy appears to be normal, severe complications can occur due to some of the syndrome's symptoms, such as seizures and aspiration pneumonia (12).

To our knowledge, no reports to date have described COVID-19 in patients with AS. Several studies have reported that the signs and symptoms of COVID-19 in children are similar to those in adults, but milder (15). In a recent report, abnormalities in chest CT images were detected among all patients upon admission (16). Typical images included bilateral multiple lobular and subsegmental areas of consolidation, as well as bilateral ground-glass opacity (16). These findings corroborate those found in our patient. In the same study, the average time between hospitalization and admission to the ICU was 3 days (16), which was about the time at which our patient showed symptom worsening and required mechanical ventilation.

Studies have shown lymphopenia is the most common hemogram finding in adults with COVID-19, occurring in as

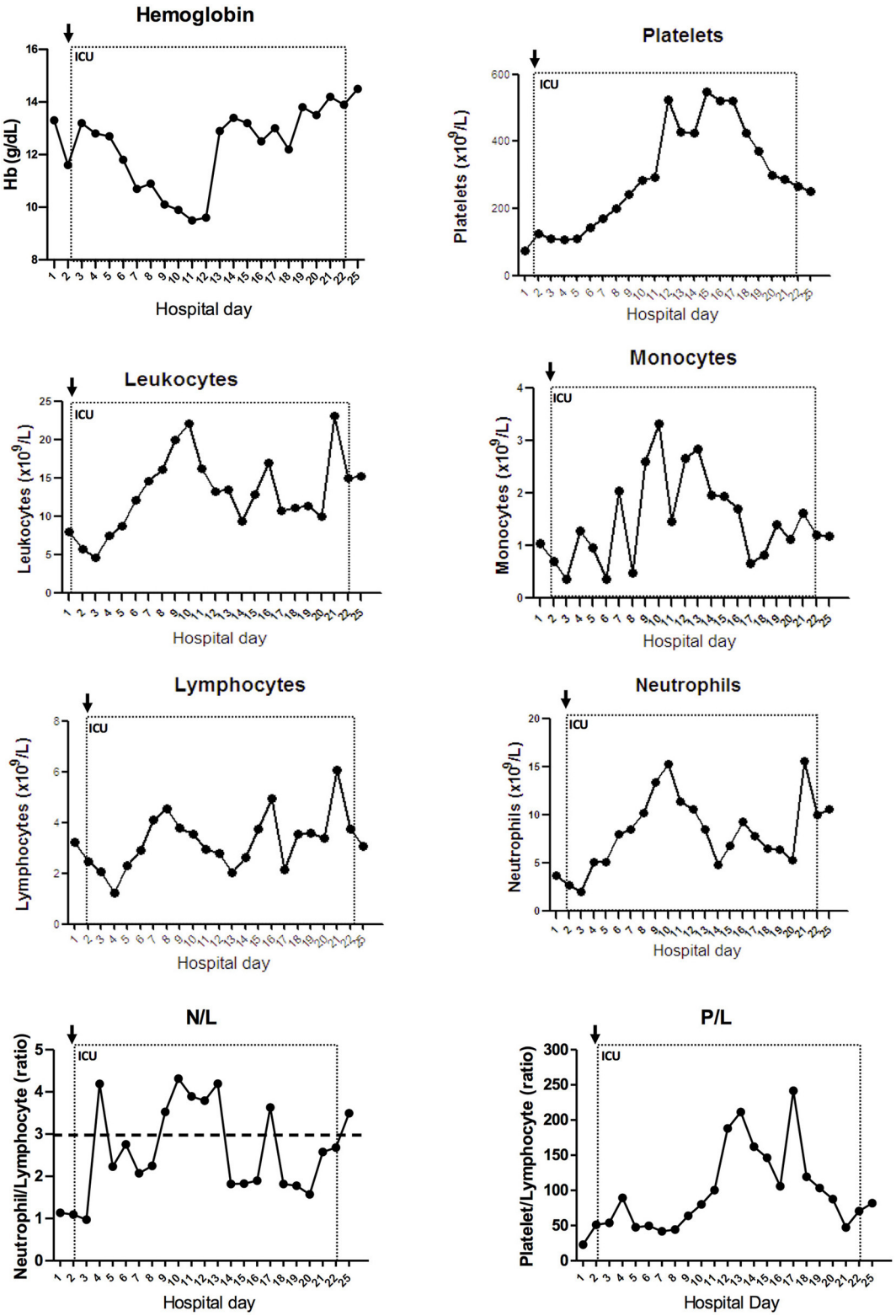


FIGURE 2 | Evolution of blood count markers during hospitalization. Hemogram was evaluated along the hospital stay. Hemoglobin, platelet, total leukocytes, monocyte, lymphocyte, neutrophil count, and the ratios neutrophil-to-lymphocyte (N/L) and platelet-to-lymphocyte (P/L) are represented with connected scatterplot. ICU, intensive care unit. Dotted line represents the ICU stay and the arrow indicates ICU admission. Dashed line indicates N/L threshold with ratio over three associated with severe illness in adults (7).

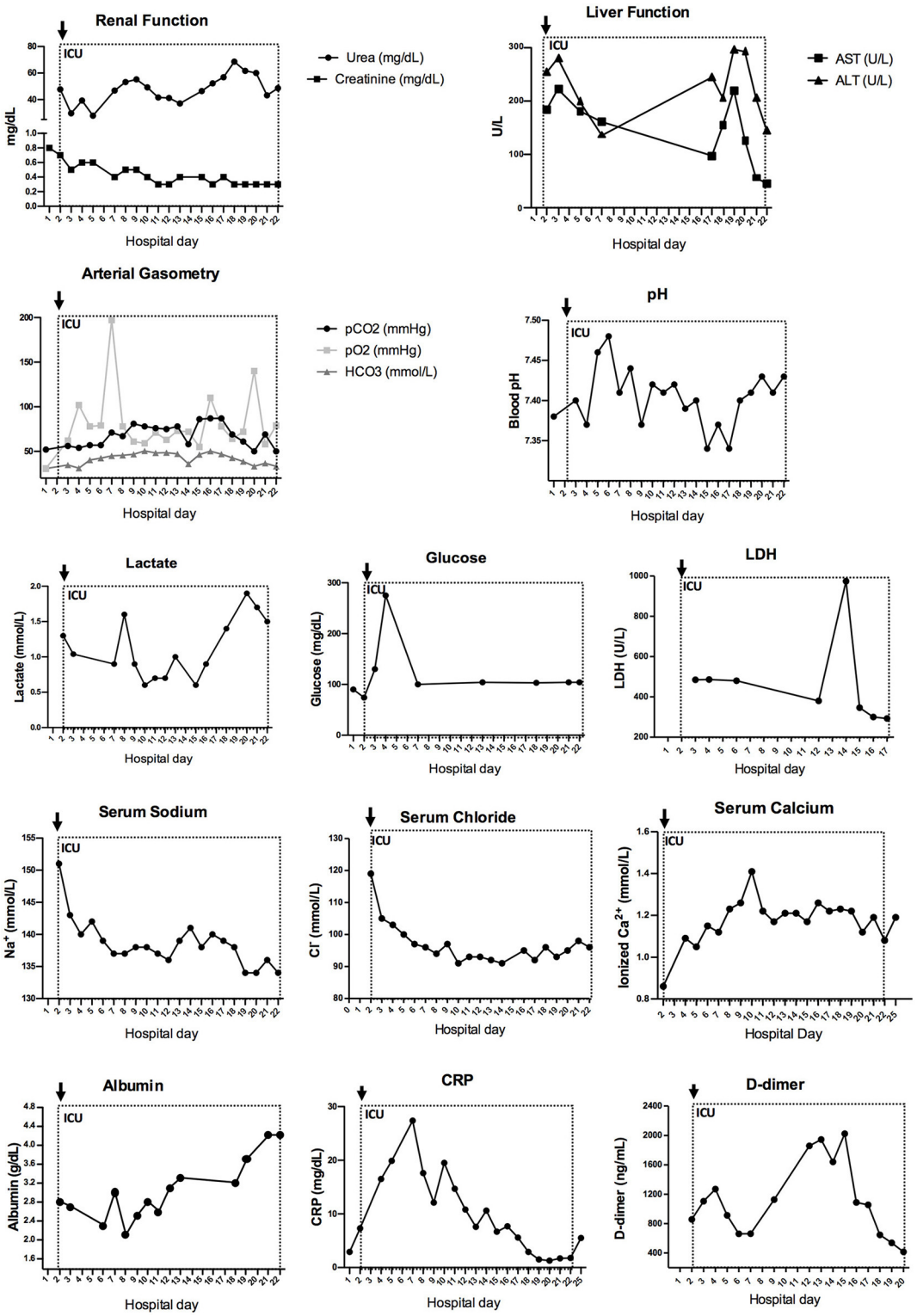


FIGURE 3 | Increased inflammatory profile is observed in arterial gasometry and biochemical markers during hospitalization. Urea and creatinine are combined to represent renal function. AST and ALT are combined to resepresent liver function. Arterial gasometry is shown in pCO2, pO2, and HCO3 combined graph and pH measures. Together with lactate, glucose, LDH, serum sodium, serum chloride, serum calcium, albumin, CRP and D-dimer, these connected scatterplot show the phisiological evolution of the patient during ICU stay. AST, aspartate transaminase; ALT, alanine aminotransferase; pCO2, partial pressure of carbon dioxide; pO2, partial pressure of oxygen; HCO3, serum bicarbonate; CRP, C-reactive protein; ICU, intensive care unit. Dotted line represents the ICU stay and the arrow indicates ICU admission.

TABLE 1 | Long-term plasma cytokines.

Cytokines (pg/mL)	Patient	Control
IL-2	0	0.33 (0.00–0.82)
IL-4	0.11	0.12 (0.01–0.18)
IL-6	0	0.44 (0–2.29)
IL-8	3.37	8.58 (3.62–18.07)
IL-10	0	2.21 (0–12.06)
IFNg	0.35	0.47 (0.30–1.16)
TNFa	14.55	36.00 (16.1–60.57)

Parentheses in Control group are minimum and maximum value.

many as 70.3–83% of hospitalized patients (15), while only being observed in 9.8% of cases (15). This is consistent with the findings in our patient, who showed neither leukopenia nor lymphopenia upon admission to the ICU. In contrast, an N/L ratio > 3.13 has been reported as a risk factor for developing severe illness in adults (8), and our patient presented peaks of N/L ratio over 3. In children, there is likely a negative correlation between LDH levels and severity (17). The literature is still sparse regarding the immune system of patients with AS, although the close relationship with autism may suggest that these patients have a dysfunctional immune response (18). In adulthood, males are prone to infectious illnesses (19). Moreover, one report described a 5-year old boy with AS who had recurrent respiratory infections, hyperactivity, and sleep disturbances; he died of upper airway obstruction due to infectious mononucleosis (20). This case supports the idea that the immune response is compromised in patients with AS. More recently, UBE3A was found to regulate interferon regulatory factor (IRF); lack of UBE3A leads to AS, and interferon cytokines are essential to the anti-viral immune response, so this may intimate a mechanism for the impaired immune response in patients with AS (21). In the present study, we demonstrated that the humoral immune response had been activated, since the level of nAbs for SARS-CoV-2 was detected in the patient's plasma 80 days after discharge. Curiously, the levels of inflammatory cytokines were lower than in the control. Further studies are required to determine whether this was a consequence of the infection or was related to the patient's AS.

The overall levels of acute phase reactants in COVID-19 correlates with disease severity and death. According to a recent study involving 140 hospitalized patients, higher levels of D-dimer and CRP were associated with severe disease (2). In the present case, the patient presented with a slight elevation in CRP and D-dimer levels upon admission to the ICU; the levels progressively increased subsequently, reaching a peak of more than four times the normal value on day 15 of hospitalization, probably because of the excessive inflammatory process accompanied by SARS-CoV-2 infection (22–24). D-dimer is also a marker of coagulation problems, which are also a feature of severe COVID-19 (23). As reported, on the fifth day of hospitalization, echocardiogram detected an image suggestive of a thrombus in the inferior vena cava, and anticoagulant treatment was initiated.

Neurological diseases can increase the risk of lower respiratory tract infection (5). In the present case, the patient had significant cognitive impairment, epilepsy, and sleep disturbance. Cognitive impairment in particular can delay diagnosis, because the child cannot communicate well with the caregiver. Moreover, changes in circadian cycle in the present case may have predisposed the patient to infection. Recent data have shown reciprocal connections between the central nervous system, sleep, and the immune system, as well as the importance of healthy sleep to maintain immune defenses (25). In addition, chronic sleep deprivation leads to sustained activation of the inflammatory response and increased risk of infectious disease, with major impairment of the antiviral response.

Several studies and meta-analyses have suggested asthma is unrelated to COVID-19 infection. In fact, the Th2 immunity in patients with asthma may be protective (26). Allergic sensitization seems to be inversely associated with ACE2 expression in the nasal epithelium, which is the main receptor through which SARS-CoV-2 enters the cell (26). However, another study showed that severe asthma can be a risk factor for increased mortality in hospitalized patients (27). In the present case, the patient's asthma was controlled, so it probably did not contribute to the severity of his condition.

Although the patient had produced a large amount of nAbs, it is not possible to determine whether these antibodies were responsible for his recovery or simply a consequence of intense, symptomatic disease. The attempt to use convalescent plasma to treat COVID-19 in clinical trials could shed light on this question; initial studies have presented positive data in this regard (28, 29). However, in one study including 228 patients with COVID-19 and pneumonia, some of the patients were treated with convalescent plasma, but the therapy showed no significant clinical benefit (30). Although it is not yet clear how much the nAbs contribute to recuperation from COVID-19, the use of pharmacological treatments has been further investigated.

When the patient was admitted to the hospital, azithromycin was thought to be effective against SARS-CoV-2, since it is commonly used to treat bacterial respiratory infections. Advances in research showed that the drug did not improve clinical outcomes over standard care (31).

Methylprednisolone was probably a main contributor to the patient's recovery, since several clinical trials have shown that administration of corticoids (dexamethasone, hydrocortisone, or methylprednisolone) is associated with lower mortality in critically ill patients with SARS-CoV-2, regardless of whether they are receiving mechanical ventilation (32).

The knowledge of COVID-19 infection in children is still sparse, and future studies must report the interaction between COVID-19 and particular syndromes. In particular, severe cases that are relevant to a specific subpopulation should be reported. To that end, in the present study, we presented the first documented case of a teenager with AS who had severe symptoms of COVID-19. The report might contribute to the understanding of COVID-19 and viral infection in patients with AS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by HMIMJ 223 4.042.665, HSL 4.045.959, ICB-USP 4.076.553. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AL, DG, FO, DB, and MA designed the study. AL, CC, TB, and MA wrote the report. DG, FO, NC, and DB revised and added intellectual content. AL, AF, JB, RB, AB, and LM assisted with infection management of the patient and proofread the report. CC, TB, RB, GP, AS, LD, IA, DO, EB-J, LB-A, and LH provided the collection of data and biological sample from the patient. CC, TB, DO, RM, LT, and MH performed the experimental analyses. All authors contributed to the article and approved the submitted version.

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First Movers in Molecular Detection: Case Comparison on Harnessing Research and Development, Industry, and Entrepreneurship

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The current unprecedented COVID-19 pandemic underscores the importance of diagnostic assays in health security preparedness and readiness. Advancing new technologies for rapid molecular detection of high consequence infectious pathogens is an ongoing challenge that requires ingenuity and vision. Sustainment of a robust supply chain for materials and the logistics of timely product delivery further challenge diagnostic kit and device manufacturers. Business economists often characterize technology companies that discover unique breakthroughs in their field and are first to bring related products to market as first movers. From a market perspective, three first mover characteristics include: having the knowledge and capability to address a unique breakthrough, excellent technological leadership, and the ability to capitalize on the opportunity. Current mainstays for molecular detection include using Taq DNA Polymerase enzyme and fluorescent chemistry for quantitative PCR (qPCR). A newer and promising technology uses CRISPR-Cas proteins for nucleic acid detection. Our panel discussion from the 2020 ASM Biothreats conference, which included members from two prototypical first mover companies, explored their respective corporate experiences. Both companies were selected for the discussion based on their revolutionary innovations and similarities in their research and development, corporate culture and trajectory. One company, established over 20 years ago, became a market leader in the biothreat detection market by advancing air thermocycling qPCR across multiple product families. The second company is a rapidly growing start-up and a scientific pioneer in establishing next generation CRISPR technologies. Here we discuss their technology development, product deployment, and customer markets to draw lessons learned for researchers, end users, and funders.

Keywords: COVID-19, molecular detection, CRISPR, qPCR, first mover

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Like the technological revolution and the Industrial Revolution before it, the biological revolution will reshape how we interact with and understand the world around us[1]

- Former United States Senator Cory Gardner.

[1]https://www.floridadaily.com/marco-rubio-bioeconomy-research-and-development-act-will-help-with-vaccines/.

BACKGROUND

As part of a panel discussion at the 2020 American Society for Microbiology's (ASM) Biothreat Conference, we discussed and contrasted experiences on technology and business with representatives from two prototypical first mover companies in the biotechnology industry. The corporate panel participants and major topics are described in **Figure 1**. This event, inspired by a similar panel discussion on "Molecular Detection in the Field" at the 2019 ASM Biothreats conference, is part of a continued collaboration to develop and publish current findings on the importance of rapidly detecting and identifying high consequence pathogens. Presenting a panel discussion to a diverse audience that includes researchers, end users, funders, and programmers is a unique and important forum to stimulate new ideas (1). Our findings are relevant across science and business sectors especially to reinforce public and private partnerships.

The clinical and biodefense diagnostics markets are each valued at over 1.5 billion dollars per year (2, 3). As a result, a significant number of laboratories are involved in molecular detection including product manufacturers, test and reference laboratories, academic institutes, and commercial research organizations. While maintaining country preparedness and readiness to respond to infectious disease outbreaks—whether intentional (bioterrorism), accidental, or naturally occurring—is typically a national priority, the niche markets created for biodefense, are expensive for commercial entities and may not be profitable due to the relatively low frequency of these events. Real-world events catalyze niche markets as in the case of the 2001 anthrax letter attacks where the United States (US) Government response had the effect of continuing biodefense related activities and establishing that market as an enterprise. Prior to this event, national laboratories and research organizations typically performed research and development (R&D) in biodefense. More recently, the biodefense market attracts those in other fields contributing to an expanded range of products (3). The availability of national biodefense funding for public corporations and private institutes allows them to remain viable in their respective markets (4). Achieving a return on investment is especially challenging for companies that develop cutting-edge technologies and define new market segments involving diagnostic assays, medical countermeasures, and protective equipment. There are additional challenges to take into account when addressing outbreaks caused by emerging and re-emerging pathogens; SARS-CoV, MERS-CoV, Zika virus, and SARS-CoV-2 are prime examples of viruses with significant human and economic impacts.

Detection and identification of microbiological pathogens including bacteria, fungi, and viruses requires the presence of the organism, or some part of it such as nucleic acid material. Pathogenic microbes are often categorized into Risk Groups 1–4 according to their "capability to cause disease in a susceptible human or animal host, virulence as measured by severity of the disease and the availability of effective preventative measures and treatments" (5). Risk Group 3 and 4 pathogens that infect animals, humans, and plants may also be classified as biological threat agents (biothreats) due to the potential for nefarious use (6, 7). Designing molecular detection methods such as qPCR and reverse transcription qPCR (RT-qPCR) relies on an *a priori* knowledge of the biological agent of interest such as the sequence of the DNA or RNA, respectively (8, 9). The advent of more affordable and available sequencing and bioinformatics platforms has enabled the determination and publication of microbial sequences with a fast turn-around time. For example, the first sequence data for SARS-CoV-2 (Risk level 3) came weeks after the first cases of a novel infectious disease were identified and the virus was isolated in Wuhan, China (10). This enabled the US Centers for Disease Control and Prevention and commercial SARS-CoV-2 RT-qPCR assays to be designed, tested and deployed with Emergency Use Authorization (EUA) by the US Food and Drug Administration (FDA) in record time.

First movers include companies and organizations that have developed a discriminator or first mover advantage, which is usually a process or product derived from intellectual property that places them ahead of their competitors. This discriminator often confers a technological edge for a first mover and early market entry, but does not guarantee market share and sustainable growth as competitors can still catch up later (11). In marketing theory, as described by Lieberman et al., first movers are often validated by leading their respective markets in sales and market share (12). First movers are also recognized by their ability to develop and commercialize leading technologies that help create new markets (13). There are several first mover theories and depending on the business case, it can be argued that although first movers have an early advantage, second movers (also called fast followers) also have advantages (14). The COVID-19 pandemic has created a niche market for first movers, fast followers, and others reacting to the need for diagnostics to inform medical care and immunological assays necessary for the research and development of vaccines and therapeutics.

In addition to pioneering a technological edge, we considered two first mover advantages by Lieberman: possessing technological leadership, and capitalizing on a feasible opportunity (12). Breakthrough technology is an innovation into a specific market space and measured according to market share. Technological leadership is sustained through discovery of those innovations that can be patented and processes and products which result in cost advantages (12). A feasible opportunity may be created among the success of that company's (and their competitors) breakthrough technology, skill, and serendipity.

Our first company, BioFire (formerly Idaho Technology, Inc.), developed a breakthrough technology using air-thermocycling to perform real time qPCR in 40 minutes which was half the time of the conventional Peltier heat block thermocyclers (15). When combined with probe chemistries such as fluorescent dyes, rapid analysis and identification of target nucleic acids can be performed. We refer to technological leadership as the action of a company visionary, often the founder, whose contribution can be measured by the number of peer-reviewed publications and patents and the ability of the company to maintain its vision. Biofire was founded in 1990 by Mr. Kirk Ririe while he was at the University of Utah, based on a feasible opportunity that arose to develop technology for the rapid detection of biowarfare

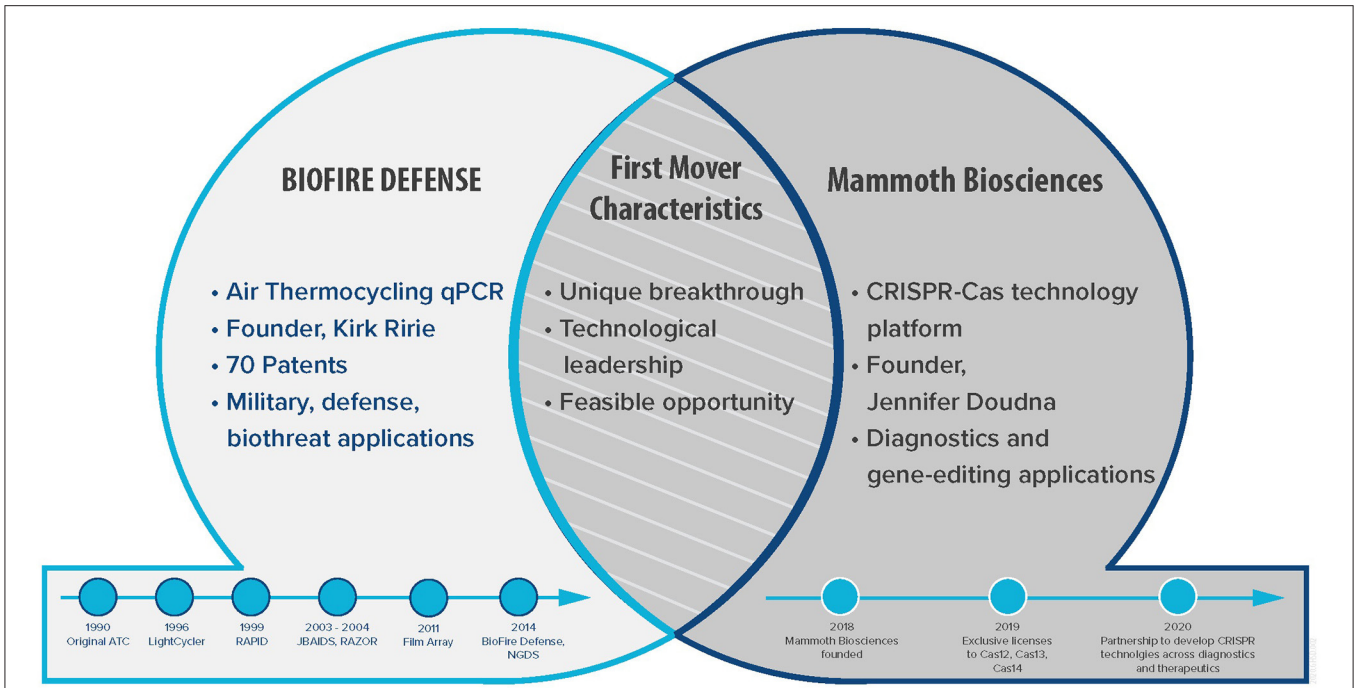

FIGURE 1 | First Movers. This Venn diagram illustrates the two prototypical first mover companies in biotechnology.

agents. Together with the US Air Force as a product champion, they optimized an existing laboratory-based qPCR platform to enable development of an instrument with a rugged form factor, freeze dried reagents, automatic data analysis, and a simplified user interface. This product became the R.A.P.I.D.® System that Idaho Technology, Inc. further developed under a major DOD contract called the Joint Biological Agent Identification and Diagnostic System (JBAIDS). JBAIDS became the first biologic identification platform with FDA 510(k) clearance and a benchmark for DOD acquisition of medical devices (16). Successful performance of this contract afforded corporate growth and Idaho Technology Inc. was renamed BioFire Defense which is now an LLC of Compagnie Merieux Alliance based in the US, and BioFire Diagnostics, a subsidiary of bioMerieux. Overall, BioFire Defense has developed and deployed four different military field-forward qPCR systems (R.A.P.I.D.®, RAZOR™, JBAIDS, and FilmArray®). In addition, FilmArray® is now a Clinical Laboratory Improvement Amendments (CLIA) waived system.

Similarly, Mammoth Biosciences launched in 2018, based on CRISPR technologies developed in the lab of CRISPR pioneer Professor Jennifer Doudna from the University of California, Berkeley. She and Professor Emmanuelle Charpentier were recently awarded the 2020 Nobel Prize in chemistry for this work. The company was founded with the vision of harnessing the natural diversity of CRISPR to enable a multitude of diagnostics and therapeutics applications across biotechnology. Further advancing molecular-based detection, Mammoth Biosciences discovers and develops novel CRISPR proteins, coupled with programmable guide RNAs (gRNA), to search through genetic material from a sample for matching DNA or RNA sequences. Their DNA Endonuclease Targeted CRISPR *Trans* Reporter (DETECTR™) platform leverages specific properties of Cas protein variants that trigger indiscriminate cleavage of any nearby single-stranded nucleic acid when target sequences are recognized (17). DETECTR™ has been configured into reagents kits offering high analytical precision for use on laboratory instrumentation[2]. The company is also leveraging the DETECTR™ platform in the development of diagnostic tests for use in decentralized settings. Their mission is to improve lives by reading and writing the code of life, with a key focus on developing next-generation diagnostics that address needs from centralized to decentralized testing environments.

DISCUSSION

Real World Events as Feasible Opportunities

Capitalizing on a feasible market opportunity according to Lieberman, such as a real world event, is considered a first mover advantage. In reality, investment in R&D and long-term relationships with product champions prepared both companies to respond effectively to extraordinary real world events. In 2001, following the anthrax letter attacks, BioFire Defense answered the call to provide military and civil support end users field-forward systems for rapid biodetection. A similar scenario occurred in early 2020 when the COVID-19 outbreak was just

[2]https://www.nibib.nih.gov/covid-19/radx-tech-program/radx-tech-phase2-awards.

emerging and efforts by clinicians and scientists to contain and understand the disease were underway. BioFire Defense and Mammoth Biosciences, among many others, were positioned well to swiftly respond with diagnostic assays to detect SARS-CoV-2. BioFire Defense leveraged their FilmArray® platform to support COVID-19 testing for their clients in the military and defense market. The Mammoth Biosciences team developed a prototype point-of-care device for SARS-CoV-2 analogous to a standard at-home pregnancy test where the result can be visualized on a lateral flow strip (18). As of August 2020, the FDA has issued over 170 EUAs for *in vitro* diagnostics including molecular tests by BioFire Defense on March 23, 2020 and Mammoth Biosciences for their SARS-CoV-2 DETECTR™ Reagent Kit on August 31, 2020[3].

Shared Similarities

During our discussion, both companies expressed similar experiences and shared values in their workforce development and corporate growth. Instilling a can-do attitude and get-it-done discipline is important for start-up and small companies with limited resources along with maintaining mission focus. Strong and supportive leadership, to direct limited resources and create the vision for a long-term unique product differentiator, and the ability to develop strategic partnerships were also cited. For example, BioFire was founded with internal and external funding and expanded consistently from 40 employees in the 1990's to over 500 in 2015 when they were acquired by bioMerieux. Today, they have over 2,000 employees and the company is part of a large international company. Mammoth Biosciences founded in 2018 has raised $45M in series B venture funding, grown to over 70 employees and recently moved from an incubator laboratory space to larger facilities in the South San Francisco area. Mammoth Biosciences raised venture capital early in the company's trajectory while BioFire's early funding was from internal and external small business innovation research programs. Both companies agreed that defining priorities and focusing on project execution continues to be important and recognizing that early limited resources will be less effective when divided among competing priorities. Mammoth Biosciences also pointed out the importance of developing strong partnerships where the partners can leverage each other's strengths and technical toolboxes.

Thriving as First Movers

Continuing research activities to discover revolutionary technologies and seeking those that are true differentiators was an attribute for both companies. Lieberman and others describe one advantage of first mover technological leadership as the ability to develop intellectual property using R&D expenditures. Anecdotally, when companies invest more of their resources toward internal R&D they tend to be more successful than those who do not. Identifying product champions especially those considered early market adopters is critical for gaining insights on product use and market behavior. While close collaborations with product champions help strengthen scientific validity and reinforce market credibility, the product champion is not necessarily a customer. It is important to distinguish between a product champion and a primary customer early on to define the expectations of each and avoid conflicts. Another observed characteristic is that business leaders tend to be very mission focused especially those at first mover companies who tend to self-invest in R&D first and acquire external funding later.

Observations From the Panel Discussion

During the discussion "Harnessing R&D, Industry, and Entrepreneurship," our panel aimed to link first mover advantages with our two prototypical companies. From our discussion that included one audience question that was timely and based on the need for a novel SARS-CoV-2 detection assay, we note the importance of reinforcing awareness and the need for continual funding in the area of biodefense technologies, including those that contribute to outbreak preparedness. Similar comparisons can be made from medical countermeasures, therapeutics, and vaccines and are beyond the scope of our discussion. When our panel developed this discussion, we recognized this limitation and only focused on two biotechnology companies harnessing revolutionary detection technology.

We categorized these current challenges according to first mover advantages below:

1) Current challenges exist to develop assays for emerging agents quickly, especially for those targets that are in less market demand. Some of these challenges are technical and may offer opportunities for first movers.

- Due to the *a priori* approach for designing molecular assays, there is a constant need to revise assays and test them against newly discovered strains. This so-called "signature erosion" is more apparent in agents that are rare and have significant genomic variation between isolates. The assays must be continually adapted to maintain their sensitivity and specificity when new strains arise. DETECTR™ may address this because guide RNAs are rapid to design and manufacture.
- Working on an assay for a new or rare infectious disease is complicated by the fact that it is difficult to get enough sample numbers for verification and validation testing. Contrived samples must be used in this case. With the FDA EUA, companies have more options to validate their assays in a timely and affordable manner. In addition, insurance reimbursements in clinical diagnostic market further complicates decisions on which assays to develop.
- The diagnostics market is looking for rapid molecular tests. For example, point-of-care tests to obtain a result while the patient is still in the office. For COVID-19, high-throughput molecular and serological tests are needed to determine who may be infected and to provide serological surveillance of recovered patients. There is also a demand for point-of-care tests and screening assays that can differentiate SARS-CoV-2 from other viral respiratory diseases such as influenza, other coronaviruses, and respiratory syncytial viruses.

[3]https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas.

- Perhaps someday technical breakthroughs will enable assays to be developed more quickly and address smaller markets that have been less emphasized such as those neglected tropical diseases often mentioned in global health.

2) The COVID-19 pandemic highlights the continual need to prioritize diagnostic assay development and strategic funding to invest in preparedness. Recent US Government priorities such as Health and Human Services "Operation Warp Speed" and the National Institutes of Health "RADx" create further feasible opportunities.

- Noting that private and commercial sector funding is important, US Government funding agencies such as Biomedical Advanced Research Authority (BARDA), DARPA, and USAID have incorporated cost sharing to offset investment; associated funding needs to include incentives that create and encourage private-public partnerships.
- In some respects, the clinical diagnostics market like inkjet printers is a razor and blades business model. Instruments and assays are required and complement each other where assays like razor blades and inkjet cartridges are highly consumed. Biofire Defense noted that their development and production of these multiplex assays was strengthened by an effective logistics and supply chain, highlighting their successes in markets driven by major real world events such as the outbreak of Ebolavirus in 2014 and the ongoing COVID-19 pandemic in 2020.
- US Government defense spending is valuable because it does not dilute the value of the company. Merging two funding streams is useful is the projects are overlapping e.g., COVID-19.
- Stock (shareholders) and Venture capital investment can impinge on how an assay is conceived. This is also true for government funding. Government vs. private funding also effects company evolution.
- Future technology being investigated by BioFire Defense includes "Extreme PCR," which is a phrase coined by its inventor, Dr. Carl Wittwer (one of Idaho Technology's and BioFire's founders), and refers to techniques that can shorten qPCR runs from 10 minutes to 10 seconds (19).
- Mammoth is discovering and developing novel Cas proteins, expanding the CRISPR toolbox, and forging new partnerships in multiple applications areas across healthcare and non-healthcare verticals.

CONCLUSIONS

The biopharmaceutical industry has a "valley of death" due to the cost, technical, and regulatory challenges associated with clinical trials. The challenges are similar in the clinical diagnostics industry and risk aversion favors the prioritization of products with higher profit margins. Outbreaks of novel infectious diseases, although relatively rare, will continue to set the stage for new first movers in diagnostics, and funding strategies with incentives to encourage industry to engage is critical. In addition, consumer demand for easy to use, point-of-care clinical diagnostics with low cost and environmental burden will continue to increase. Additionally, novel approaches to speed up testing and to reduce the environmental burden of testing, such as reusable and recyclable materials, are predicted to become an area of innovation within diagnostics.

Life science enterprises such as biopharma and biotechnology, including diagnostics, are part of the US bioeconomy that is estimated to be 2% of the US Gross National Product (20). Initiatives that support the bioeconomy include the goal of nurturing, supporting, and sustaining a domestic biosciences industry (21). During the COVID-19 pandemic, the need for diagnostic assays for molecular and serological surveillance and the demand for scalable diagnostic capabilities from both central laboratories and point-of-need locations continues to increase. First mover companies such as BioFire Defense and Mammoth Biosciences were well-positioned to tailor their molecular diagnostic assays and started developing within weeks of identification of the SARS-CoV-2 viral sequences. Biosurveillance and rapid reliable diagnostics in the midst of a pandemic ultimately saves lives. The economic consequences resulting from the COVID-19 pandemic are a reminder that the bioeconomy, particularly biotechnology and synthetic biology, remains an important component and driver of the global economy. Continual investment in R&D and successful business practices will promote resilience in this sector.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KY developed the panel discussion with MS, JM, and GO. KY moderated the panel discussion with MS. JM provided notes from the panel discussion. KY drafted the manuscript with contributions from MS, JM, and GO. All authors reviewed and agreed with the manuscript.

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ORIGINAL RESEARCH
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Analysis of Epidemiological and Clinical Characteristics of COVID-19 in Northwest Mexico and the Relationship Between the Influenza Vaccine and the Survival of Infected Patients

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The first cases of unexplained pneumonia were reported in Wuhan, China, in December of 2019. Later, a novel coronavirus (SARS-CoV-2) was identified as the causal agent of pneumonia. This virus has since spread to more than 180 countries and has been declared a pandemic by the World Health Organization. Herein, we aimed to determine the epidemiological and clinical characteristics of symptomatic patients with coronavirus disease 2019 (COVID-19) and the relationship between the influenza vaccine with a lower risk of severe COVID-19 infection in the state of Sinaloa. We collected demographic and clinical data of 4,040 patients with acute respiratory infections across Sinaloa state hospitals from February 28 to May 15, 2020. The prevalence of COVID-19 among hospitalized patients with respiratory symptoms in Sinaloa showed 45.2% of men were more affected than women ($p < 0.001$), and people aged 40–49 years were the most affected. The main symptoms of COVID-19 infection were cough and fever ($p < 0.001$), while hypertension, obesity, and type 2 diabetes were the chronic diseases associated with COVID-19 than non-COVID-19 ($p < 0.003$). Healthcare workers were most likely to be infected compared to other occupations ($p < 0.001$). The general lethality rate was 14.1%, and males >62 years were the ones who had a higher lethality rate ($p < 0.001$); the aforementioned chronic diseases were related to higher lethality of COVID-19 ($p < 0.001$). Likewise, higher lethality was seen in housewives and patient retirees/pensioners compared with other occupations ($p < 0.001$). Finally, we found there was a relationship

between influenza vaccination and a lower risk of severe COVID-19 infection and mortality ($p < 0.001$). These findings showed that healthcare workers, men >62 years with chronic diseases, and retired people were most affected. Furthermore, the influenza vaccine could decrease the severeness of COVID-19 cases.

Keywords: COVID-19, Mexico, influenza vaccine, clinical characteristics, epidemiological

INTRODUCTION

Coronavirus belongs to a family of viruses that cause symptoms related to respiratory diseases. Although these viruses predominantly have animal hosts, they can be transmitted to other species via different mechanisms to infect new hosts, including humans. In December 2019, Wuhan, China, reported the first cases of pneumonia caused by an unknown pathogen. Later, on December 30, 2019, a novel virus was isolated from the bronchoalveolar lavage fluid of patients with acute pneumonia in the Jinyintan Hospital (1).

Soon after, the World Health Organization (WHO) identified a novel coronavirus (2019-nCOV) as a causal agent of pneumonia reported in China (2). This new coronavirus was identified as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses (3). SARS-CoV-2 is highly contagious and the causative agent for coronavirus disease 2019 (COVID-19). Globalization and international mobility facilitated the spread of the disease to more than 160 countries, leading to more than 179,112 infections and 7,426 deaths; accordingly, the WHO declared COVID-19 as a pandemic on March 11, 2020 (4).

COVID-19 has also affected Sinaloa; this state housed the first COVID-19 diagnosed case in Mexico on February 28, 2020. The case was a 41-year-old man who had traveled to Italy. This person was confined to a hotel until he recovered from the infection. After that, COVID-19 spread to the rest of Mexico.

The importance of this study in Sinaloa lies in the large proportion of the population that suffers from chronic diseases, such as obesity, type 2 diabetes, and hypertension. In fact, Sinaloa is one of the 10 main states with obesity and diabetes cases in Mexico (5). The association among chronic diseases and COVID-19 severe infection is well-studied (6). Mexico is one of the main countries with obesity worldwide; Sinaloa is one of the states with a higher burden of this disease in the country, and the new COVID-19 disease could thus affect the population more in this state. Here, we reported the prevalence and epidemiological and clinical characteristics of COVID-19 cases in Sinaloa: one of the first states in Mexico with reported SARS-CoV-2 infections. We also analyzed the association between comorbidities and non-communicable diseases (NCD) with COVID-19 lethality, taking into account their high burden in Mexico. Finally, we analyzed the relationship between influenza vaccination and survival among patients with COVID-19.

MATERIALS AND METHODS

Region of Study

This study was carried out in Sinaloa State, located in the Northwest of Mexico. This state has a population of 2,966,321 subjects. In all, 50.6% (1,502,236) of them are women and 49.4% (1,464,085) are male. In terms of age, 44.8% (1,330,837) of the population in Sinaloa are between 0 and 24 years old, and 33.4% (992,097) are 40–85 years old (Instituto Nacional de Estadistica y Geografia, www.inegi.org.mx).

Source of Data

Demographic data (sex, age, social security, and occupation), clinical information (comorbidities, signs and symptoms, history of influenza vaccination, and clinical outcome) from patients who sought care for respiratory symptoms, and the result of SARS-CoV-2 polymerase chain reaction (PCR) testing were collected between February 28 (date of first COVID-19 case in Sinaloa) and May 15, 2020; we identified 6,933 patients from different health institutions of Sinaloa state. Among them, the Mexican Social Security Institute (Instituto Mexicano del Seguro Social, IMSS), Hospitals of Health Services Sinaloa (Servicios de Salud, SSA), Institute of Security and Social Services for State Workers (Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado, ISSSTE), private hospitals, and hospitals from other government agencies were included in this cross-sectional and exploratory study.

COVID-19 was confirmed based on positive qRT-PCR results for SARS-CoV-2 and upon presentation of characteristic symptoms of the disease, whereas influenza H1N1, respiratory syncytial virus, influenza A H3 and B, enteroviruses, and rhinovirus infections were diagnosed according to Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza and the Institute of Epidemiological Diagnosis and Reference (Instituto de Diagnóstico y Referencia Epidemiológicos, InDRE) standards (7). Patients negative for SARS-CoV-2 but who were affected by other respiratory viruses were classified as non-COVID-19.

Of 6,933 patients who sought care for respiratory symptoms (inclusion criteria), 4,040 were tested by qRT-PCR to identify SARS-CoV-2 infection. Samples that did not meet quality standards or triage criteria (symptoms of COVID-19) were excluded ($n = 2,893$).

Statistical Analysis

Pearson's chi-square tested differences between groups and categories (age-group, sex, PCR test result, symptoms, clinical outcome, comorbidities, vaccination history, occupation, lethality, or survival).

Pearson's chi-square was also used to compare the effects of underlying diseases, prior vaccinations, and occupation on lethality. Moreover, prior vaccination was analyzed by a multivariate test, which was adjusted by sex. A $p \leq 0.05$ was considered to indicate statistical significance; a Bonferroni correction was applied to avoid alpha 1 error. The data were

FIGURE 1 | Distribution of COVID-19 cases by age range. The 1,826 COVID-19-positive patients were divided by age range.

analyzed using the statistical package SPSS® Statistics version 24 (IBM Corp., Armonk, NY, USA); the graphs were constructed with the SigmaPlot version 12 program (SYSTAT, CA, USA).

Ethical Approval

The study was approved by the Ethics Committee of The Women's Hospital, Secretariat of Health (No. 202005-03) and was conducted following the ethical principles of the World Medical Association Declaration of Helsinki. Patients' personal identities and other private information were anonymized; therefore, the need for informed consent was waived in accordance with local legislation and national guidelines (NOM-012-SSA3-2012).

RESULTS

Characteristics of Study Population and Distribution of COVID-19 Cases

From February 28 (date of the first case of COVID-19 registered in Sinaloa), 4,040 patients with symptoms related to COVID-19 were included in this study in which 49.8% were female and 50.2% were male. The mean age of male and female patients was 46.5 and 43.9 years, respectively ($p < 0.001$). The prevalence of SARS-CoV-2 was 45.2%, 0.6% influenza H1N1, and 0.6% other viruses (respiratory syncytial virus, influenza A H3 and B, enteroviruses, and rhinovirus). COVID-19 infection was more common in male (52.2%) than female (44.8%) patients ($p < 0.001$); additionally, the mean age of male patients was higher than that of female patients (49.7 vs. 47.3 years, respectively, $p < 0.002$). The youngest patient with COVID-19 was a 15-day-old male infant, while the oldest was a 97-year-old man. The most affected age groups were 40–49 years followed by 30–39 years and 50–59 years (**Figure 1**).

Symptoms and Comorbidities in People With Respiratory Diseases

A comparison of the COVID-19 and non-COVID-19 symptoms showed that cough, fever, arthralgia, poor general health, dyspnea, chills, chest pain, diarrhea, polypnea, and cyanosis were associated with COVID-19 infection ($p < 0.01$), while myalgia, headache, runny nose, and odynophagia were predominantly associated with non-COVID-19 infections (**Table 1**). The remaining symptoms were found in similar proportions in all patients. Moreover, hypertension (28.3%), obesity (21.7%), and type 2 diabetes (17.8%) were the chronic diseases more prevalent

TABLE 1 | Clinical characteristics related to COVID-19 and non-COVID-19 patients.

Symptoms	COVID-19 % (1,826)	*Non-COVID-19 % (2,214)	*p*-value
Cough	82.8 (1,511)	75.4 (1,669)	< 0.001
Fever	79.0 (1,441)	61.8 (1,368)	< 0.001
Headache	75.8 (1,381)	76.3 (1,686)	0.71
Myalgia	58.5 (1,057)	76.3 (1,082)	< 0.001
Arthralgia	55.3 (55.3)	45.3 (993)	< 0.001
Poor general health	51.5 (51.5)	40.2 (881)	< 0.001
Dyspnea	45.6 (832)	31.8 (703)	< 0.001
Chills	39.3 (711)	31.6 (693)	< 0.001
Sudden onset of symptoms	38.2 (662)	32.5 (618)	< 0.001
Irritability	35.8 (653)	35.5 (719)	0.02
Odynophagia	35.6 (643)	37.3 (816)	0.25
Chest pain	34.4 (623)	28.6 (626)	< 0.001
Runny nose	31.7 (570)	34.0 (746)	0.1
Diarrhea	18.9 (345)	12.2 (270)	< 0.001
Polypnea	15.9 (288)	11.2 (246)	0.001
Abdominal pain	11.3 (204)	10.6 (232)	0.48
Conjunctivitis	8.4 (152)	9.8 (214)	0.14
Vomiting	7.6 (138)	5.9 (130)	0.03
Cyanosis	2.9 (53)	1.7 (38)	0.01

**Non-COVID-19 includes others virus and those with negative real-time RT-PCR test results. Pearson's chi-squared test was performed with Bonferroni correction to check for statistical significance. p < 0.002.*

TABLE 2 | Underlying diseases related to COVID-19 and non-COVID-19.

Underlying diseases	COVID-19 % (1,826)	*Non-COVID-19 % (2,214)	*p*-value
Hypertension	28.3 (517)	18.1 (400)	0.001
Type 2 diabetes	17.8 (324)	11.1 (245)	0.001
Obesity	21.7 (395)	18.0 (398)	0.003
Smoking	6.5 (119)	7.3 (162)	0.31
Cardiovascular disease	5.0 (91)	3.8 (84)	0.06
Chronic kidney disease	3.2 (58)	2.3 (51)	0.08
COPD	2.9 (52)	3.0 (66)	0.43
Immunosuppression	2.1 (38)	2.3 (50)	0.7
Asthma	2.7 (49)	6.2 (137)	0.001
HIV	0.5 (10)	0.7 (15)	0.6

*COPD, chronic obstructive pulmonary disease; HIV, human immunodeficiency virus. *Non-COVID-19 includes other viruses and those with negative real-time RT-PCR test results. Pearson's chi-squared test with Bonferroni correction to check for statistical significance. p < 0.002.*

in COVID-19 patients ($p < 0.001$), whereas asthma was more common in non-COVID-19 patients (6.2%, $p < 0.001$; **Table 2**).

Clinical Outcomes

Disease progression was classified as follows: (i) if the patient continued with treatment, (ii) if the patient was transferred to another hospital, or (iii) if the patient died. Comparisons showed that a higher proportion of non-COVID-19 patients were discharged than COVID-19 patients (23.1 vs. 11.4%, $p < 0.001$), while a greater number of patients with COVID-19 infections died than those with non-COVID-19 infections (14.1 vs. 3.4%, $p < 0.001$; **Figure 2**).

Occupational Risk for SARS-CoV-2 Infection

We found that healthcare workers, including nurses, lab workers, doctors, dentists, and workers from hospital-support areas, were the most infected (27%). Further, other employees (any employment not mentioned above, 18.5%) and housewives (15.6%) were more infected than retirees, students, farmers, drivers, teachers, merchants, and managers or business owners ($p < 0.001$; **Figure 3**).

Lethality of COVID-19 in Sinaloa

Of 1,826 hospitalized patients diagnosed with COVID-19 included in this study, 57.9% were ambulatory, and 42.1% needed hospitalizing. The general lethality rate was 14.1% (258/1,826): 6.6% (17/258) were ambulatory, and 93.4% (241/258) were hospitalized patients. Overall, 67.4% of deaths occurred in male and 32.6%, in female patients ($p < 0.001$). With respect to age, lethality in COVID-19 patients ranged from 1 to 95 years. The mean age of deceased patients was 62.37 years, and the age-group most affected was 70–79 years (24%), followed by 50–59 years (23.6%).

The first COVID-19 case in Sinaloa was registered on February 28, 2020. However, it was not until March 25, 2020, that the number of cases increased gradually to reach 10 deaths per day on April 10 (42 deaths in 16 days). Then, a slight dip occurred until April 16 (3 deaths per day) followed by a further increase to 10 deaths per day on April 26 (135 deaths in all). On May 5, a new spike was recorded with 14 deaths occurring in a day. Finally, on May 15, 258 deaths were confirmed according to the Ministry of Health report (**Figure 4**).

Clinical Outcomes in COVID-19 Patients

We found a higher burden of the following underlying chronic diseases in deceased patients than those that survived, respectively: hypertension (52.1 vs. 24.4%, $p < 0.001$); type 2 diabetes (36.6 vs. 14.7%, $p < 0.001$); obesity (31.9 vs. 20%, $p < 0.002$); cardiovascular diseases (12.5% vs. 3.8%, $p < 0.001$); chronic obstructive pulmonary disease or COPD (8.6 vs. 1.9%, $p < 0.001$); and chronic kidney disease (8.2 vs. 2.4%, $p < 0.001$) (**Figure 5**). Furthermore, lethality was higher in housewives at 26.4% (68/258), followed by retirees/pensioners at 24% (62/258) and others employees with 16.3% (42/258) ($p < 0.001$), in comparison with others occupations (**Figure 5**).

Influenza Vaccination and Its Relation With COVID-19 Survival

Finally, we analyzed the relationship between influenza vaccination history and COVID-19 cases who were deceased and those who survived. Of the 331 COVID-19 patients that were vaccinated against influenza, 7% (23 patients)

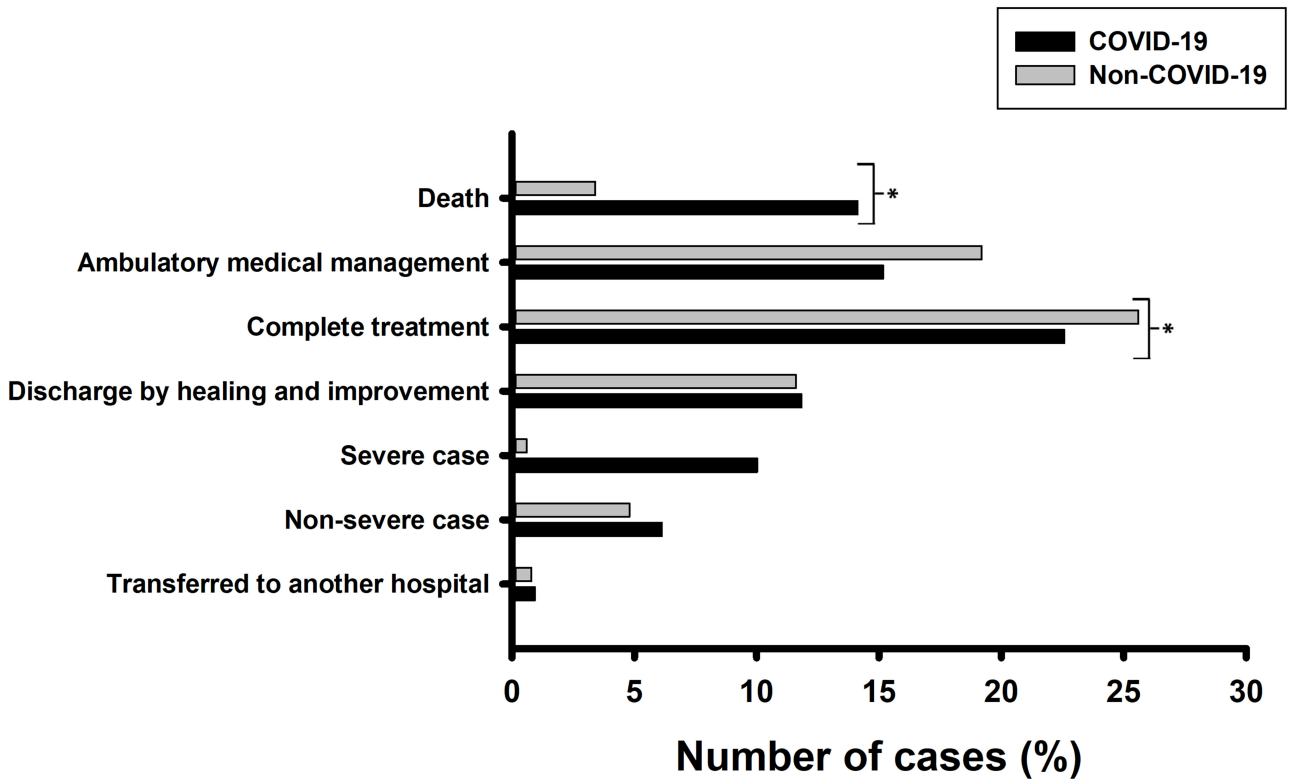

FIGURE 2 | Monitoring of patient evolution. A total of 1,826 COVID-19 patients and 2,214 non-COVID-19 patients were analyzed. Pearson's chi-squared test was performed with Bonferroni correction to check for statistical significance. *$p < 0.008$.

died of COVID-19, while 93% (308 patients) survived. In the 1,406 patients that did not receive the influenza vaccine, 15% (206 patients) died, and 85% (1,200 patients) survived ($p < 0.001$).

DISCUSSION

Since the first reports of the novel coronavirus SARS-CoV-2 infection were confirmed in December 2019, COVID-19 spread rapidly worldwide. At present, 188 countries are affected with >5 million positive cases and >330,000 confirmed deaths worldwide. The US, UK, and Italy are the most affected countries (8). The confirmed cases and deaths by COVID-19 in Mexico continued to increase throughout the country, with Sinaloa State being the most affected state with the highest prevalence rate and lethality (9).

To the best of our knowledge, this study is the first report of the epidemiologic profile of COVID-19 in Mexico with a special focus on symptomatic patients in Sinaloa state. We found that, from February 28 to March 15, the prevalence of COVID-19 was 45% and lethality 14.1%. Men with chronic diseases and healthcare workers were the most infected groups. Finally, we found that influenza vaccination was related to COVID-19 survival when compared to unvaccinated people.

Consistent with other reports, our study also showed that men were more affected by SARS-CoV-2 infections and COVID-19-related deaths than women (10–12). This could likely be explained by differences in immune responses; recently Takahashi et al. (13) showed that males with SARS-CoV-2 infection produce high levels of pro-inflammatory innate immunity chemokines and cytokines, such as IL-8, IL-18, and CCL5, correlating with higher non-classical monocytes, while women produce a robust immune system response with T cells—in particular activated CD8 T cells. Therefore, the poor T cell responses were associated with future worsening of COVID-19 in male patients (13).

Regarding age, SARS-CoV-2 infections were more frequent in the age group of 40–49 years, similar to that reported in countries such as Spain, Canada, and The Netherlands, wherein the highest numbers of infection were in people older than 45 years (14). We also found a high proportion of positive cases in patients aged between 30 and 39 years, consistent with reports from China and Germany. In Germany, most infected people were older adolescents and younger adults. Golstein and Lipsitch (15) hypothesized that this age range was more prone to ignoring social distancing than older adults, thereby facilitating the faster spread of the virus (15).

The high proportion of COVID-19 cases among young adults could be attributed to this age group, as the majority are workers,

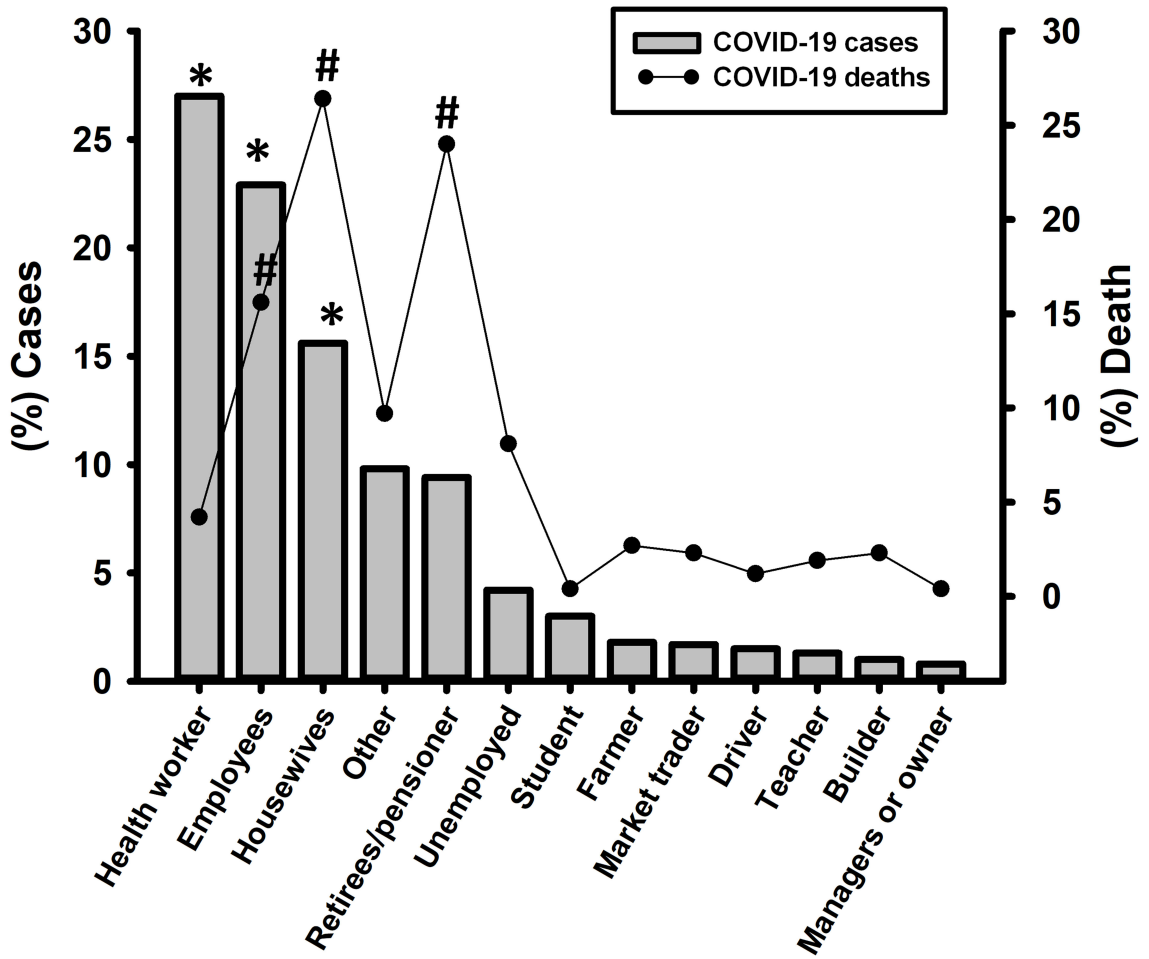

FIGURE 3 | Distribution of COVID-19 cases and deaths depending on the occupation of COVID-19 patients. Pearson's chi-squared test was performed with Bonferroni correction to check for statistical significance. *$p < 0.003$ in COVID-19 cases; #$p < 0.003$ in COVID-19 deaths.

and consequently could have less adherence to social distancing, which is similar to Germany's observations. Further, the high prevalence of chronic diseases in young adults in Mexico, such as hypertension, obesity, and type 2 diabetes, could increase the susceptibility to develop severe COVID-19 disease. This fact demonstrates that a change of lifestyle is necessary for adults and children, which includes avoidance of a western diet (diet based on obesity-inducing processed foods: foods high in saturated fats and sugars), following a healthy and well-balanced diet, and increase physical activity to prevent this phenomenon (16–18).

Moreover, the main symptoms and comorbidities related to COVID-19 reported in Mexico were similar to those reported in other countries (2, 19–21). Additionally, our data showed that healthcare workers were the most affected group because of the increased frequency of contact with suspected or infected cases of SARS-CoV-2. Previous work showed that both physical examination and nebulizer treatment increased the risk of infection among healthcare workers (22). Thus, it is necessary to improve training on the use of personal protective equipment (PPE) to reduce infections (23, 24).

The high numbers of positive COVID-19 among healthcare workers could be attributed to different facts. One of them is the lack of appropriate protective gear or the training to use it correctly. The other fact that could be contributing to the high prevalence of infection in healthcare workers is that, similarly to the general population, they also have a high proportion of chronic diseases. Vázquez-Martínez et al. (25) reported that 70% of the female staff of IMSS were obese or overweight (25). In another study, it was reported that healthcare workers in Mexico presented with diverse chronic diseases such as obesity or being overweight, high blood pressure, type 2 diabetes, and COPD (26, 27).

At present, the global lethality of COVID-19 is 6.45%; in this study, we observed a lethality rate of 14.1%, which was lower than Belgium (16.41%) and France (15.54%), similar to Italy (14.27%) and the UK (14.21%), but higher than the Netherlands (12.87%), Spain (12.19%), and Sweden (12.02%) (8) (Accessed May 23). Changes to this rate in each country depend on their epidemiologic profile (mean age, population structure, and the burden of chronic diseases), availability of

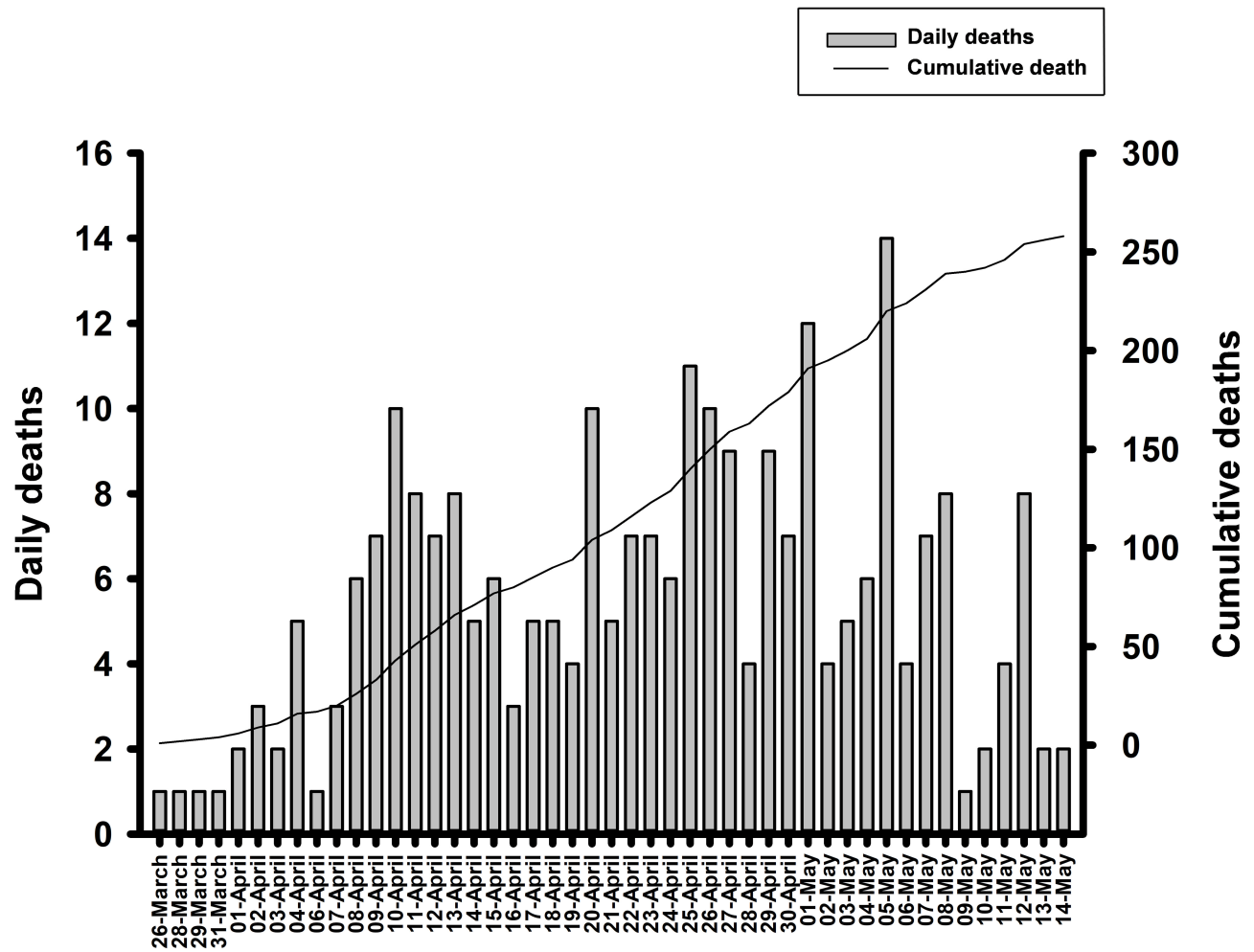

FIGURE 4 | Daily deaths and cumulative deaths by COVID-19.

health services, and adherence to the guidelines to reduce virus transmission (28). Moreover, the presence of genomic variants of SARS-CoV-2 in each country could impact COVID-19 survival (29).

Consistent with other studies, patients >65 years and with underlying chronic diseases such as hypertension, obesity, and type 2 diabetes showed a strong correlation with COVID-19 lethality (30–32). The occupations with a higher lethality rate were housewives, and retirees/pensioners. Age could be more related to COVID-19 mortality than occupation.

Moreover, we found a relation of lower risk of COVID-19 severe infection and mortality in COVID-19 patients vaccinated against influenza than those who were unvaccinated, which is consistent with previous studies (33, 34). The relation of lower risk of COVID-19 severe infection and influenza vaccine could be related to some features shared by both viruses, e.g., both viruses enter the pulmonary cells through ACE-2 receptors, and the hemagglutinin-esterase protein is very similar in both viruses. Furthermore, the spike protein has similar features to the class 1 viral membrane fusion protein of the influenza virus (35–38). Due to these similarities, the immune system response induced by the influenza vaccine could be beneficial to COVID-19 patients. Influenza vaccines also can protect against other viruses such as parainfluenza, RSV, and non-influenza virus coinfections. On the other hand, an influenza vaccine could increase the risk of other viruses, such as metapneumovirus and other coronaviruseses, by virus interference (39); however, more studies are needed to corroborate this observation.

To our best knowledge, this is the first paper to have identified the main epidemiological and clinical characteristics of patients infected with COVID-19 in one of the most affected states of México. We also highlighted the situation of healthcare workers in relation to the spread of COVID-19 in Mexico. Last, we showed a correlation between influenza vaccines and the survival of patients infected with COVID-19 in the Mexican population.

The limitation of this study is the sample size and the asymptomatic patients were not included, this study was based on patients presenting to the aforementioned hospitals only.

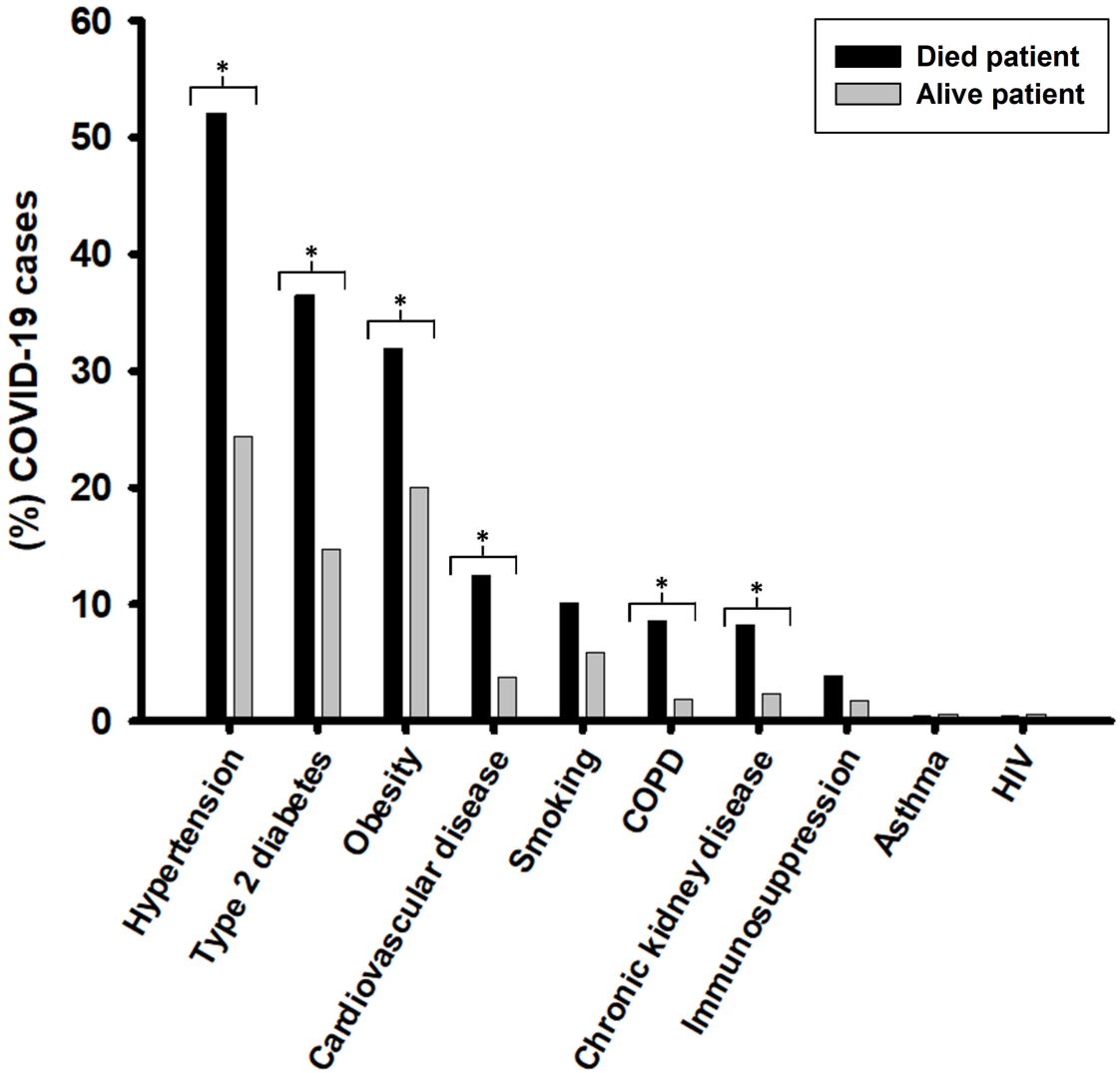

FIGURE 5 | Relationship between underlying diseases and COVID-19 deaths. Pearson's chi-squared test was performed with Bonferroni correction to check for statistical significance. *$p < 0.005$.

Hence, patients attending private clinics/laboratories, those with suspected infections that did not get tested were not considered.

CONCLUSIONS

This study provides evidence regarding the high prevalence of COVID-19 disease in symptomatic patients from healthcare institutions in Sinaloa, Mexico. It also identified risk groups (men aged 40–49 years, people with chronic diseases, and healthcare workers) that showed a greater propensity for being infected with SARS-CoV-2. The lethality registered in this study was high (14.1%), as patients >65 years with chronic diseases had a higher mortality rate than other demographics. Finally, we found a possible relationship between influenza vaccination and lower risk of COVID-19 severe infection.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of The Women's Hospital, Secretariat of Health (No. 202005-03). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

UA-Z, FM-V, NL-S, and AdC-R conceived and designed the study. HF-V, JV-R, JA-R, and JM-S collected the data. UA-Z, FM-V, NL-S, AdC-R, and JM-G analyzed and interpreted the data. UA-Z, FM-V, and AdC-R drafted the manuscript. JA-F, FU, SM-A, JS-C, JA-R, and AbC-R critically revised the manuscript. AdC-R, JA-F, AbC-R, and UA-Z approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Distinguishing Coronavirus Disease 2019 Patients From General Surgery Emergency Patients With the CIAAD Scale: Development and Validation of a Prediction Model Based on 822 Cases in China

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Background: During the epidemic, surgeons cannot identify infectious acute abdomen patients with suspected coronavirus disease 2019 (COVID-19) immediately using the current widely applied methods, such as double nucleic acid detection. We aimed to develop and validate a prediction model, presented as a nomogram and scale, to identify infectious acute abdomen patients with suspected COVID-19 more effectively and efficiently.

Methods: A total of 584 COVID-19 patients and 238 infectious acute abdomen patients were enrolled. The least absolute shrinkage and selection operator (LASSO) regression and multivariable logistic regression analyses were conducted to develop the prediction model. The performance of the nomogram was evaluated through calibration curves, Receiver Operating Characteristic (ROC) curves, decision curve analysis (DCA), and clinical impact curves in the training and validation cohorts. A simplified screening scale and a management algorithm were generated based on the nomogram.

Results: Five potential COVID-19 prediction variables, fever, chest CT, WBC, CRP, and PCT, were selected, all independent predictors of multivariable logistic regression analysis, and the nomogram, named the COVID-19 Infectious Acute Abdomen Distinguishment (CIAAD) nomogram, was generated. The CIAAD nomogram showed good discrimination and calibration, and it was validated in the validation cohort. Decision curve analysis revealed that the CIAAD nomogram was clinically useful. The nomogram was further simplified as the CIAAD scale.

Conclusion: We established an easy and effective screening model and scale for surgeons in the emergency department to use to distinguish COVID-19 patients. The algorithm based on the CIAAD scale will help surgeons more efficiently manage infectious acute abdomen patients suspected of having COVID-19.

Keywords: COVID-19, infectious acute abdomen, prediction model, nomogram, prediction scale

BACKGROUND

Since the outbreak of coronavirus disease 2019 (COVID-19), which was characterized as a pandemic by the World Health Organization on March 11, 2020, the virus has rapidly spread globally (1, 2). Millions of people have been infected, resulting in tens of thousands of deaths (3). The ongoing pandemic is not only an enormous threat to public physical health but also an acid test for the medical systems in both developed counties and developing countries (4). In addition to prevention, the quick and accurate recognition of COVID-19 is currently one of the most important tasks.

The medical management of other diseases has been critically disturbed, especially in diseases characterized by fever, which is a typical symptom of COVID-19 (5). There are numerous high-risk people who are coming into close contact with confirmed COVID-19 patients. Disrupting transmission is the most effective way to control the epidemic of COVID-19. Under the current situation, an infectious acute abdomen is still one of the most common surgical emergencies. Patients with acute abdomen infection often display fever and similar changes in routine blood and other biochemistry tests as those observed in patients with COVID-19, which would cover up the signs of COVID-19 (6). Currently, the diagnosis of COVID-19 mainly depends on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) nucleic acid detection (7). However, that standard procedure is to double-check, which is time-consuming and still has the risk of false-negative results (8), and this hinders efforts to perform emergency operations in a timely manner and to prevent cross-infection in the hospital. Therefore, there is a pressing need for an easier and more feasible method to help surgeons preliminarily distinguish COVID-19 patients from other infectious acute abdomen patients who have symptoms mimicking those of COVID-19 and take proper precautions in emergency operations.

Using the clinical data of 822 patients, 584 with COVID-19 and 238 with infectious acute abdomen cases, we compared the demographic, clinical, imaging, and laboratory characteristics to identify the significant predictors of COVID-19. Furthermore, a prediction model to distinguish between the two diseases was generated based on machine learning and is presented in the form of a nomogram, which had good discrimination performance in both the training and validation cohorts. Ultimately, we offer a practical screening scale, named the CIAAD scale, and an algorithm, with accompanying precautionary advice for surgeons treating infectious acute abdomen patients.

Abbreviations: ACE2, angiotensin-converting enzyme 2; AUC, area under the curve; CIAAD, COVID-19 infectious acute abdomen distinguishment; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; CT, computed tomography; LASSO, least absolute shrinkage and selection operator; PCT, procalcitonin; ROC, receiver operating characteristic; RT-PCR, reverse transcription-polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WBC, white blood cell.

METHODS

Patients

Ethics approval was obtained from the Ethics Committees of A Hospital (Note. a hospital in Wuhan, name omitted for review) and the B Hospital (Note. a hospital in Beijing, name omitted for review) for this retrospective study. We included 584 COVID-19 patients seen at the A Hospital between January 2, 2020, and February 15, 2020, in our study. The diagnostic criteria for COVID-19 were positive RT-PCR results for SARS-CoV-2 or viral gene sequencing results that were highly homologous with SARS-CoV-2 using respiratory or blood samples (7). Since the routine medical treatment of other diseases in Wuhan was severely disturbed by the epidemic, the clinical data of 283 infectious acute abdomen patients undergoing emergency operations at B Hospital between February 28, 2019, and April 3, 2020, were collected. The definition of infectious acute abdomen cases was an acute abdomen with a primary infectious cause, such as acute appendicitis, or with secondary infectious peritonitis, such as perforation, obstruction, and bleeding. The inclusion criteria were as follows: (1) fever; (2) abnormal routine blood results or other infection indicators; or (3) signs of pneumonia. The patients with infectious acute abdomen admitted after January 20, 2020, were all tested for SARS-CoV-2 and none of them were positive. Those admitted before January 20 were not tested for SARS-CoV-2 and assumed as negative as the epidemic had not broken out yet.

Data Collection and Definitions

Demographic, clinical, laboratory, treatment, and outcome data from the COVID-19 and infectious acute abdomen patients were extracted from the electronic medical records system of the A Hospital and B Hospital, respectively. The ranges of normal values and definitions for different variables were the same.

Fever was defined as an axillary temperature of at least 37.3°C. The chest CT scores were graded retrospectively by two radiologists in sequence. Each lung was divided into the upper, middle, and lower lobes, and the scoring criteria were as follows: $<$1/3 of the lung infected, 0 points; 1/3–2/3 of the lung infected, 1 point; $>$2/3 of the lung infected, 2 points. The classification of COVID-19 severity was based on the Chinese management guidelines for COVID-19 (version 7.0) published by the National Health Commission of China (7).

Potential Predictor Selection

The primary cohort of all 822 patients was randomly divided into a training cohort and a validation cohort at a ratio of 2:1. The least absolute shrinkage and selection operator (LASSO) method, one of the most effective methods of regularized regression with substantial advantages when managing multicollinear data, was used to select the most useful predictive variables for COVID-19 in the training cohort (9).

Development and Validation of a Prediction Model

We conducted multivariate logistic regression with the potential predictors to further verify their predictive efficacy and then

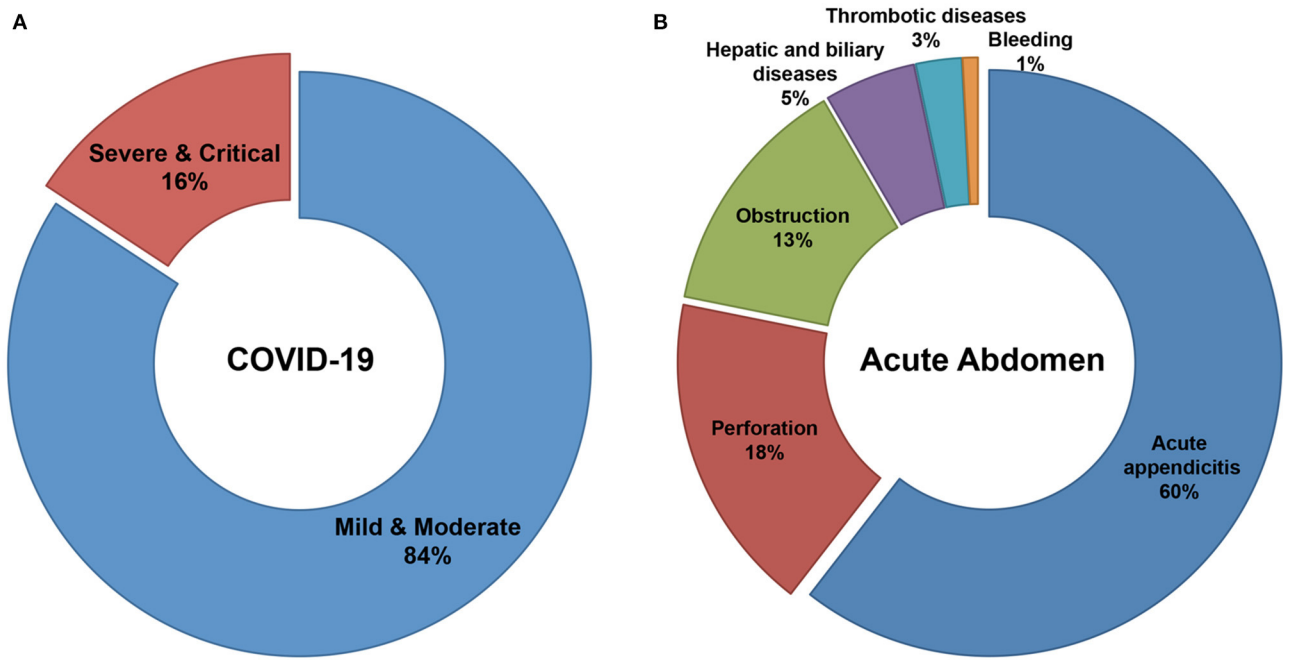

FIGURE 1 | Distribution of severity in COVID-19 patients and disease spectrum in infectious acute abdomen patients. **(A)**. 16% of enrolled COVID-19 patients were severe or critical and the left 84% were mild or moderate. **(B)**. The disease spectrum of enrolled infectious acute abdomen patients showed the top three causes for emergency operations were acute appendicitis (60%), gastrointestinal perforation (18%), and bowel obstruction (13%).

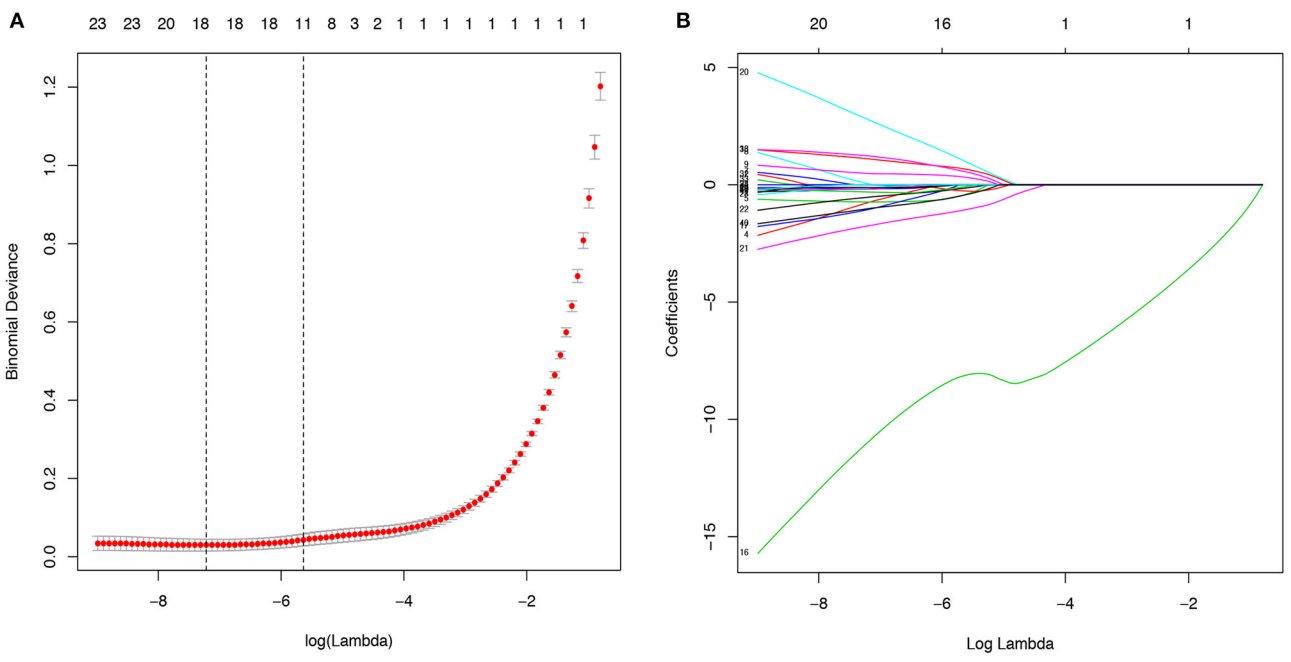

FIGURE 2 | Potential predictors selection using the least absolute shrinkage and selection operator (LASSO) regression model. **(A)**. The binomial deviance curve was plotted versus log(λ). Dotted vertical lines were drawn at the optimal values by using the minimum criteria and the one standard error of the minimum criteria (the 1-SE criteria). **(B)**. LASSO coefficient profiles of the 40 alternative variables. A coefficient profile plot was produced against the log (λ) sequence.

TABLE 1 | Demographic, clinical, imaging, and laboratory characteristics of patients on admition or first to emergency.

Clinical variables	All patients (n = 822)	COVID-19 (n = 584)	Acute abdomen (n = 238)	*P*-value
Age (years), No. (%)	53.0(36.0–66.0)	55.5(38.0–67.0)	46.5(33.8–64.0)	0.001
Gender				0.012
Male, No. (%)	386(47.0)	258(44.2)	128(53.8)	-
Female, No. (%)	436(53.0)	326(55.8)	110(46.2)	-
Chronic diseases				
Chronic obstructive pulmonary disease, No. (%)	47(5.7)	39(6.7)	8(3.4)	0.063
Hypertension, No. (%)	251(30.5)	190(32.5)	61(25.6)	0.051
Diabetes mellitus, No. (%)	104(12.7)	86(14.7)	18(7.6)	0.005
Cardiovascular and cerebrovascular diseases, No. (%)	97(11.8)	78(13.4)	19(8.0)	0.011
Renal failure, No. (%)	39(4.7)	33(5.7)	6(2.5)	0.056
Symptoms				
Fever, No. (%)	545(66.3)	468(80.1)	77(32.4)	<0.001
Shortness of breath, No. (%)	232(28.2)	228(39.4)	4(1.7)	<0.001
Fatigue, No. (%)	254(30.9)	213(36.5)	41(17.2)	<0.001
Muscle pain, No. (%)	154(18.7)	147(25.2)	7(2.9)	<0.001
Diarrhea, No. (%)	66(8.0)	52(8.9)	14(5.9)	0.148
Chest CT				<0.001
0, No. (%)	492(63.4)	313(55.1)	179(86.1)	-
1, No. (%)	148(19.1)	120(21.1)	28(13.5)	-
2, No. (%)	136(17.5)	135(23.8)	1(0.5)	-
Infection-related biomarkers				
CRP level, median (IQR), mg/L, NR* 0–8	19.0 (4.2–48.0)	16.3 (3.6–44.3)	32.0(12.0–120.8)	<0.001
PCT level, median (IQR), ng/mL, NR 0–0.5	0.06(0.04–0.12)	0.05(0.04–0.09)	0.78(0.11–10.25)	<0.001
Blood routine				
Leucocytes, median (IQR), $\times 10^9$, NR 3.50–9.50	5.7(4.1–9.9)	4.9(3.8–6.5)	12.1(9.2–15.5)	<0.001
Neutrophils, median (IQR), $\times 10^9$, NR 2.00–7.50	4.0(2.6–7.9)	3.3(2.2–4.8)	10.3(7.0–13.7)	<0.001
Lymphocytes, median (IQR), $\times 10^9$, NR 0.80–4.00	1.0(0.7–1.4)	1.0(0.7–1.4)	1.1(0.7–1.8)	0.02
Platelets, median (IQR), $\times 10^9$, NR 100–350	189.0(147.0–243.0)	178.0(134.0–224.8)	227.0(181.0–274.8)	<0.001
Hemoglobin, median (IQR), g/L, NR 120–160 (male), 110–150 (female)	131.0(120.0–143.0)	128.0(119.3–140.0)	137.0(120.8–153.0)	<0.001
Blood biochemistry				
Alanine aminotransferase, median (IQR), U/L, NR 9–50 (male), 7–40 (female)	18.0(12.0–30.4)	19.7(13.2–32.7)	15.0(9.0–24.0)	<0.001
Total bilirubin, median (IQR), μmol/L, NR 5.1–22.2	9.7(7.1–14.4)	8.6(6.4–11.9)	14.1(9.9–21.5)	<0.001
Blood urea nitrogen, median (IQR), mmol/L, NR 2.78–7.14	4.3(3.3–5.9)	4.1(3.2–5.5)	5.1(3.8–6.9)	<0.001
Serum creatinine, median (IQR), μmol/L, NR 59–104 (male), 45–84 (female)	67.1(54.3–82.3)	64.8(51.8–78.3)	74.0(64.0–93.5)	<0.001
Coagulation function				
Fibrinogen, median (IQR), g/L, NR 1.80–3.50	3.1(2.5–3.7)	3.0(2.5–3.5)	3.5(2.8–4.6)	<0.001
D-dimer, median (IQR), mg/L, NR 0–0.55	0.6(0.3–1.5)	0.5(0.3–1.1)	0.8(0.4–3.2)	<0.001

**NR: normal range.*

built a nomogram to distinguish COVID-19 patients from infectious acute abdomen patients on the basis of the results of the multivariable logistic analysis in the training cohort. We named the nomogram the COVID-19 and Infectious Acute Abdomen Distinguishment (CIAAD) nomogram. Calibration curves were plotted to assess the calibration of the CIAAD nomogram, and the C-index was measured to quantify its discrimination performance. Then, the CIAAD nomogram developed using data from the patients in the training cohort was applied to the patients in the validation cohort, and the calibration curve and C-index were derived on the basis of the regression analysis. The ability of the CIAAD nomogram to distinguish between the two groups of patients in both the training and validation cohorts was also assessed by calculating the area under the receiver operating characteristic curve (AUC).

Clinical Usefulness Assessment

Decision curve analysis was performed and clinical impact curves generated to evaluate the clinical practicability of the CIAAD nomogram by quantifying the net benefits at different threshold probabilities in both the training and validation datasets.

Development of Screening Scale

The score for each item in the CIAAD nomogram was divided by 25 and then rounded to obtain a simplified score. The simplified scores were verified to have the same efficacy as the original nomogram. We subdivided the risk of COVID-19 into low (<0.3), moderate (0.3–0.7), and high (>0.7) risk. The CIAAD Scale was generated on the basis of the simplified scoring criteria and risk classification.

Statistical Analysis

Categorical variables are expressed as numbers and percentages. Continuous variables are expressed as medians with interquartile ranges. The chi-square test and Mann-Whitney U-test were used to evaluate categorical and continuous data, respectively. Statistical analysis was conducted with R software (version 3.6.1; http://www.Rproject.org) and the SPSS statistical software package (version 25.0). $P < 0.05$ was considered statistically significant.

RESULTS

Demographic and Clinical Characteristics

A total of 822 patients, 584 COVID-19 patients without infectious acute abdomen and 238 infectious acute abdomen patients without COVID-19, were included in this study (**Table 1**). Nearly 16% of the COVID-19 patients had severe or critical COVID-19 (**Figure 1A**). The infectious acute abdomen patients primarily had acute appendicitis (60.5%), perforation (17.6%), and obstruction (13.4%) (**Figure 1B**). Compared with the COVID-19 patients, the infectious acute abdomen patients were younger ($p = 0.001$) and had fewer chronic diseases, such as diabetes ($p = 0.005$) and cardiovascular and cerebrovascular diseases ($p = 0.011$). Fever, as reported before, was the most common symptom in the COVID-19 patients (80.1%), and nearly one-third of the infectious acute abdomen patients got abnormal body temperature as well. COVID-19 was associated with a larger infected proportion of the lung ($p < 0.001$). Nevertheless, the abdominal infection causing acute abdomen resulted in more abnormal laboratory test results, such as CRP, PCT, WBC, neutrophils, and fibrinogen, in infectious acute abdomen patients than in COVID-19 patients ($p < 0.001$).

Potential Predictor Selection

The 38 variables collected were reduced to five potential predictors (abdominal pain, fever, chest CT, CRP, PCT, and WBC) with non-zero coefficients in LASSO regression on the basis of the data from the 547 patients in the training cohort (**Figure 2**). The AUC of the five variables, namely, fever, chest CT, CRP, PCT, and WBC, was obtained in the training cohort and validation cohort (**Supplementary Figures 1, 2**). CRP got the highest AUC in both training and validation cohorts of 0.835 and 0.854.

TABLE 2 | Multivariable logistic regression of potential predictors for screening COVID-19 in infectious acute abdomen patients (training cohort).

Variables and Intercept	β*	Odds Ratio (95%CI)	*P*-value
Fever	1.915	6.788 (3.314–13.904)	<0.001
Chest CT	1.753	5.773 (3.172–10.507)	<0.001
CRP	−2.508	0.081 (0.043–0.152)	<0.001
PCT	−0.8	0.449 (0.281–0.739)	0.001
WBC	−1.836	0.160 (0.092–0.278)	<0.001
Intercept	10.104		

*β: *the regression coefficient.*

Development of a Prediction Model

To simplify the model, the concrete values of CRP, PCT, and WBC were transformed into categorical variables (CRP and PCT: 1 for normal, 2 for high, 3 for undetermined; WBC: 1 for low, 2 for normal, and 3 for high), and fever was also defined as 0 for "no" and 1 for "yes." Multivariate logistic regression analysis was performed with the five variables, and all of these potential predictors have substantial value with regard to distinguishing COVID-19 from infectious acute abdomen (**Table 2**). A risk score formula was preliminarily built to predict the probability of COVID-19 as follows: Logit (P = COVID-19) = 10.104 + 1.915 × fever + 1.753×chest CT + (−2.508) × CRP + (−0.8)×PCT + (−1.836) × WBC. The CIAAD nomogram was generated on the basis of the above result (**Figure 3A**).

Performance of the Nomogram in the Training and Validation Cohorts

The calibration curve of the CIAAD nomogram for the prediction of the risk of COVID-19 demonstrated good agreement between prediction and reality in the training cohort (**Figure 3B**). The C-index value for the prediction nomogram was 0.981 (95% CI, 0.963 to 0.999) in the training cohort. Good calibration was also observed in the validation cohort, with a C-index value of 0.966 (95% CI, 0.960 to 0.972) (**Figure 3C**). The ROC analysis in the training and validation cohorts yielded AUC values of 0.970 (95% CI, 0.961 to 0.982) and 0.966 (95% CI, 0.957 to 0.975), which suggested that the predictive performance was good (**Figures 3D,E**).

Clinical Use and Development of a Simplified Scale

Decision curve analysis was performed and clinical impact curves were generated for the CIAAD nomogram in both the training and validation cohorts (**Figure 4**), demonstrating a high net clinical benefit that was almost greater than the entire threshold probability.

To make our prediction model more concise and practical in the context of emergency surgery, we simplified the scoring criterion of the CIAAD nomogram and created a new scale, named the CIAAD scale (**Figure 5**). The lowest and highest

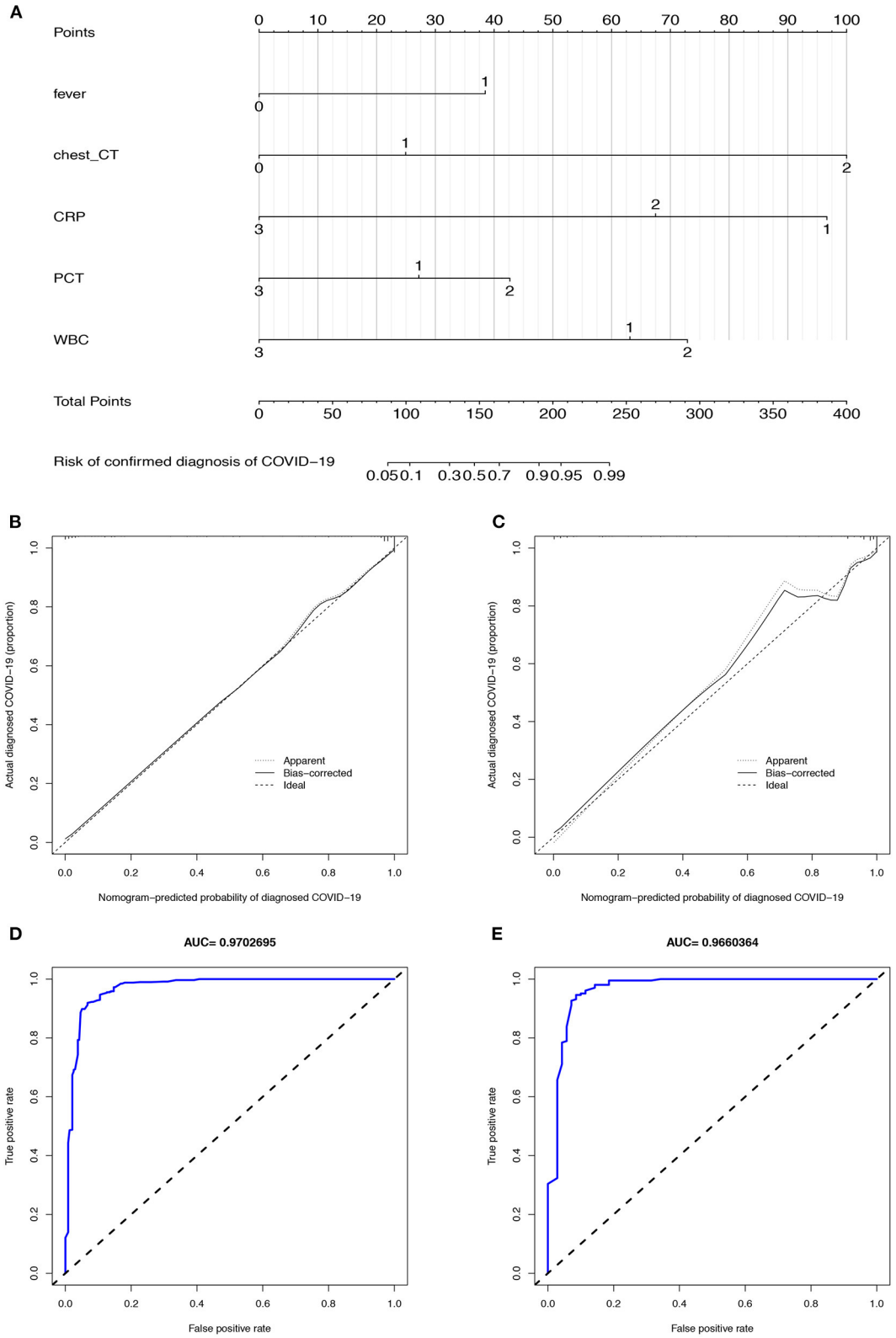

FIGURE 3 | The CIAAD nomogram and its discrimination performance in training and validation cohort. **(A)**. The CIAAD nomogram was developed in the training cohort based on fever, chest CT, CRP, PCT, and WBC. **(B)**. Calibration curve of the CIAAD nomogram in the training cohort. Calibration curves depict the calibration of

(Continued)

FIGURE 3 | each model in terms of the agreement between the predicted risks of COVID-19 and observed outcomes of a confirmed diagnosis of COVID-19. The Y-axis represents the actual COVID-19 rate. The X-axis represents the predicted COVID-19 risk. The diagonal dotted line represents a perfect prediction by an ideal model. The black solid line represents the performance of the CIAAD nomogram, of which a closer fit to the diagonal dotted line represents a better prediction. **(C)**. Calibration curve of the CIAAD nomogram in the validation cohort. **(D)**. ROC curve of the CIAAD nomogram in the training cohort. **(E)**. ROC curve of the CIAAD nomogram in the validation cohort.

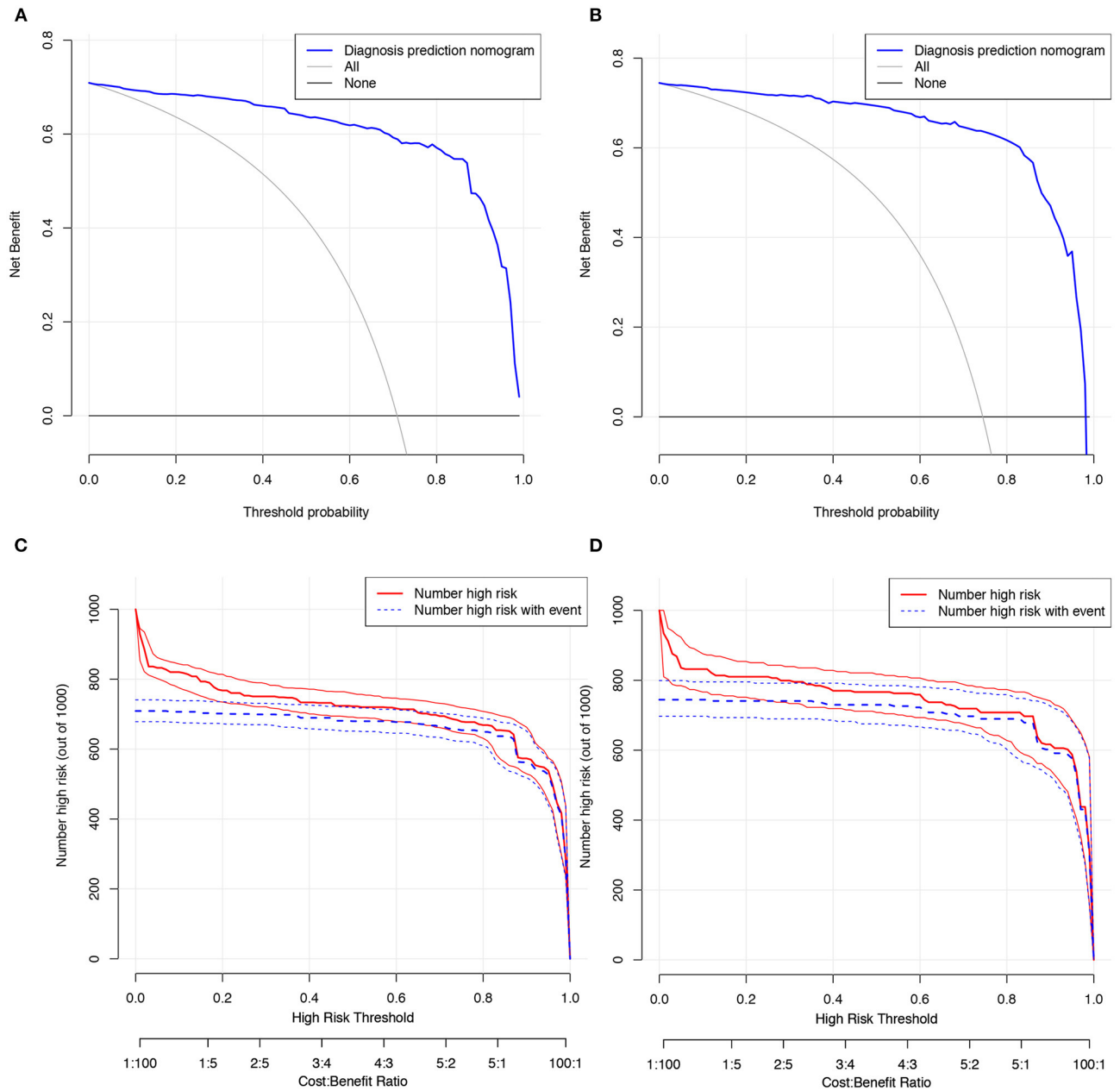

FIGURE 4 | Decision curve analysis and clinical impact curves for the CIAAD nomogram in training and validation cohort. **(A)**. Decision curve analysis for CIAAD nomogram in the training cohort. The Y-axis measures the net benefit. The blue line represents the CIAAD nomogram. The gray line represents the assumption that all patients are COVID-19 patients. The black line represents the assumption that there are no COVID-19 patients. **(B)**. Decision curve analysis for the CIAAD nomogram in the validation cohort. **(C)**. Clinical impact curve for the CIAAD nomogram in the training cohort. The red curve (Number high risk) indicates the number of people classified as positive (high risk) by nomogram under each threshold probability. The blue curve (Number high risk with event) is the number of truly positive people under each threshold probability. **(D)**. Clinical impact curve for CIAAD nomogram in the validation cohort.

COVID-19 Infection-Induced Acute Abdomen Distinguishment (CIAAD) Scale			
Item	**Assessment**	**Scoring Criteria**	**Score**
Fever	No	0	
	Yes	1.5	
Chest CT*	None or Slight	0	
	Moderate	1	
	Severe	4	
CRP	Normal	4	
	High	2.5	
PCT	Normal	1	
	High	2	
WBC	Low	2.5	
	Normal	3	
	High	0	
Risk Assessment			
Total score	Total score=3.5	Risk-free	
	3.5＜Total score＜5	Low risk	
	5＜Total score＜7	Moderate risk	
	Total score＞7	High risk	
*Chest CT: ＜1/3 lung infected was defined as slight, 1/3-2/3 lung infected was defined as moderate, ＞2/3 lung infected was defined as severe.			

FIGURE 5 | The COVID-19 Infectious Acute Abdomen Distinguishment Scale based on the CIAAD nomogram. Patients with a total score of <5 were considered of low risk of true SARS-CoV-2 infection, five to seven were of moderate risk, and more than seven meant high risk.

scores on this scale are 3.5 and 14.5, respectively. Items with higher scores are more common in COVID-19 patients, such as fever, abnormal chest CT, and normal levels of CRP and WBC. If the total score is 3.5, surgeons can regard the acute abdomen patient as COVID-19 risk-free. If the total score of a patient is <5, he/she has a low risk (<30%) of having confirmed COVID-19, and the risk increases to more than 70% as the total score reaches 7.

DISCUSSION

As the global outbreak of COVID-19 continues, the latest total of infected patients has exceeded 20 million, and humankind will face the threat of this disease for the foreseeable future (2). Vast amounts of medical resources have been expended to control the spread of the virus and treat affected individuals, resulting in the postponement of treatment for patients with many other diseases. However, for surgeons confronted with patients with infectious acute abdomen who urgently need to undergo emergency operations, it is necessary to accurately distinguish COVID-19 patients from those with similar and misguiding symptoms in the shortest possible time. To prevent the cross-infection of medical staff, such as doctors and nurses, and other patients in outpatient clinics, wards, and operating rooms, high-level precautions are necessary when managing patients who are strongly suspected of having COVID-19. Excessive precautions could waste substantial amounts of precious medical resources. In contrast, neglecting necessary screening would increase the risk of cross-infection of medical staff. The current screening procedures, such as nucleic acid detection and CT, have the disadvantages of inadequate accuracy and time-consuming operation. Consequently, a more convenient, efficient, economical, and effective COVID-19 screening method is desired by surgeons. To our knowledge, this study provides the first screening model and scale for surgeons to use to distinguish infectious acute abdomen patients from suspected COVID-19 patients in the emergency department by retrospectively comparing demographic, clinical, and laboratory characteristics of the two groups of patients. The CIAAD nomogram and scale have satisfactory performance with regard to the prediction of COVID-19 and great potential to help medical institutions resume routine medical work during the epidemic.

Challenges and Opportunities

Current reports of COVID-19 patients show that respiratory symptoms, such as fever, cough, and dyspnoea, are the main clinical manifestations (10). Nevertheless, digestive symptoms, such as diarrhea, nausea, vomiting, and abdominal pain, are gradually being reported as early-onset symptoms, which deserves more attention (11, 12). A meta-analysis revealed that nearly half of the patients had positive results for SARS-CoV-2 in stool samples, and another bioinformatics analysis provided a probable theoretical basis for the digestive symptoms: angiotensin-converting enzyme II (ACE2) is highly expressed in the esophagus, ileum, and colon (13, 14). The mixture of fever and some digestive symptoms mimics the symptoms of infectious acute abdomen to a large extent. Similarly, if the patient is older or the abdominal infection develops into a systemic infection, signs of pneumonia can emerge in infectious acute abdomen patients as well. In patients with infectious acute abdomen, increased morbidity and mortality associated with a delay in the treatment of many of the surgical causes suggest the need for an aggressive and expeditious surgical approach (15). During the epidemic, quick and accurate screening of infectious acute abdomen patients suspected of having COVID-19 is vitally important.

The definite diagnosis of COVID-19 still mainly depends on a positive RT-PCR result for SARS-CoV-2 (16), which has the disadvantage of the possibility of false-negative results due to disease stage, viral load, and sample quality. In our COVID-19 cohort, the positive rate at the first nucleic acid test was merely 43.7% (255/584). Meanwhile, chest CT is also considered a good screening tool. However, the existence of mild cases not associated with pneumonia, atypical imaging findings, and substantial dependence on physicians' experience limit the screening value of chest CT. A study enrolled 1,014 COVID-19 patients from Wuhan and showed that the positive rate for chest CT was 88%, compared with 59% for RT-PCR8. Several prediction models based on the integration of demographic, clinical, imaging, and laboratory variables have been developed to evaluate the disease risk or prognosis (17). Unfortunately,

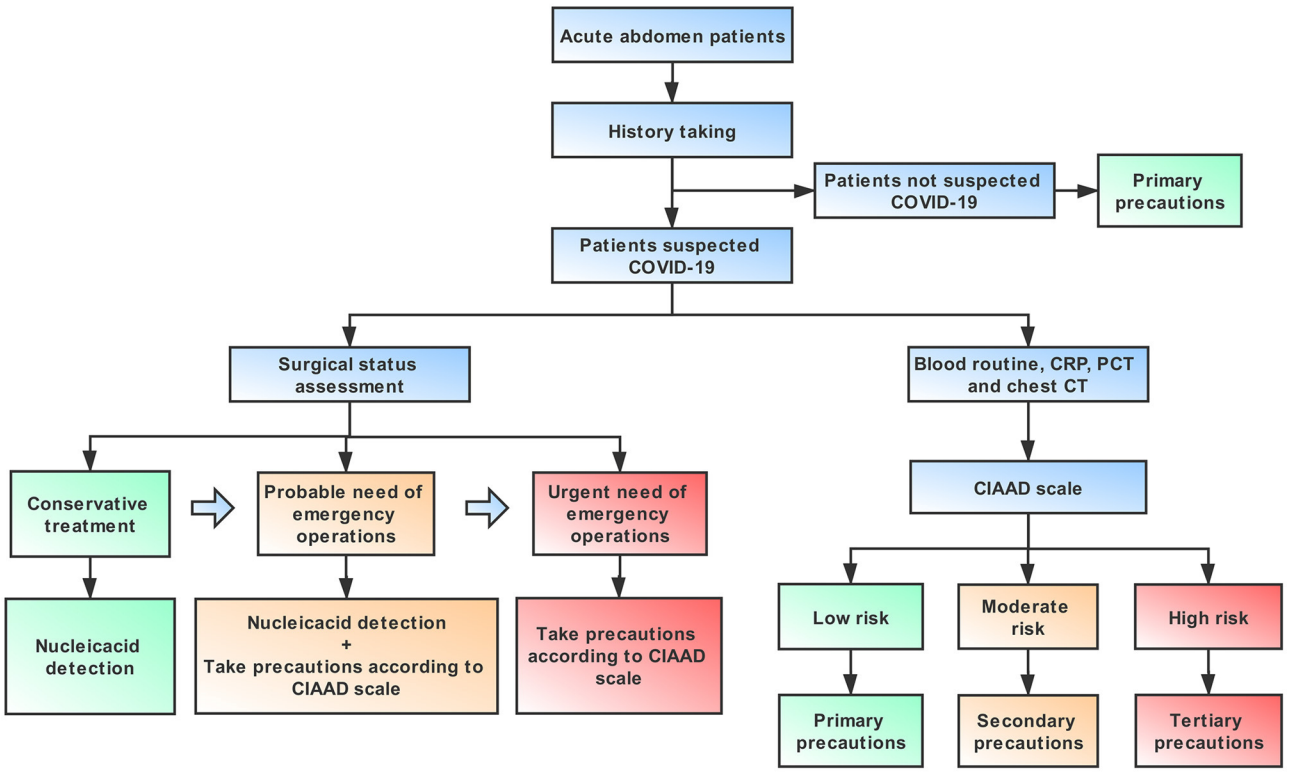

FIGURE 6 | An algorithm help surgeons to manage infectious acute abdomen patients suspected COVID-19 in the emergency department (ED). Key procedures in this algorithm are history taking, surgical status assessment, and the CIAAD scale. Primary precautions are needed for all patients in ED during the epidemic.

the target populations of the published diagnostic models were patients presenting at fever clinics or ordinary patients suspected of having COVID-19 (17). It is not appropriate for surgeons to borrow these models directly to screen infectious acute abdomen patients, and our CIAAD nomogram and scale fill the existing gap.

Strengths and Limitations of This Study

The strength of our model is the strong relevance. The recommended user of the CIAAD scale is a surgeon in the emergency department, and the recommended population to be assessed is infectious acute abdomen patients suspected of having COVID-19. To this end, we collected first-hand and high-quality data from COVID-19 patients and used strict enrolment criteria for infectious acute abdomen patients. In addition, via LASSO regression analysis, five quantifiable indicators were successfully selected. Although many variables, such as diabetes, cough, and D-dimer level, varied considerably between COVID-19 and infectious acute abdomen patients, they were ruled out by LASSO regression analysis as being too heavily weighted or causing the prediction model to be cumbersome. The selected indicators were all included in previous prediction models, which further verified the prediction capacity of these variables (17). We also clarified the differences of specific significant clinical indexes between COVID-19 patients and infectious acute abdomen patients. For example, COVID-19 patients often have no rise in CRP, PCT, and WBC, which was in accordance with the results of other studies. On the contrary, the infectious acute abdomen enrolled in our study suffered more severe inflammation, which resulted in more abnormality in the above-mentioned indexes. The discrepancy of infection indicators between two groups of patients truly reflected the difference in types of infection.

However, there was a limitation that cannot be evaded in our study. The disturbance of routine medical work by the epidemic resulted in the lack of patients with both COVID-19 and acute abdomen. As the number of emergency operations for acute abdomen decreased sharply in Wuhan, the data pertaining to acute abdomen patients were from B Hospital, a renowned hospital in China. We did not obtain patients with both COVID-19 and infectious acute abdomen, who would be the best study objects out of our research aim. The 822 patients were the most suitable two cohorts to gain distinguishment nomogram and scale. In spite of the limitation, patients with a score of 3.5 according to the CIAAD scale could be regarded as COVID-19 risk-free, which was of definite prediction accuracy.

Implications for Practice and Future

An algorithm that is helpful for allowing both a focused workup and expeditious therapy is provided in this article, including necessary advice regarding prevention for medical staff, based on the guidelines published by the World Health Organization (18) (**Figure 6**). It is important to note that standard precautions are needed for all patients. For an infectious acute abdomen patient suspected of COVID-19, the first step is to evaluate his/her surgical status and assess the patient with the CIAAD scale. The degree of urgency of the patient's need for surgical intervention

determines whether the medical staff should wait for the results of the nucleic acid test or take precautions according to our algorithm. The level of precautions adopted should be informed by the degree of risk of COVID-19 according to the results of screening with the CIAAD scale.

As the scale is harmless and has a net benefit over nearly the entire threshold probability, according to the decision analysis curves, we strongly recommend that surgeons worldwide use our CIAAD scale and the accompanying algorithm. With its wide use in a larger population, the efficacy of the CIAAD scale will be further prospectively tested.

CONCLUSION

With the aim of distinguishing COVID-19 patients from infectious acute abdomen patients, we established an easy and effective screening model and scale for use by surgeons in the emergency department. The algorithm based on the CIAAD scale can help surgeons manage infectious acute abdomen patients suspected of having COVID-19 more efficiently and help prevent cross-infection.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committees of the Peking Union Medical College Hospital and the Central Hospital of Wuhan. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZBB, WYX, SWW, and QC contributed equally to this article. WWB, WYJ, ZBB, WYX, and SWW designed the study. ZBB, WYX, SWW, ZXT, LTH, CHT, and WWB collected assembled the data. ZBB, WYX, SWW, QC, and WZH analyzed and interpreted the data. ZBB, WYX, and SWW wrote the first draft, which all authors revised for critical content. All authors approved the final manuscript. WWB and WYJ are the guarantors. The corresponding authors attest that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

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SUPPLEMENTARY MATERIAL

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Molecular Epidemiology and Drug Resistant Mechanism of Carbapenem-Resistant *Klebsiella pneumoniae* in Elderly Patients With Lower Respiratory Tract Infection

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Infection by carbapenem-resistant *Klebsiella pneumoniae* (CRKp) hampers the treatment of elderly patients with lower respiratory tract infection (LRTI); however, relevant data with respect to the characteristics of CRKp in elderly patients with LRTIs are limited. In the present study, *K. pneumoniae* isolated from elderly patients with LRTIs was collected and identified by VITEK-MS. VITEK 2 compact was used for drug sensitivity test to screen CRKps, and broth dilution method was used for drug sensitivity of tigecycline and colistin. The resistance genes, virulence genes, and serotypes of CRKps were detected via polymerase chain reaction. The homology of CRKps was analyzed via PFGE and MLST. Moreover, plasmid conjugation experiment was carried out to determine the transferability of carbapenem resistance. PCR-based replicon typing (PBRT) and S1 nuclease-PFGE were conducted for plasmid profiling. From January 2019 to August 2019, 258 elderly patients with LRTIs caused by *K. pneumoniae* were observed; of these, 31 (12.02%) infections were caused by CRKp strains. Majority of the patients were admitted to the intensive care unit and neurosurgery wards. Intracranial hemorrhage and pneumonia were the most common underlying diseases. Furthermore, 29 patients infected by CRKp had been exposed to various antimicrobial drugs before the positive culture. All isolates exhibited high resistance to β-lactam antibiotics. The predominant carbapenem resistance gene was bla_{KPC-2}, and CRKps carrying bla_{KPC-2} were all ST11 type. Two bla_{NDM-5} carrying isolates were assigned to ST307 and ST1562, respectively. Conjugative assays revealed that plasmids harboring bla_{NDM-5} gene were self-transmissible. Plasmid analysis suggested that two bla_{NDM-5} were located on a ∼45 kb IncX3 type plasmid. The high incidence of CRKp in elderly patients with LRTIs indicates the urgent need for further surveillance and strict infection control measures.

Keywords: *K. pneumoniae*, carbapenemase, elderly, drug resistance, lower respiratory tract infection

INTRODUCTION

Carbapenem-resistant *Enterobacteriaceae* (CRE) presents an urgent public health concern worldwide due to rapidly rising resistance rates and subsequent high mortality. Report from China CRE Network revealed that most CRE cases were caused by *Klebsiella pneumoniae* (73.9%) (1). *K. pneumoniae* is a common causative pathogen of various nosocomial infections, including pneumonia, urinary tract infection, abdominal infection, and bacteremia. Despite improvements in hospital infection control and antimicrobial scientific stewardship, carbapenem-resistant *K. pneumoniae* (CRKp) is still on the rise (2, 3). Surveillance of antibiotic resistance by CHINET in China revealed that 3.0 and 2.9% of *Klebsiella spp.* were resistant to imipenem and meropenem, respectively, in 2005, compared to 25.3 and 26.8%, respectively, in 2019 (http://chinets.com/Data/GermYear). This antibiotic resistance poses a greater challenge in clinical treatment of infection caused by CRKp.

Lower respiratory tract infections (LRTIs) are a leading cause of mortality and morbidity worldwide (4). Community- or hospital-acquired LRTIs are highly prevalent in the elderly. In developing countries, the situation is more complicated, and management is often difficult due to the identification of etiological agents and administration of an appropriate treatment in cases requiring antibiotic therapy. *K. pneumoniae* is an important pathogen causing LRTIs in the elderly (5). A report from China CRE Network revealed that 65.4% of the CRE patients presented LRTIs, and it is more serious in the elderly (1). Infection by CRKp makes the treatment of elderly patients with LRTIs face greater challenges.

Although CRKp has led to wide global disseminations and serious clinical outcomes, limited data is available on the molecular epidemiology of this pathogen in elderly patients with LRTIs. Therefore, we conducted this study to investigate the resistance profiles, molecular epidemiology, and clinical characteristics of CRKp isolates obtained from elderly patients with LRTIs.

MATERIALS AND METHODS

Bacterial Isolates and Patients

K. pneumoniae strains were collected from lower respiratory tract specimens, including bronchoalveolar lavage fluid, sputum, and pleural effusion, obtained from individual elderly patients (age $\geq$ 60 years) admitted to Taian City Central Hospital in China from January 2019 to August 2019. Elderly patients who presented at least two of the following symptoms were included in the study: fever, cough, dyspnoea, wheezing, chest pain, or sore throat. Any prior antimicrobial treatment taken by the patient was also recorded before microbiological investigations. Patients diagnosed with pulmonary tuberculosis or with infections other than LRTIs were excluded. Patient information including age, gender, diagnosis, treatment, and outcomes was obtained from the Electronic Medical Records. The methods in this study were approved by the Ethics Committee of Taian City Central Hospital and were carried out in accordance with the approved guidelines. The VITEK-MS (bioMérieux, France) was used to identify the bacterial strains. CRKp was defined as the minimal inhibition concentration (MIC) of ertapenem $\geq$2 μg/mL or the MIC of imipenem and meropenem $\geq$ 4 μg/mL (6). Only the first episode of CRKp-associated LRTIs was included.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by VITEK 2 compact system using GN13 cards (bioMérieux, France) according to the manufacturer's instructions. The MICs of imipenem, meropenem, and ertapenem were determined by an *E*-test (bioMérieux); whereas, MICs of tigecycline and colistin were determined by broth microdilution method (Biokont, China). *Escherichia coli* ATCC25922 and *K. pneumoniae* ATCC700603 (American Type Culture Collection Center, Manassas, VA, USA) acted as the quality controls. All antibiotics were administered according to the 2019 European Committee on Antimicrobial Susceptibility Testing breakpoint (www.eucast.org/clinical_breakpoint). Susceptibility data were analyzed using WHONET 5.6 software recommended by the World Health Organization.

PCR and DNA Sequence Analysis of Drug Resistance Genes, Serotype, and Virulence Genes

Samples were screened for the presence of carbapenem resistance genes (*bla*$_{KPC}$, *bla*$_{SME}$, *bla*$_{IMI}$, *bla*$_{NMC}$, *bla*$_{GES}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, *bla*$_{SPM}$, *bla*$_{NDM}$, and *bla*$_{OXA-48like}$), other β-lactamase genes (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{MOX}$, *bla*$_{FOX}$, *bla*$_{DHA}$, *bla*$_{CIT}$, *and bla*$_{EBC}$), and integron structures (Int1, Int2, Int3) (7, 8). For CRKp isolates that were resistant to quinolones or aminoglycoside, plasmid-mediated quinolone resistance genes [*qnrA, qnrB, qnrC, qnrD, qnrS, qepA*, *and aac(6)-Ib-cr*] or 16S rRNA methyase gene *(rmtA, rmtB, rmtC, rmtD, npmA*, and *armA*) were screened (9, 10). Primers used in the present study were listed in **Supplementary Table 1**.

The isolates were serotyped for serotypes K1, K2, K5, K20, K54, and K57, and moreover, 12 virulence-associated genes, including *rmpA, aerobactin*, *wcaG, ybtA, iucB, iroNB, ureA, uge, kfuBC, fim, wabG*, and *allS,* were screened using PCR as previously reported (11). Nucleotide sequences were analyzed and compared using BLAST (http://www.ncbi.nlm.nih.gov/blast).

Pulse-Field Gel Electrophoresis

An overnight bacterial culture was suspended in cell suspension buffer [100 mM EDTA, 100 mM Tris-HCl (pH 8.0)] and adjusted to an optical density of 4.0 at a wave length of 600 nm. The suspension was mixed with equal volumes 2% solution of low-melting agarose in Tris-EDTA [TE: 1 mM EDTA, 10 mM Tris-HCl (pH 8.0)]. After cooling, the agarose sections were incubated for 4 h at 54°C in cell lysis buffer [50 mM Tris-HCl, 50 mM EDTA (pH 8.0), 0.01 g/ml N-lauroyl-sarcosine, sodium salt, 0.1 mg/ml proteinase K]. Thereafter, the sections were washed thoroughly with TE buffer and digested overnight with XbaI restriction endonuclease (Takara Bio, Inc., Otsu, Japan). Genomic DNA was separated in 0.5× Tris/borate/EDTA (TBE) buffer in a PFGE system (CHEF Mapper; Bio-Rad Laboratories, Inc., Hercules,

CA, USA) at 14°C, using a voltage of 6 V/cm, a switch angle of 120°, and a switch ramp of 6–36 s for 21 h.

Multilocus Sequence Typing

MLST of *K. pneumoniae* was performed according to protocols available on the MLST Pasteur website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). Seven conserved housekeeping genes (*gapA, infB, mdh, pgi, phoE, rpoB*, and *tonB*) were amplified, sequenced, and compared with those in the MLST databases.

Conjugation Assay and Analysis of Plasmids

Conjugation was performed using the mixed broth method. Briefly, *E. coli J53* Azi^R was used as the recipient strain and 31 clinical CRKps served as the donors. Transconjugants were selected on Mueller Hinton agar supplemented with meropenem (0.5 μg/mL) and sodium azide (100 μg/mL). The transconjugants were identified by VITEK-MS. Antimicrobial susceptibility test of the transconjugant was carried out as described for the clinical strain.

Plasmid incompatibility types of the transconjugants were identified via PCR-based replicon typing, as reported previously (8). The size and amount of plasmids carried by the 31 clinical isolates and transconjugants were evaluated using S1-pulsed-field gel electrophoresis (S1-PFGE), as previously described (12). The genome of *Salmonella* H9812 digested with XbaI was used as the marker.

RESULTS

Clinical Characteristics of Patients With LRTIs Caused by CRKp

From January 2019 to August 2019, 258 elderly patients with LRTIs caused by *K. pneumoniae* were observed; of these, 31 (12.02%) were caused by CRKp strains. The median age of 31 patients was 75.38 years (range: 60–92 years), and 58.06% (18/31) were male patients. Moreover, 13 patients (41.94 %) were hospitalized in the intensive care unit, and 9 patients in the neurosurgery ward. Intracranial hemorrhage and pneumonia were the most common underlying diseases. Furthermore, 22 (70.97%) patients received endotracheal intubation. The baseline clinical characteristics of the patients are presented in **Table 1**.

Additionally, 29 patients were exposed to various antimicrobial drugs before the positive culture (**Table 1**). The main antibiotics used included β-lactam/β-lactamase inhibitor combinations, cephalosporins, carbapenems, quinolones, vancomycin, and linezolid. Based on *in vitro* susceptibility testing results, 80.65% (25/31) of the patients received inappropriate empirical treatment. Of these, 41.94% (13/31) switched to appropriate drugs such as tigecycline, levofloxacin, and amikacin after susceptibility results were available. Antimicrobial treatment adjustment and outcomes are presented in **Table 1**. Eventually, 16 patients improved, 14 patients were discharged automatically or transferred to other hospitals, and only one patient died.

Susceptibility Results of CRKp Isolates and Drug Resistance Genes

The antimicrobial susceptibility profiles of the CRKp isolates are listed in **Table 2**. Only Kp22 was sensitive to aztreonam. Other isolates exhibited high resistance to cephalosporin, β-lactam/β-lactamase inhibitor combinations, and carbapenems. Kp27 was sensitive to levofloxacin and ciprofloxacin. The resistance rate of 31 isolates to gentamicin and amikacin was 19.35%. Among the 31 isolates, 27 strains (87.10%) were susceptible to trimethoprim-sulfamethoxazole and all isolates were susceptible to colistin and tigecycline (100%).

The predominant carbapenem resistance gene was bla_{KPC-2} (93.55%, 29/31). Two isolates carried bla_{NDM-5}. In addition to carbapenem resistance genes, we examined other types of β-lactamase genes, including ESBLs and AmpC genes. The distribution patterns of resistance genes in these strains are listed in **Table 3**. We identified bla_{TEM-1}, bla_{SHV-1}, and $bla_{CTX-M-1group}$ in 31 (100%), 28 (90.32%), and 27 (87.10%) isolates, respectively. Moreover, 30 isolates carried class I integron. Among the 25 aminoglycoside resistant CRKps, 23 isolates carried the *rmtB* gene, and Kp23 also carried *armA*. Kp3 and Kp9 carried only *armA*.

Serotype and Virulence Genes

The serotype and 12 virulence-associated genes of CRKp isolates were analyzed. For the six serotypes closely related to hypervirulent *K. pneumoniae*, all strains exhibited negative results. Among the 12 virulence-associated genes, *fim, uge, mrkD, and wabG* were harbored by all 31 isolates. In addition, *rmpA* and *iucB* were also harbored by 74.19% (23/31) and 80.65% (25/31) of the strains, respectively; however, the other six genes including, *aerobactin*, *wcaG, ybtA, iroNB, ureA, kfuBC,* and *allS* were not detected in any of the 31 isolates. The detailed results are listed in **Table 3**.

PFGE and MLST Analysis of CRKp Isolates

MLST analysis revealed that 29 CRKps carrying bla_{KPC-2} were ST11 type, which was the most common type of CRKp found in China. Moreover, bla_{NDM-5} carrying Kp22 and Kp27 was assigned to ST307 and ST1562, respectively. For PFGE, a cluster was defined as strains with homology >80%. The results revealed that the homology of 29 isolates of ST11 was more than 80%, indicating a cluster; however, no homology between the two strains carrying bla_{NDM-5} (**Figure 1**). Notably, the eight CRKps from neurosurgery, between April 6, 2019 and May 19, 2019, showed high homology (≥95%).

Conjugation and Plasmid Analysis

Our results revealed that bla_{NDM-5} was successfully transferred to *E. coli J53* from Kp22 and Kp27 isolates. The corresponding transconjugants are termed J22 and J27, respectively. Regrettably, the bla_{KPC-2} gene failed to be transferred. Compared to the recipient strain *E. coli J53*, J22, and J27 exhibited significantly reduced carbapenem susceptibility (**Table 2**). The sensitivities of cephalosporin, β-lactam/β-lactamase inhibitor combinations, and carbapenems were similar to those of the donor strains,

TABLE 1 | Clinical characteristics of 31 elderly patients with LRTIs by CRKp.

No.	Gender	Age	Ward	Clinical diagnosis	Specimen	Antibiotic therapy(Before culture)	Antibiotic therapy(After culture)	Admission date	Isolate date	Discharge date	Endotracheal intubation	Prognosis
P1	F	86	ICU	Severe pneumonia	Pleural effusion	MEM, LZD, SCF, TZP, MFX	TGC	2019/1/24	2019/2/15	2019/3/15	Yes	Automatic discharge
P2	F	66	General Medical Ward	Multiple system atrophy	BALF	TZP, AK	AK	2019/1/3	2019/2/16	2019/3/10	Yes	Automatic discharge
P3	F	85	Respiratory	Pneumonia	BALF	VAN, SCF		2019/2/11	2019/2/19	2019/3/5	Yes	Automatic discharge
P4	M	81	Cardic department	Heart failure	Sputum	TZP, LEV, IMP	TGC	2019/3/1	2019/3/19	2019/3/27	No	Got better
P5	M	77	EICU	COPD	Sputum	TZP, SCF	TGC	2019/3/7	2019/3/18	2019/3/28	Yes	Got better
P6	F	60	Neurosurgery	Intracranial hemorrhage	Sputum			2019/4/4	2019/4/11	2019/4/22	Yes	Transfered
P7	M	68	Neurosurgery	Intracranial hemorrhage	Sputum	TZP	TGC	2019/4/3	2019/4/11	2019/4/24	Yes	Got better
P8	F	69	Neurosurgery	Intracranial hemorrhage	Sputum	TZP	TGC	2019/4/9	2019/4/18	2019/4/28	Yes	Got better
P9	F	85	Respiratory	Pneumonia	BALF	MEM		2019/2/26	2019/4/24	2019/5/8	Yes	Transfered
P10	M	82	ICU	Fever	BALF	MEM, VRC, TZP		2019/4/4	2019/4/23	2019/5/8	No	Got better
P11	M	61	Nephrology	chronic renal failure	BALF			2019/2/12	2019/4/28	2019/5/4	No	Got better
P12	F	62	Respiratory	COPD and pneumonia	Sputum	SCF, IMP, TGC, VRC	TGC	2019/4/21	2019/4/28	2019/5/2	No	Got better
P13	M	65	Neurosurgery	Intracranial injury	Sputum	TZP, TGC	TGC	2019/4/24	2019/4/29	2019/5/19	Yes	Got better
P14	M	76	Neurosurgery	Intracranial hemorrhage	Sputum	TZP, FOX		2019/5/2	2019/5/11	1905/7/11	Yes	Got better
P15	F	61	Neurosurgery	Intracranial hemorrhage	BALF	TZP	TGC	2019/4/27	2019/5/11	2019/5/25	Yes	Got better
P16	F	82	Neurosurgery	Intracranial hemorrhage	Sputum	SCF, TZP		2019/5/5	2019/5/19	2019/5/24	Yes	Transfered
P17	F	71	ICU	Jaundice	Pleural effusion	TZP, LZD		2019/7/11	2019/8/6	2019/8/7	Yes	Automatic discharge
P18	M	79	ICU	Cardiac insufficiency	Pleural effusion	MEM, LEV, IMP	TGC	2019/1/25	2019/5/25	2019/6/14	Yes	Automatic discharge
P19	F	92	ICU	Epilepsy	Sputum	LEV, MEM, LZD	AK	2019/5/10	2019/5/26	2019/6/12	Yes	Got better
P20	M	79	ICU	Bellyache	Sputum	MEM		2019/5/29	2019/5/30	2019/6/10	No	Got better
P21	M	76	ICU	Abdominal distention	Sputum	SCF, VRC	TGC	2019/5/20	2019/6/10	2019/6/19	No	Automatic discharge
P22	M	84	Nephrology	chronic renal failure	BALF	TZP, LZD, CAZ		2019/5/15	2019/6/12	2019/6/27	No	Automatic discharge
P23	M	74	ICU	Cerebral vascular disease	Sputum	CAZ, MEM, LEV, SCF, LZD, TGC	TGC	2019/5/8	2019/6/13	2019/8/22	Yes	Got better
P24	M	91	ICU	Intracranial injury	Sputum	CAZ, TZP	TGC	2019/6/6	2019/6/21	2019/12/30	Yes	Death
P25	M	64	ICU	Shock	Sputum	MEM, TZP, AK	AK	2019/6/17	2019/6/26	2019/8/9	No	Got better
P26	F	87	Respiratory	Pneumonia	BALF	TZP, SCF	TGC	2019/4/23	2019/7/1	2019/7/15	Yes	Automatic discharge
P27	M	84	ICU	Jaundice	BALF	TZP, IMP, FEP, LEV	LEV	2019/6/5	2019/7/8	2019/7/15	Yes	Automatic discharge
P28	M	91	ICU	Intracranial injury	BALF			2019/6/27	2019/7/11	2019/8/9	Yes	Transfered
P29	F	70	Neurosurgery	Intracranial injury	Sputum	TZP	TGC	2019/3/30	2019/4/6	2019/4/23	Yes	Got better
P30	M	68	Neurosurgery	Intracranial hemorrhage	Sputum	CAZ, LEV, TZP	TGC	2019/7/25	2019/8/4	2019/9/17	Yes	Got better
P31	M	61	Nephrology	Chronic renal failure	Sputum	TZP, LZD		2019/4/10	2019/4/13	2019/5/7	No	Automatic discharge

F, female; M, man; N, no; Y, yes; ICU, intensive care unit; AK, amikacin; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; IMP, imipenem; LEV, levofloxacin; LZD, linezolid; MEM, meropenem; MFX, moxifloxacin; SCF, cefoperazone/sulbactam; TGC, tigecycline; TZP, piperacillin-tazobactam; VAN, vancomycin; VRC, voriconazole.

TABLE 2 | The MIC of 31 CRKp isolates and two transconjugants (μg/mL).

No.	SAM	TZP	ATM	CRO	CAZ	FEP	FOX	ETP	IMP	MEM	SXT	CIP	LEV	CN	AK	TGC	CO
Kp1	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≥16/304	≥4	≥8	≤1	≤2	1	0.25
Kp2	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≤1	≤2	0.5	0.25
Kp3	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.25	1
Kp4	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≥16/304	≥4	≥8	≥16	≥64	0.5	0.5
Kp5	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.5	0.5
Kp6	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.25	0.5
Kp7	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.5	0.5
Kp8	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	1	0.5
Kp9	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	1	1
Kp10	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≥16/304	≥4	≥8	≥16	≥64	0.25	0.5
Kp11	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.5	1
Kp12	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.5	0.25
Kp13	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.5	0.25
Kp14	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.25	0.5
Kp15	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.25	0.5
Kp16	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.25	0.5
Kp17	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.25	0.25
Kp18	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.5	0.25
Kp19	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≤1	≤2	0.5	0.5
Kp20	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.25	0.5
Kp21	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≤1	≤2	0.5	0.5
Kp22	>16/8	≥128	≤4	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	1	0.5
Kp23	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	1	0.5
Kp24	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.25	1
Kp25	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.5	0.5
Kp26	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≥16/304	≥4	≥8	≥16	≥64	0.5	1
Kp27	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≤0.25	≤0.25	≤1	≤2	1	0.25
Kp28	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.5	0.25
Kp29	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.25	1
Kp30	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≥16	≥64	0.5	0.5
Kp31	>16/8	≥128	>16	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≥4	≥8	≤1	≤2	0.5	0.5
J22	>16/8	≥128	≤4	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≤0.25	≤0.25	≤1	≤2	0.125	0.125
J27	>16/8	≥128	≤4	≥64	≥64	≥64	>16	>32	>32	>32	≤1/19	≤0.25	≤0.25	≤1	≤2	0.125	0.125

MIC, Minimum inhibitory concentration; SAM, ampicillin sulbactam; TZP, piperacillin/tazobactam; ATM, aztreonam; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; ETP, ertapenem; IPM, imipenem; MEM, meropenem; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; LEV, levofloxacin; CN, gentamicin; AK, amikacin; TGC, tigecycline; CO, colisin.

TABLE 3 | The drug-resistance genes and virulence genes of 31 CRKp isolates.

	Drug-resistance genes								Virulence genes					
	β-lactamases						Aminoglycoside		Capsule			Fimbriae		Iron acquistion
No.	*bla*$_{KPC-2}$	*bla*$_{NDM-5}$	*bla*$_{SHV-11}$	*bla*$_{TEM-1}$	*bla*$_{CTX-M-1\ group}$	Class I integrons	rmtB	armA	rmpA	wabG	uge	fimH	mrkD	iucB
Kp1	*		*	*		*				*	*	*	*	
Kp2	*		*	*		*				*	*	*	*	
Kp3	*		*	*	*	*		*		*	*	*	*	*
Kp4	*		*	*	*	*	*			*	*	*	*	*
Kp5	*		*	*	*	*	*		*	*	*	*	*	*
Kp6	*		*	*	*	*	*		*	*	*	*	*	
Kp7	*		*	*	*	*	*		*	*	*	*	*	*
Kp8	*		*	*	*	*	*		*	*	*	*	*	*
Kp9	*		*	*	*	*		*		*	*	*	*	*
Kp10	*		*	*	*	*	*		*	*	*	*	*	*
Kp11	*		*	*	*	*	*		*	*	*	*	*	*
Kp12	*		*	*	*	*	*		*	*	*	*	*	*
Kp13	*		*	*	*	*	*		*	*	*	*	*	*
Kp14	*		*	*		*	*		*	*	*	*	*	*
Kp15	*		*	*	*	*	*		*	*	*	*	*	*
Kp16	*		*	*	*	*	*		*	*	*	*	*	*
Kp17	*		*	*	*	*	*		*	*	*	*	*	*
Kp18	*		*	*	*	*	*		*	*	*	*	*	*
Kp19	*			*	*	*				*	*	*	*	
Kp20	*		*	*	*	*	*		*	*	*	*	*	*
Kp21	*		*	*	*	*	*		*	*	*	*	*	*
Kp22		*		*			*			*	*	*	*	*
Kp23	*		*	*	*	*	*	*	*	*	*	*	*	*
Kp24	*		*	*	*	*	*		*	*	*	*	*	*
Kp25	*		*	*	*	*	*		*	*	*	*	*	*
Kp26	*		*	*	*	*	*			*	*	*	*	
Kp27		*		*	*	*		*	*	*	*	*	*	
Kp28	*		*	*	*	*	*		*	*	*	*	*	*
Kp29	*		*	*	*	*	*		*	*	*	*	*	*
Kp30	*		*	*	*	*	*		*	*	*	*	*	*
Kp31	*		*	*	*	*	*	*	*	*	*	*	*	*

: positive; blank: negative.

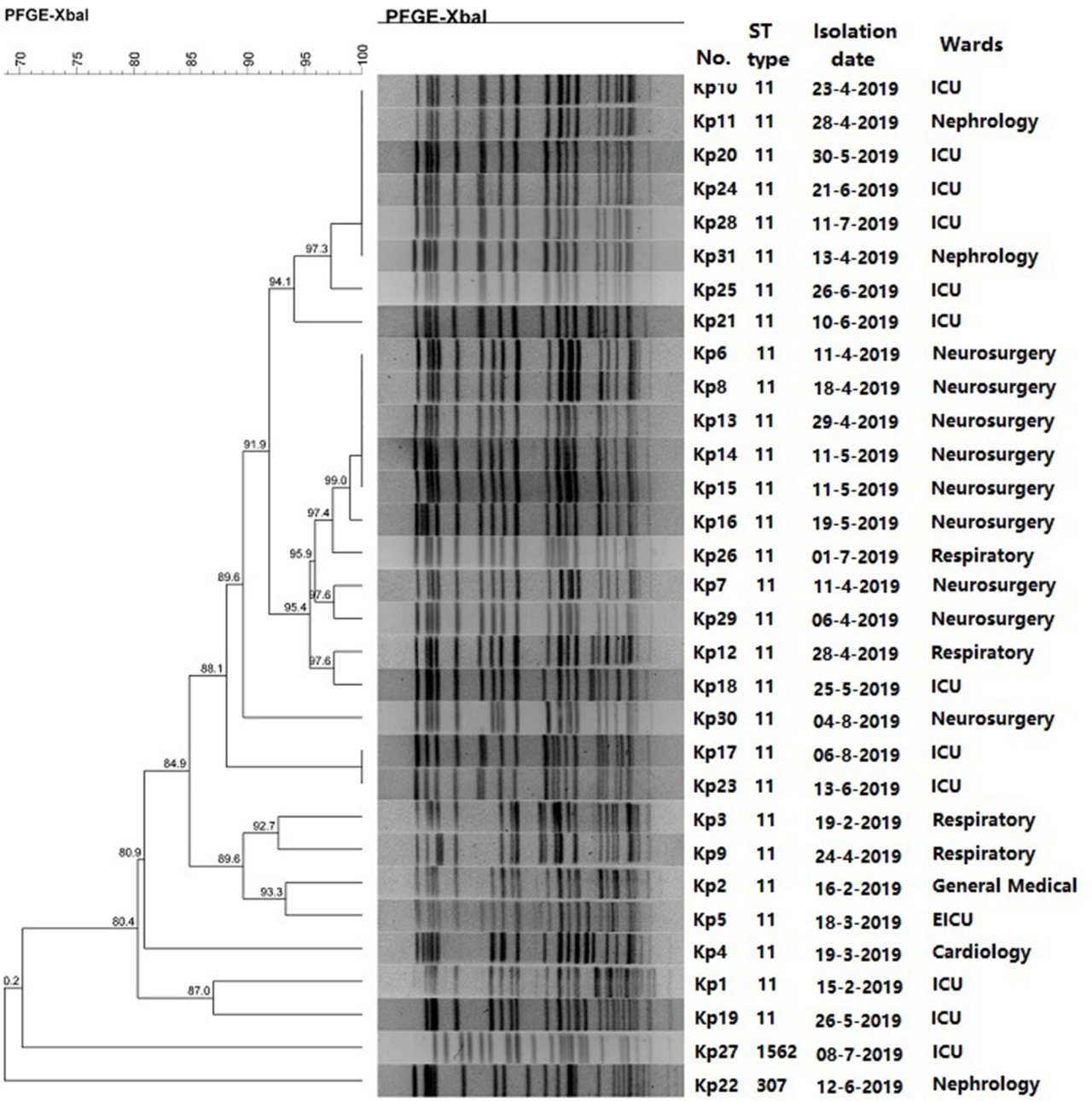

FIGURE 1 | PFGE results and MLST typing for 31 CRKp isolates.

and the sensitivity of J22 and J27 to aztreonam, trimethoprim-sulfamethoxazole, quinolones, aminoglycosamines, tigecycline, and colistin were similar to those of *E. coli J53*. Moreover, S1-PFGE revealed that 31 CRKp isolates carried 1–4 plasmids (**Supplementary Figure 1**). Both Kp22 and Kp27 contained two plasmids. After conjugation, J22 and J27 contained only one plasmid, about 45 kb in size (**Figure 2**). Furthermore, PCR-based replicon typing revealed that these two plasmids belong to the IncX3 incompatibility group.

DISCUSSION

Respiratory tract infections can occur at any age; however, LRTIs is more common in the elderly people. Moreover, pneumonia is a leading cause of illness and death in the elderly (13). Viruses are responsible for a large proportion of LRTIs but antibiotics are often unnecessarily prescribed for their treatment without any laboratory testing and can contribute to the emergence of antimicrobial resistance (14). *K. pneumoniae* is the predominant

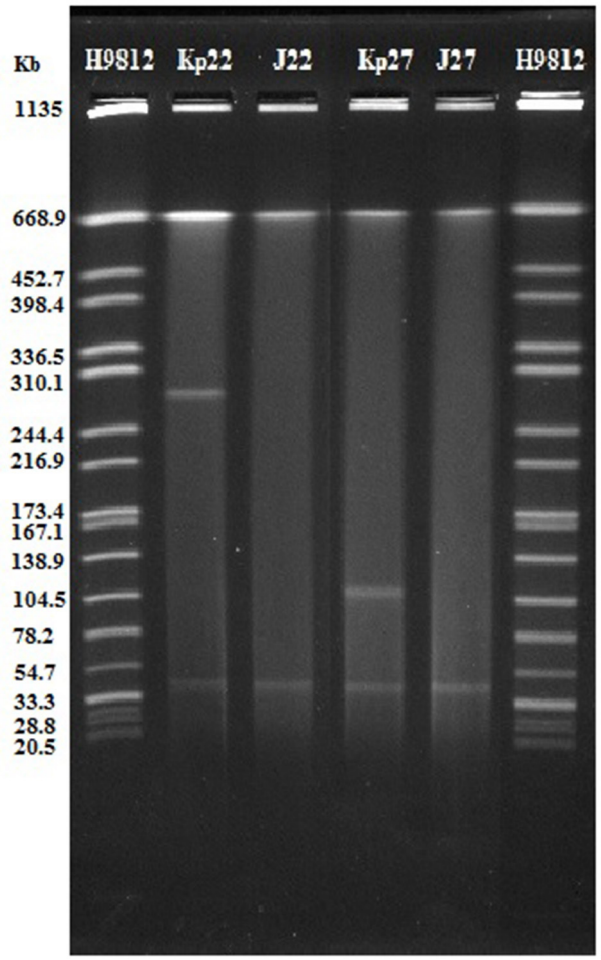

FIGURE 2 | S1-PFGE results for Kp22 and Kp27 and their transconjugants.

bacterial pathogen of LRTIs, and the positive rate of CRKp is increasing year by year, particularly for elderly patients (15, 16). Analysis of the molecular characteristics and drug resistance mechanism of CRKp from elderly patients with LRTIs can prove beneficial in treating this kind of infection.

In China, KPC-2 is the predominant carbapenemase in CRKp, and ST11 is the major sequence type of CRKp (17, 18). Similarly, the majority (93.55%) of CRKp isolates in the present study were KPC-2-producing ST11. We isolated two strains of $bla_{\mathrm{NDM-5}}$ carrying *K. pneumoniae* in the present study. In addition to *bla*KPC-2 and $bla_{\mathrm{NDM-5}}$, all the 31 isolates harbored *bla*TEM-1. Moreover, majority of these isolates carried $bla_{\mathrm{SHV-11}}$ and $bla_{\mathrm{CTX-M-1}}$, which was in accordance to the results of the previous reports (19, 20). These resistance genes led to the resistance of 31 isolates to β-lactams. Furthermore, most isolated strains were resistant to aminoglycosides and quinolones, which made the clinical fewer antibiotics choice. Fortunately, all strains were sensitive to tigecycline and colistin.

The emergence and spread of New Delhi metallolactamase (NDM)-producing *Enterobacteriaceae* has posed a serious public health concerns. The NDM-5 carbapenemase differs from NDM-1 by only two amino acid substitutions (Val88Leu and Met154Leu) and exhibits increased resistance to carbapenems and expanded-spectrum cephalosporins (21). NDM-5 has been identified mostly in *E. coli* but has rarely been described in *K. pneumoniae* and other *Enterobacteriaceae* isolates. In the present study, two *K. pneumoniae* isolates carrying $bla_{\mathrm{NDM-5}}$ came from different wards. They were assigned to be ST307 and ST1562. NDM-5 carried ST307 *K. pneumoniae* has been reported previously (21, 22). To the best of our knowledge, this is the first report of ST 1562 types with $bla_{\mathrm{NDM-5}}$. The $bla_{\mathrm{NDM-5}}$ gene was located on a conjugative IncX3 plasmid. In 2019, Ziyan Kong reported a nosocomial outbreak of NDM-5-producing *K. pneumoniae* in a neonatal unit in China (8). They demonstrated that all $bla_{\mathrm{NDM-5}}$ genes were located on a ∼45 kb IncX3 type plasmid. The study of Zhu et al. (21) showed that the IncX3 plasmid facilitated the dissemination of $bla_{\mathrm{NDM-5}}$ among multiclonal *K. pneumoniae* strains and that conjugal transfer contributed significantly to IncX3 plasmid stability within *K. pneumoniae*. Hence, there is an urgent need for effective infection control measures to prevent $bla_{\mathrm{NDM-5}}$ variants from causing epidemic in the future.

Over the past decade, carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKP) has gained immense attention (23). In this study, the serotypes closely related to hypervirulent *K. pneumoniae* and 12 virulence-associated genes of 31 CRKp isolates were analyzed. Six virulence-associated genes, *fim, uge, mrkD, wabG, rmpA,* and *iucB* were harbored by almost 31 isolates. These virulence genes are related to capsule synthesis, flagella movement, and iron acquisition, which are virulence factors of *K. pneumonia* (24). Fortunately, several serotypes of hypervirulent *K. pneumoniae* remained undetected. Eventually, 31 patients revealed good prognosis, and only one patient died.

The main risk factors of CRKp infection include immunosuppression, ICU admission, antibiotics exposure, surgery, mechanical ventilation, and central venous catheterization (25, 26). Elderly patients with LRTI often present non-specific symptoms, which are often covered by primary diseases, leading to irrational use of antibiotics (27). In the present study, 29 patients were exposed to various antimicrobial drugs before the positive culture. Piperacillin/tazobactam was the most commonly used antibiotic in 31 patients. Based on *in vitro* susceptibility testing results, majority of these patients changed to appropriate drugs such as tigecycline, levofloxacin, and amikacin. Importantly, nervous system diseases including intracranial hemorrhage and injury are the most common underlying diseases in this study. Most of these patients are unconscious and were subjected to mechanical ventilation. This is in accordance with the results of previous studies (26). Admission to neurosurgery is also a risk factor for CRKp infection. In addition, CRKp strains from neurosurgical patients have high homology, which indicates that there may be an outbreak of nosocomial infection of CRKps in neurosurgery; however, as this study was retrospective, we could not perform in-depth analysis of the patient's surrounding environment and the source of the strain.

In summary, we reported the characteristics of CRKp isolates collected from elderly patients with LRTIs. The high incidence of CRKp highlights the urgent need for further surveillance and strict infection control measures, particularly for ICU patients and immune-compromised elderly patients. One bla_{NDM-5} carrying CRKp with ST1562 were first reported.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Taian City Central Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MJ and FZ designed the experiments and revised the manuscript. CS carried out the experiments and wrote the manuscript. WW and SL analyzed the data. ZZ contributed to experiment conception. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.669173/full#supplementary-material

Supplementary Figure 1 | S1-PFGE results for 31 CRKp isolates.

Supplementary Table 1 | Primers used in the present study.

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Toward a Country-Based Prediction Model of COVID-19 Infections and Deaths Between Disease Apex and End: Evidence From Countries With Contained Numbers of COVID-19

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The complexity of COVID-19 and variations in control measures and containment efforts in different countries have caused difficulties in the prediction and modeling of the COVID-19 pandemic. We attempted to predict the scale of the latter half of the pandemic based on real data using the ratio between the early and latter halves from countries where the pandemic is largely over. We collected daily pandemic data from China, South Korea, and Switzerland and subtracted the ratio of pandemic days before and after the disease apex day of COVID-19. We obtained the ratio of pandemic data and created multiple regression models for the relationship between before and after the apex day. We then tested our models using data from the first wave of the disease from 14 countries in Europe and the US. We then tested the models using data from these countries from the entire pandemic up to March 30, 2021. Results indicate that the actual number of cases from these countries during the first wave mostly fall in the predicted ranges of liniar regression, excepting Spain and Russia. Similarly, the actual deaths in these countries mostly fall into the range of predicted data. Using the accumulated data up to the day of apex and total accumulated data up to March 30, 2021, the data of case numbers in these countries are falling into the range of predicted data, except for data from Brazil. The actual number of deaths in all the countries are at or below the predicted data. In conclusion, a linear regression model built with real data from countries or regions from early pandemics can predict pandemic scales of the countries where the pandemics occur late. Such a prediction with a high degree of accuracy provides valuable information for governments and the public.

Keywords: coronavirus, COVID-19, mortality, pandemic, prediction, infectious disease, death

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INTRODUCTION

Disease modeling and prediction are important but difficult because of the great variations among infectious diseases (1). Multiple models have been developed to predict the total number of infections and deaths from COVID-19. Examples include the model by the U.S. Center for Disease Control and Prevention (CDC) (https://www.cdc.gov/coronavirus/2019-ncov/covid-data/forecasting-us.html), by the Institute for Health Metrics and Evaluation (IHME) (http://www.healthdata.org/covid/updates), the model at University of Washington (2), and at the Johns Hopkins coronavirus resource center (https://coronavirus.jhu.edu/). These models are useful, but changes had to be made constantly on their predictions based on new developments of COVID-19 (3, 4). Therefore, using real data from countries nearing the end of the pandemic to build prediction models may be an effective way to predict infections and deaths in countries where the pandemic is still ongoing.

Real data to build the predictions models were from countries including China, South Korea, and Switzerland, in which the COVID-19 pandemic has largely been controlled and its apex has already passed. Although the country-based conditions and pandemic situations in each country are very different, we believe that a careful analysis of the situations in these countries will help with predictions for countries where the pandemic is still developing and endemic (5). Although in some countries, the prevalence and incidence rate of the 2019 novel coronavirus (COVID-19) has passed its apex, and countries such as Italy and the UK are partially lifting restrictions, the decision to return to normal is still based on the trajectory of the pandemic and the accurate prediction of the pandemic's nadir (6).

In this study we tested whether a country-based model using data from China, South Korea, and Switzerland is useful for predictions for other countries. We conducted a comprehensive analysis of these data to build the model and then used the model to make predictions about the countries where the pandemic is still currently prevalent.

METHODOLOGICAL APPROACH

Data Collection

Data for COVID-19 disease prevalence and mortality from cities and provinces in China and other countries were obtained from public websites (7). Data were collected on daily cumulative total number of patients, new cases, cumulative total deaths, and new deaths. Data from China were collected from the period beginning Jan 19, 2020 up to March 19, 2020, when the daily new domestic case fell to zero. Data from other countries begins with the date of the first report of the number of COVID-19 patients through May 10 for establishment of the predictive model. Data for the model testing were collected before and up to March 30, 2021 from https://www.worldometers.info/coronavirus/. The newly updated data from all countries were collected from WHO daily situation report on COVID-19 at https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports.

Characterization of the COVID-19 Pandemic

Data were uploaded into an Excel spreadsheet and characterized with different parameters. For the cities and provinces in China, patterns of COVID-19 were defined by the parameters as follows. The time of the beginning of the COVID-19 pandemic is defined as the day the first COVID-19 patient was reported. The end of the pandemic was defined as the 1st day of zero new patients reported that was followed by no new patients reported continuously for the next 14 days. The whole pandemic period is defined as the day of the first reported COVID-19 patient to the day of the end of the pandemic (8–11). For data from China and other countries, the weighted numbers of patients and deaths were also calculated in intervals of 3, 5, and 7 days for estimating the apex day of the pandemic.

Relationship Between the Number of Patients at Apex Days, Death Ratio, and the Length of the Period From Apex to the End

One important statistic is the length of time from the disease apex day to the end of the disease pandemic (as defined by the metric described above). Once we obtained the parameters above, we calculated the days from the apex day to the end of the pandemic for cities and provinces in China. The days from the "first report" day and from the end day to the apex day, which is defined as the day with the largest number on the average of 3, 5, and 7 days, were then calculated. The relationship between the days from apex day to first report day, and days from apex day to end day, was characterized by regression modeling. For infected people during the COVID-19 pandemic period, we divided numbers of people into two categories: the infected numbers from the beginning of the pandemic to the apex day, and infected numbers for the remaining days until the end of the pandemic period. For the relationship between numbers of people infected in these two categories, we used four regression models: linear, exponential, logarithmic, and power models. Similarly, we also divided the death numbers into the same two categories as that for infections. The relationship between the two categories was analyzed with a similar approach.

Prediction of Days to the End of the Pandemic, Number of Infections (Or Infected Patients), and Deaths in The First Wave and Entire Pandemic in Top Pandemic Countries

Based on the mathematical models, we estimated the days from apex day to the end of the pandemic period, and the predicted future infection and death rates after the apex day in the 10 countries. These 10 countries are believed to be the countries with the highest prevalence of the epidemic with relatively reliable data on COVID-19. Multiple models were tested for the initial estimation, followed by estimations for the least

TABLE 1 | Information from pandemics in different cities, provinces, and countries for model construction.

City	Days from apex day to end	Days from apex day to begin	No. of patients on peak day	Total patients	Total death	Death to apex day
Wuhan	51	25	46,904	50,333	3,869	1,444
S Korea	50	17	4,335	10,683	237	32
Xiaogan	24	13	287	3,518	129	25
Huanggang	30	13	211	2,907	125	17
JingZhou	27	13	613	1,580	52	7
Erzhou	19	19	861	1,394	59	28
SueiZhou	22	12	706	1,307	45	8
Huangshi	25	11	509	1,015	39	2
Helongjiang	44	15	35	484	13	3
Henan	23	12	86	1,273	22	2
Beijing	34	13	24	485	8	1
Hunan	27	10	65	1,018	4	0
Guangdong	38	12	95	1,391	8	0
Zhejiang	24	7	98	1,234	1	0
Anhui	23	13	63	990	6	0
Jiangxi	25	12	65	935	1	0
Shandong	43	14	33	762	7	0
Switzerland	50	27	7,474	30,344	1,854	98
Summary	579	258	62,465	111,653	6,479	1,667
Average	32	14	3,470	6,203	360	93
Ratio		2.24		1.79		3.89
R	0.56		0.99		0.92	
R (W/o Wuhan)	0.56		0.97		0.94	

and largest numbers for each of the three types of pandemic features.

The prediction made using data before May 10, 2020 was compared to predictions based on real data in the first wave of the pandemic and entire pandemic. The numbers of patients predicted based on the ratio before and after apex day and based on regression models are compared to the real numbers of patients at the end of the first pandemic and on the day of March 30, in the 14 countries. Similar comparisons were conducted for numbers of deaths.

Statistical Analyses

For the correlational analysis, we followed our previous criteria: a significant correlation was defined as an R value equal to or more than 0.7 or −0.7 for either a strong positive or negative correlation, an R value between 0.35 and 0.69 or −0.35 and −0.69 was considered a moderate correlation, and an R value between 0 and 0.35 or 0 and −0.35 was considered as no correlation between the two measures. To build models for estimating the total number of patients and total mortalities based on prevalence up to the apex day, we compared the models by testing all multiple regression models, including linear, polynomial, logistic regression, and linear with power analysis. The best fit to the distribution of real data on the plots was selected as the estimation model.

RESULTS

Characterizations of Apex Days of the COVID-19 Disease

By analyzing data from nine provinces other than Hubei, the major cities of Hubei Province, and from South Korea and Switzerland, we determined the apex incidence in each region. Then we counted the date from the beginning of the disease epidemic to the apex (hereafter referred to as before apex day) and the date from the apex to the end of the pandemic. Overall, the date from the apex to the end of the outbreak was almost twice that of the period from the beginning to the apex of the disease (see **Table 1**). The average time from the day of first case reported to the apex day was 14 days, while the time between the apex day to the day of no new patients was more than 32 days. The ratio of before and after the apex day is 1:2.24. The correlation coefficient between these two time periods was 0.56 (see **Table 1**). **Figure 1A** shows the pre- and post-onset dates in each region. **Figure 1B** shows the correlation between the early stage and the late stage. Their relationship is expressed in the form of three models using linear, polynomial, and exponential regressions. At the same time, we noticed that the apex period of presentation is not the same everywhere (see **Supplementary Figure 1**). Some areas have an obvious apex period (see **Figure 1C**), while in some areas the apex period is relatively flat and not as obvious (see **Figure 1D**). What is more interesting is that

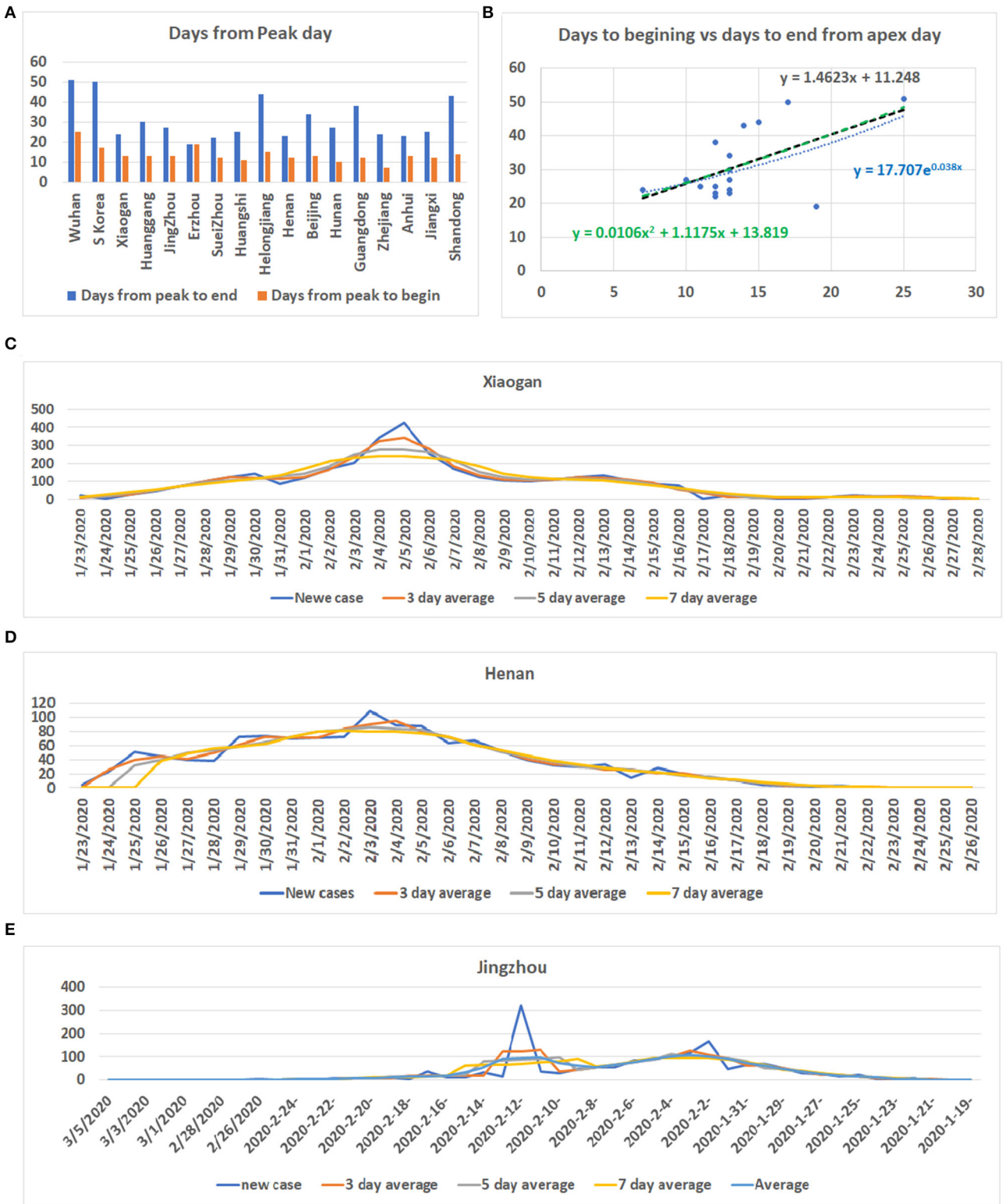


FIGURE 1 | Information for COVID-19 pandemic. **(A)** The number of days from the beginning of the COVID-19 onset to the apex of onset and the number of days from the apex of onset to no new cases in each region. Vertical axis: days, Horizontal axis: cases. **(B)** Correlation between the date before the apex of onset and the date after the apex. Vertical axis: days after apex day, Horizontal axis: cases before and including apex day. **(C)** The distribution of the number of cases in Xiaogan City during all periods measured. It shows that there is a very obvious apex. **(D)** The daily distribution of patients in Henan Province during the pandemic. The apex onset date is not obvious. **(E)** The distribution of patients in Jingzhou shows that there are two apexes of incidence.

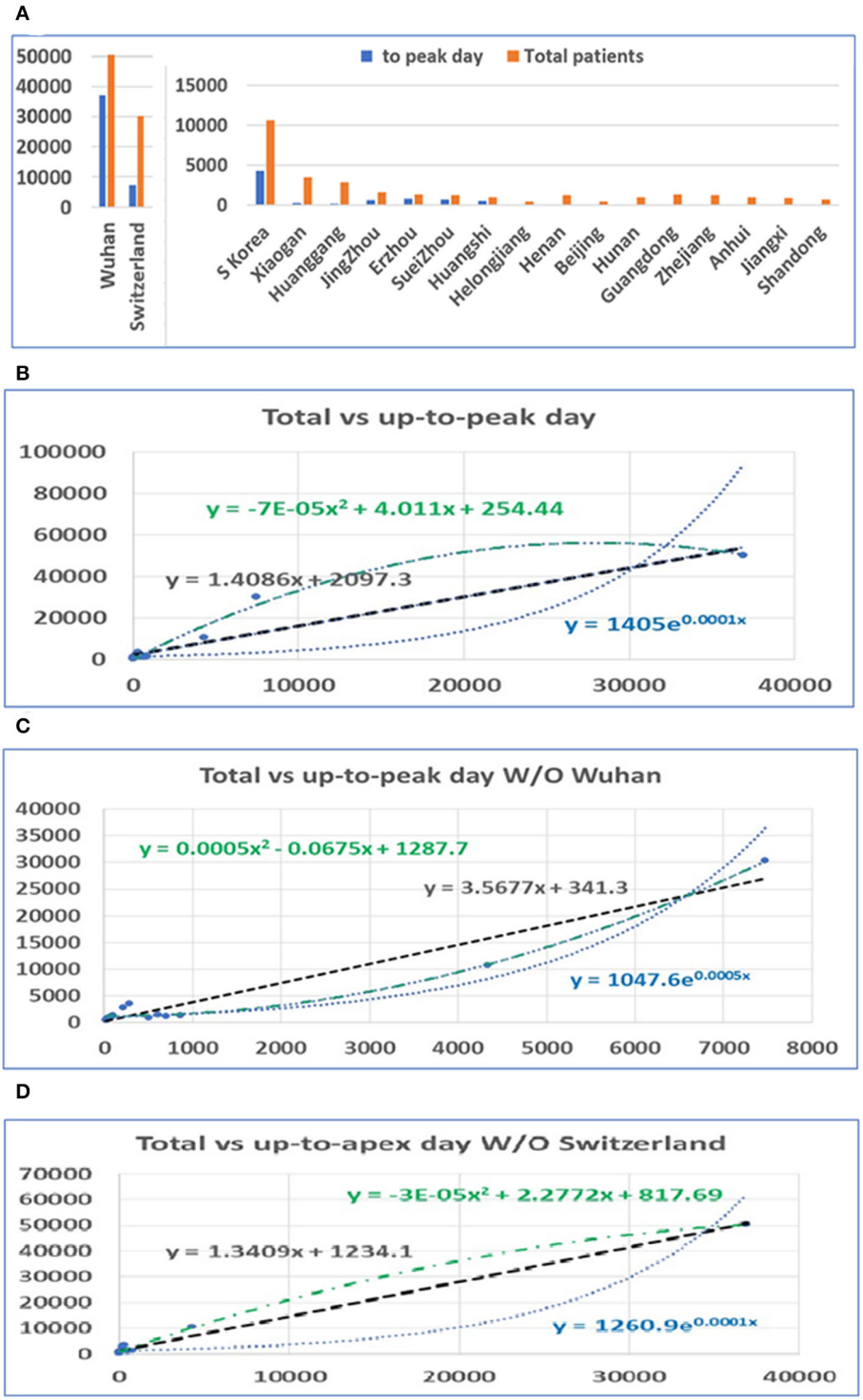


FIGURE 2 | The distribution of patients before and after the pandemic apex. **(A)** The number of patients in major cities in Hubei Province in China, the provinces with the highest incidence, those in Switzerland, and those in South Korea before and after the apex of the pandemic. **(B)** The relationship between the total number of patients and the number of patients before the apex of the disease. The number on the vertical axis is the total number of cases, and the horizontal axis is the number of cases before the apex of the pandemic. **(C)** The relationship between the total number of cases and the number of cases before the apex period, excluding data from Wuhan. **(D)** The relationship between the total number of cases and the number of cases before the apex period, excluding data from Switzerland.

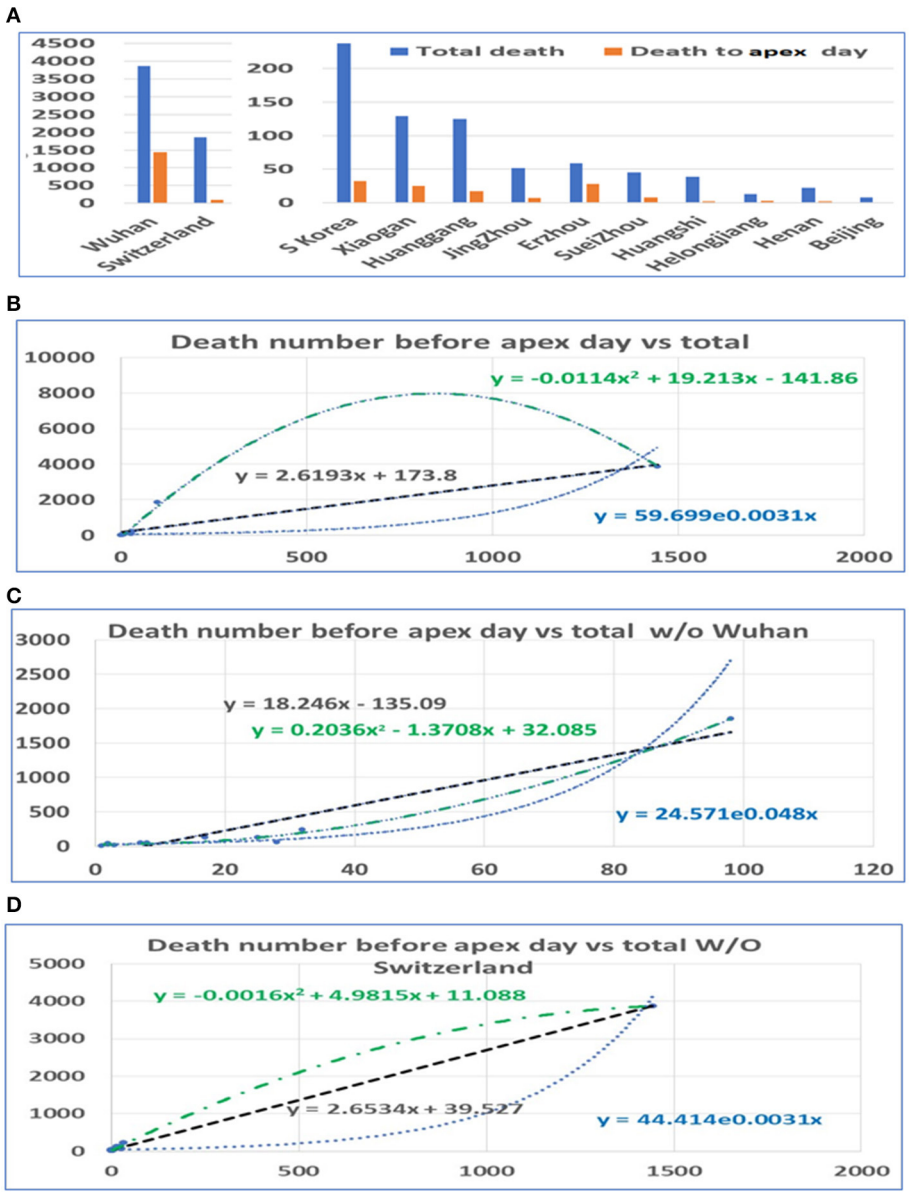


FIGURE 3 | The distribution of mortality before and after the pandemic apex. **(A)** The mortality in major cities in Hubei Province in China, the provinces with the highest incidence, those in Switzerland, and those in South Korea before and after the apex of the pandemic. **(B)** The relationship between the total mortality and the mortality before the apex of the disease. The number on the vertical axis is the total mortality, and the horizontal axis is the mortality before the apex of the pandemic. **(C)** The relationship between the total mortality and the mortality before the apex period, excluding data from Wuhan. **(D)** The relationship between the total mortality and the mortality before the apex period, excluding data from Switzerland.

some areas have a small apex period after the apical apex period (see **Figure 1E**).

Relationship of Infection Rates Before and After Apex Day

After the apex period of the COVID-19 pandemic was determined, we conducted statistical analyses of the number of patients before and after the apex period. Interestingly, the difference between the number of patients before and after the apex period is not as great as the difference between the days for the pandemic period before and after the apex day (see **Figure 2A**). Our analysis shows that when the data from Wuhan are included, the number of infected persons in the latter period of the outbreak is smaller than the number of patients before the apex period of the infection. If we exclude the data from Wuhan, in the latter period of the pandemic, the number of infected patients is slightly bigger than the previous number. However, the number of patients before the apex day and the total

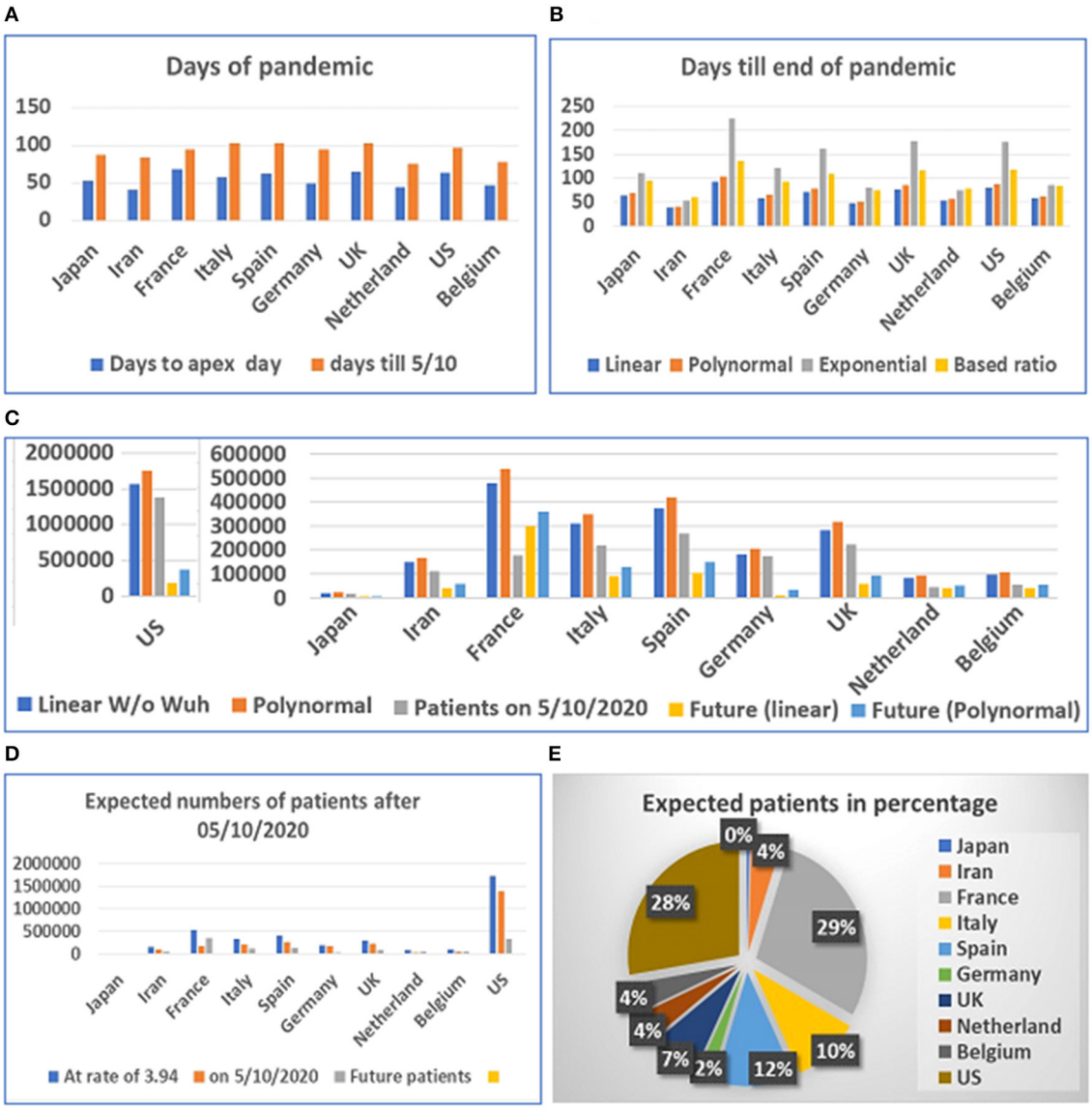
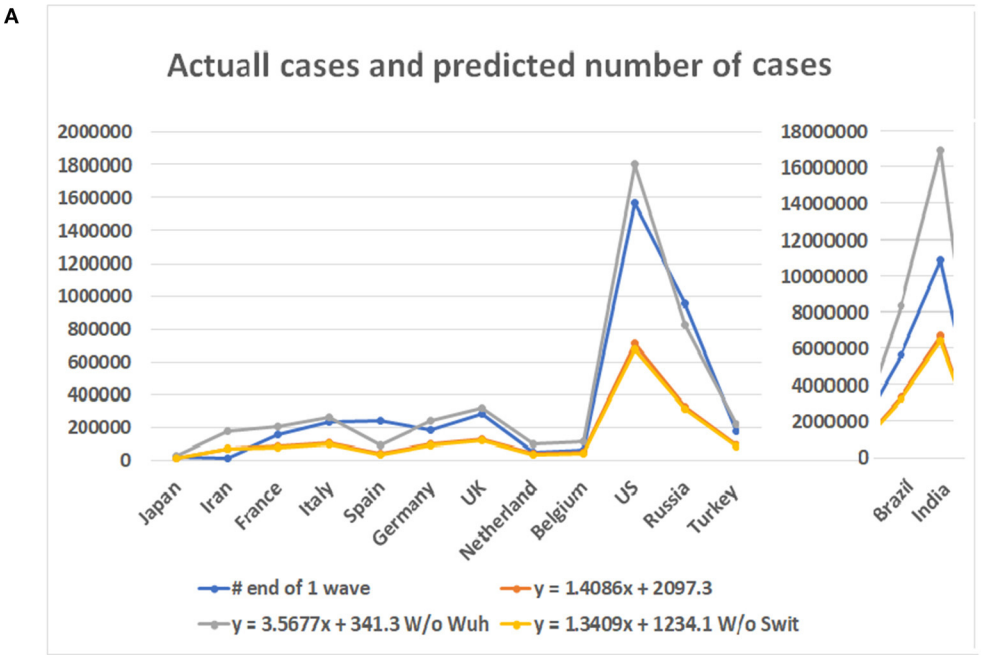


FIGURE 4 | Days and number of patients predicted after May 1, 2020 in 10 countries. **(A)** Days of pandemic from beginning to apex day and to May 1, 2020. **(B)** Days remaining before end of pandemic predicted with different models. **(C)** Future numbers of patients after May 10, 2020, predicted by linear model (W/O data from Wuhan) and polynomal model. **(D)** Proportions of expected patients in different countries based on calculated ratio before and after apex day from data from China, Switzerland, and South Korea. **(E)** Proportion of expected patients in different countries.

number of patients showed a significant correlation, regardless of whether data for Wuhan are included or not, with r values of 0.99 and 0.97 (see **Table 1**), respectively. **Figures 2B,C** show the relationship between the total number of people affected and the number of patients before apex day (see **Figure 2B**) and without data from Wuhan (see **Figure 2C**). **Figure 2D** shows the relationship between the total number of people infected and the number of patients before apex day without the data from Switzerland. The total numbers of patients before and after the apex are 62,465 and 111,653, respectively, with a ratio of 1:1.79 (see **Table 1**). However, if the data of Wuhan is excluded, numbers of patients before and after the apex are 15,561 and 61,320, respectively, and the ratio is 1:3.94. The models for these relationships were interpreted with multiple models including linear, polynomial, logarithmic power, and exponential regressions (see **Supplementary Figure 2**).

Relationship Between Death Numbers Before and After the Apex Day

In a similar way, we conducted statistical analyses of the mortalities before and after the apex of the pandemic. The mortality rates before the apex day and the total for the pandemics among cities in China and among these three countries are highly correlated, with $r = 0.92$. Unlike the number of infections, the mortalities after the apex were several times higher than that before the apex. Similarly, we performed



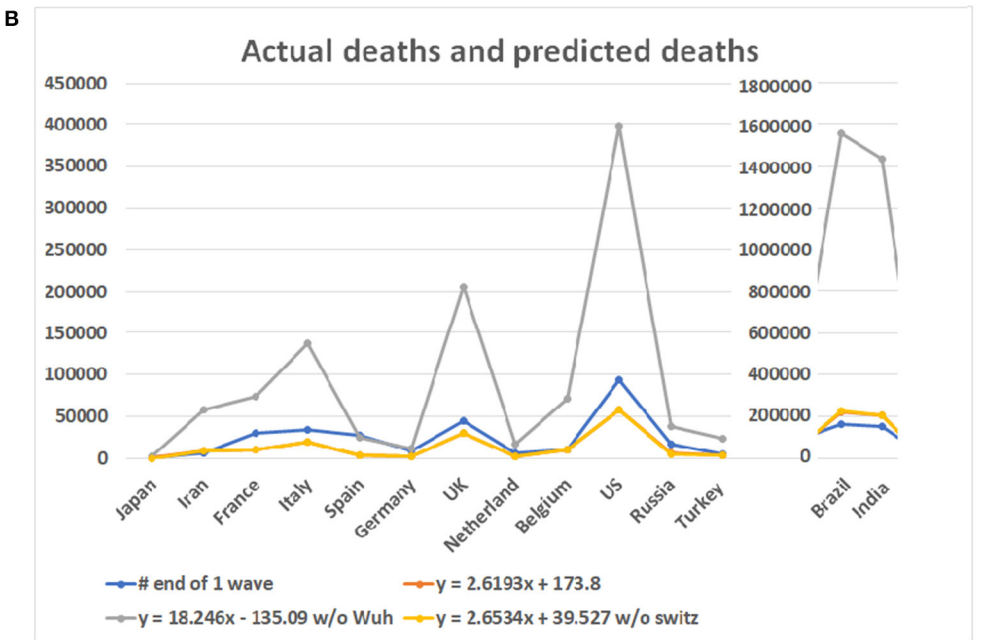


FIGURE 5 | Case numbers and mortality predicted for 14 countries and real numbers in the first wave of the disease. Numbers on Y bar are the case of death numbers. Color code: Blue for the real numbers. Red for the predicted numbers based on linear model. Gray color for the predicted numbers based on the linear model without data from Wuhan. Point for predicted numbers without data of Switzerland. **(A)** Predicted number of cases based on three linear models and real number of cases. **(B)** Death numbers predicted based on the linear models and reported numbers from 14 countries.

analyses both including and excluding data from Wuhan. The death toll in some provinces was zero before the apex. In order to prevent these provinces from being delayed for our statistics, we deleted the data from these regions in the analysis of the mortalities (see **Figure 3A**). Even so, the mortalities between the apex and the end of the outbreak are at least double the mortality before the apex. The correlation between the number of deaths before the apex day and number of total deaths is still high, with an r value of 0.92 with Wuhan, and 0.95 without Wuhan (see **Table 1**). The mortality numbers

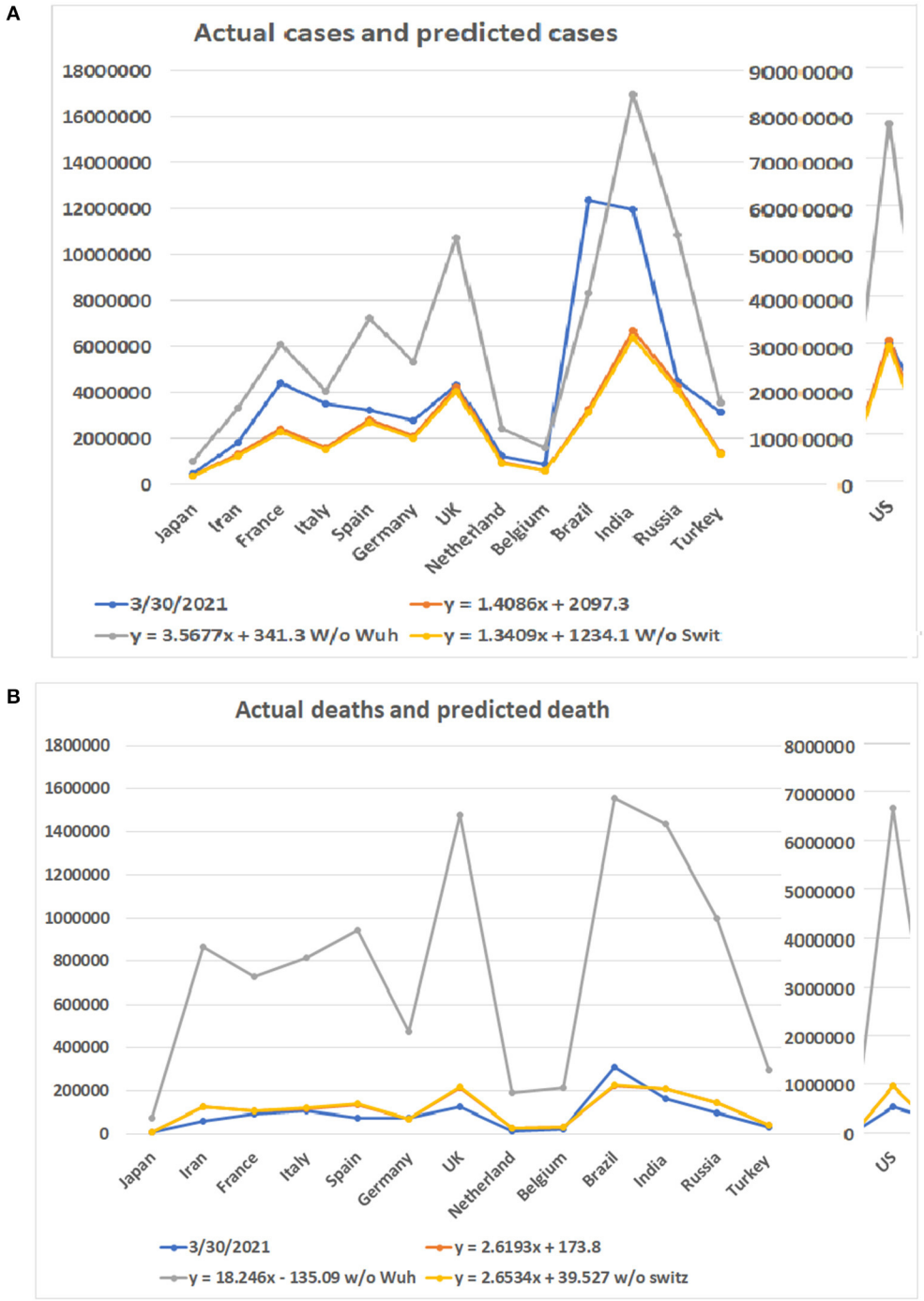


FIGURE 6 | Comparison of predicted numbers of patients and deaths on March 30, 2021 to the real numbers. Numbers on Y bar are the case of death numbers. Color code: Blue for the real numbers up to March 30, 2021. Red for the predicted numbers based on linear model. Gray color for the predicted numbers based on the linear model without data from Wuhan. Point for predicted numbers without data from Switzerland. **(A)** Predicted number of cases based on three linear models and real number of cases. **(B)** Predicted number of deaths based on three linear models and real number of deaths.

after the apex day are 3.87 and 11.58 times more than that before the apex day, with and without the data from Wuhan, respectively. Accordingly, the three types of regression models were performed between the death number before apex day and the total death numbers (see **Figures 3B,C**). Similar to the infected patient data, these models included linear, polynomial, and exponential regressions (see **Supplementary Figure 3**). The models in **Figure 3B** included the data from Wuhan while those in **Figure 3C** did not. The models in **Figure 3D** shows the relationship without the data from Switzerland. These results

indicate that many infected people who did not die before the apex day may die within a few days or even a dozen days after the apex period.

Prediction of Disease Extended Days, Infected Populations, and Deaths After the Apex Day of the COVID-19 Pandemic

Based on our estimation of the date of the pandemic before and after the apex of the COVID-19 pandemic, we predicted the future incidence of COVID-19 (beginning May 10, 2020) for several countries (see **Supplementary Table 1**). The data from some of the countries suggest that the disease has reached its apex (see **Supplementary Figure 4**). However, these data indicated that the predicted number of people infected in various countries after May 10 still ranges from at least 10,000 to tens of thousands. The maximum number of patients in the future for Japan, Iran, France, Italy, Spain, Germany, the UK, Netherland, Belgium, and the US are 5,220, 54,204, 349,134, 120,913, 142,082, 27,856, 88,157, 48,214, 51,619, and 340,713 based on simple ratio and 5,854, 57,405, 358,878, 127,308, 149,729, 31,722, 94,019, 50,109, 53,767, and 372,081 based on polynomial regression model, respectively.

The prediction of the length of the onset date after the apex period (see **Figure 4A**) was based on modeling by linear, polynomial, and exponential equations. Time was also calculated based on the ratio between days before and after the apex day. According to the results of these calculations, at least 3 weeks to 1 and 3 months are needed for the most affected countries to end the pandemic. The longest predicted pandemic durations are for France, the UK, and the US (see **Figure 4B**).

The prediction of the duration between the apex day and end day of disease was tested using multiple models. However, final predicted values were based on the linear and polynomial models (see **Figure 4C**). Our results suggest that the straight-linear equation produced negative values, while the data calculated by the exponential equation resulted in extraordinarily large numbers (see **Supplementary Table 2**). The model predicted that the number of patients in the US may exceed 1.7 million, while predicted patients in France may reach half a million before the end of the COVID-19 outbreak. We then calculated the number of patients before and after the apex day based on simple ratio, between before and after the apex day, which was found to be 1:3.94, calculated from data from China, South Korea, and Switzerland. The results predict that the US and France will have fewer than half a million more patients before the end of the pandemic (see **Figures 4D,E**).

Test the Predictability of Models With Data of the Apex Day and Accumulated Data in the First Wave of the COVID-19 Pandemic

Among the six mathematical models (see **Figures 3B–D**), only the linear models and one polynomial model converged. The first of the three linear models included the 10 locations while the second and third excluded data from Wuhan and Switzerland, respectively. The polynomial model excluded data from Wuhan. Other models produced either negative values or apparently aberrant data (see **Supplementary Table 3**). As the world failed to contain the COVID-19 disease, multiple waves of disease have occurred in many countries. We then compared the predicted case and death numbers from these three minor models and compared them to the real data in the first wave of the pandemic in 14 countries, which include the original 10 countries and four countries which were among the top 10 on the COVID-19 pandemic based on the case numbers (**Figure 5**). By comparing data from these three models, we obtained the minimum and maximum numbers of potential cases and deaths from each of these 14 countries (see **Figures 5A,B**). The maximum number of deaths in the future for Japan, Iran, France, Italy, Spain, Germany, the UK, Netherland, Belgium, the US, Brazil, India, Russia, and Turkey are 27,520, 180,395, 203,661, 265,728, 91,303, 238,967, 316,514, 95,067, 109,474, 1,805,451, 8,360,768, 16,962,457, 828,915, and 218,146, respectively. The minimum number of cases are 11,449, 68,907, 77,651, 100,978, 35,422, 90,920, 120,066, 36,836, 42,251, 679,675, 3,143,454, 6,376,350, 3,12,649, and 83,095, respectively. The actual number of cases in these countries are 16,851, 11,635, 156,156, 234,013, 242,707, 185,416, 285,420, 50,005, 60,550, 1,568,448, 5,631,181, 10,826,363, 956,749, and 175,218, respectively (**Figure 5A**). While data from most of the countries falls into the predicted range, the reported case from Iran is less than the predicted minimum number and Spain and Russia is more than the predicted maximum number of cases.

For the number of deaths, the maximum number predicted for these countries are 2,474, 57,522, 73,433, 136,765, 25,063, 11,634, 206,574, 17,454, 71,079, 398,175, 1,555,117, 1,433,745, 38,473, and 23,512, respectively. The actual numbers of deaths in all but Spain are less than the predicted maximum numbers. The minimum number predicted for these countries are 419, 8,424, 10,735, 19,826, 3,704, 1,751, 29,848, 2,597, 10,396, 57,353, 223,438, 206,014, 5,654, and 3,478, respectively. The death numbers of four countries, Iran, Belgium, Brazil, and India, are less than the predicted minimum numbers of deaths.

Test the Predictability of Models With Data of the Apex Day and Total Accumulated Data of the Updated COVID-19 Pandemic Period

As the COVID-19 pandemic continues to spread worldwide, we tested our models with updated data up to March 30, 2021. The highest apex among multiple waves was used as the apex of the entire pandemic of a country. The accumulated case and death numbers before the apex were used to predict the total number of cases and deaths. The predicted numbers than were compared to the accumulated total number of cases and deaths up to March 30, 2021. **Figure 6** shows the predicted minimum and maximum numbers of patients and deaths based on the linear models and the real numbers on March 30, 2021. The total maximum numbers of cases for these 14 countries are 1,002,062, 3,338,991, 6,116,667, 4,083,759, 7,226,932, 5,330,517, 10,765,566, 2,414,208, 1,590,804, 77,637,725, 8,360,768, 16,962,457, 10,882,711, and 3,551,883. The

predicted minimum number of cases for these countries are 377,725, 1,256,047, 2,300,021, 1,535,964, 2,717,308, 2,004,551, 4,047,283, 908,472, 599,001, 29,180,808, 3,143,454, 6,376,350, 4,091,312, and 1,336,061. A surprising finding is that the number of patients in Brazil has surpassed predicted maximum numbers by all three models (**Supplementary Table 4**). For the rest of the 13 countries, none of the numbers of patients on March 30, 2021 have surpassed the predicted maximum numbers of patients. Notably, the patient numbers of all countries have surpassed the minimum numbers of patients predicted by the regression model (see **Figure 6A**).

Regarding the number of deaths, the actual numbers of deaths in 12 out of 14 countries are less than the predicted minimum numbers of the deaths (**Figure 6B**). Only the death numbers of two countries, Germany and Brazil, are more than the predicted minimum numbers of deaths but have not reached the maximum numbers. The predicted minimum numbers of deaths in the future for Japan, Iran, France, Italy, Spain, the UK, Netherland, Belgium, the US, India, Russia, and Turkey are 1,610, 62,246, 10,666, 9,576, 61,145, 85,418, 11,106, 8,012, 415,536, 44,462, 45,914, and 11,681 (**Supplementary Table 5**).

DISCUSSION

Our data shows that real-time models and predictions are relatively reliable. The models may be utilized in the future for the case and death number prediction of pandemics of similar diseases. Due to the influence of societal factors and policies of different countries and regions, experience, or previous evidence-based models have considerable limitations in the predictions and estimations of the current COVID-19 pandemic. We tested a systematic prediction algorithm of the development of the COVID-19 disease from the pre-onset period to the turning point or apex day to after the turning point through analysis of real data from mainland China, South Korea, and Switzerland. Our analysis showed that the second half of the pandemic has more infections and deaths than the first half of the pandemic. This result suggests that the disaster caused by the COVID-19 pandemic is far from over. Unfortunately, the multiple waves of the pandemic in many countries around the world indicates such a prediction is true.

The linear model is used to predict the cases and deaths most close to the real numbers. However, we now realized that when the number of cases in a country or a region is below a single digit number for a consecutive 12 days, it indicates that a wave of the pandemic is ending but it is not necessarily the end of the whole pandemic. Therefore, we tested the models with the data from the first wave and the data of the entire pandemic period up to March 30, 2021. In both cases, the real data either fall in or close to the predicted data.

While most of the real data agrees with data predicted from the models, there are exceptions. In the test for the first wave of the disease, the reported case from Iran is less than the predicted minimum number, and Spain and Russia it is more than the predicted maximum number of cases. In the prediction of death numbers, for the first wave, the death numbers of Iran, Belgium, Brazil, and India are less than the predicted minimum numbers of deaths. The death numbers from these countries for the entire pandemic period are less than the predicted. This result is expected because the pandemic period has not yet ended, and the death apex usually comes later than the apex of case numbers. Thus, more deaths are expected before the end of the pandemic.

It is also important to note that our estimations are based on conditions of lockdowns in major cities, maintaining social distance, and wearing personal protective equipment (PPE). If the protective measures are not maintained in the later stages of the pandemic, the number of patients and deaths will be more than those predicted in our analyses. The pandemic was not ended as predicted.

Multiple social and environmental factors can influence the case and death numbers (12–15). At present, the differences between the infection rate and lethality of the 2019-nCoV among different populations are not clear. There has been no systematic analysis of any variation between infection rates and lethality of different mutations of the 2019-nCoV among different populations. In addition, we cannot rule out the possibility that different environmental and societal conditions may have an impact on viral infection rates and mortality rates. In particular, the medical system and availability of treatment methods for a disease directly affect the death rate of any infectious disease.

Initial knowledge of any novel disease is inherently limited (16–18). The data from Wuhan have been revised since the initial outbreak. Therefore, in our statistics, we evaluated pandemic data both with and without data from Wuhan. The prediction without Wuhan's data seems more accurate.

CONCLUSION

A linear regression model built with real data from early COVID-19 pandemics can predict pandemic scales of later disease waves. Such a prediction with a high degree of accuracy benefits disease control and provides valuable information for governments and the public. However, many factors may influence the predictivity of the model. The model may need to be modified based on different situations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

TG, LW, WG, XM, LA, AP, SH, and YW: conceived and designed the experiments. TG, LW, NX, and WG: performed data searching, collection, and analyzed the data. XM, YW, ZL, WG, and AP: contributed analysis tools. TG, LW, NX, XM, ZL, AP, LA, SH, WG, and YW: wrote the manuscript. All authors: revised and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.585115/full#supplementary-material

Supplementary Figure 1 | Disease infection apex and relations of different regions.

Supplementary Figure 2 | Relationship between number of infected persons before the apex day and the total number.

Supplementary Figure 3 | Death numbers and relations before apex day and total.

Supplementary Figure 4 | Patterns of numbers of infected persons and peak day of investigated countries.

Supplementary Table 1 | Days before and after apex day.

Supplementary Table 2 | Calculation of the numbers of patients from the first wave.

Supplementary Table 3 | Calculation of the numbers of deaths from the first wave.

Supplementary Table 4 | Calculation of the numbers of patients from the entire pandemic.

Supplementary Table 5 | Calculation of the numbers of deaths from the entire pandemic.

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COMMUNITY CASE STUDY
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COVID-19 Testing Experience in a Resource-Limited Setting: The Use of Existing Facilities in Public Health Emergency Management

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Introduction: Coronavirus disease 2019 (COVID-19) is a public health emergency with little testing and treatment experiences at its occurrence. Diagnostic and treatment rapidly changed in the world including Ethiopia. Haramaya University has strived to change its diagnostic capacity using existing facilities in response to the national call to the pandemic.

Objective: This summary aims to detail experiences of setting up COVID-19 testing in Haramaya University laboratories, Eastern Ethiopia.

Methods: Desktop exercise was conducted to understand the start-up and implementations of COVID-19 testing in two Haramaya University laboratories, Hararghe Health Research Partnership and Campylobacter Genomics and Environmental Enteric Dysfunction laboratories. Communication, formats, guidelines, and standards were reviewed and summarized. Discussion with those involved in the start-up and implementation of the testing were also held. Ideas were summarized to learn the experiences the COVID-19 testing exercises.

Lesson Learned: This is a huge experience for Haramaya University to participate in the national call to increase the testing platform in the management of COVID19. Close work relationship with the public health authorities at all levels demonstrated the university's commitment to public service. The university has used the opportunity to advance its molecular testing capability by training its staff and students. The University has also contributed to the capacity development for laboratories in the surrounding areas of Harar, Somali, Oromia, and Dire Dawa. The pandemic has been an opportunity in harnessing existing resource for the benefit of the public during such times of dire needs to provide critical public health laboratory interventions.

Keywords: coronavirus, COVID-19 testing, HHR, CAGED, Haramaya University, Ethiopia

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The disease was first identified in December 2019 in Wuhan, the capital of China's Hubei province and has since spread globally, resulting in the ongoing 2019–2020 coronavirus pandemic (1). The outbreak of COVID-19 was declared a public health emergency of international concern on January 30, 2020, and thereafter declared a pandemic (2, 3). Following this, the World Health Organization has activated a worldwide surveillance, quarantine, testing, isolation, and treatment of positive cases.

Coronaviruses are a large family of enveloped, positive single-stranded RNA viruses that infect humans and a wide range of animals. These viruses were first described in 1966 (4). Experts of the International Committee on Taxonomy of Viruses termed it the SARS-CoV-2 virus as it is very similar to the one that caused the SARS outbreak (SARS-CoVs) (5).

The definitive diagnosis of active COVID-19 infection is based on the detection of either the spike protein or viral genes using real-time reverse transcription–quantitative polymerase chain reaction (RT-qPCR) tests. The genetic material test is the predominant test being implemented worldwide for detection, isolation, and treatment of COVID-19 cases (6). The test helps to detect carriers of the virus, which is fundamental in public health response efforts. It ensures the isolation of COVID-19 patients to prevent local spread and more broadly informs national response measures (7).

Other tests for immunoglobulin G (IgG) and IgM and point-of-care tests have been developed and used with varying sensitivities and specificities (8).

COVID-19 is a new phenomenon, and there was no preparation and institutional capacity for the testing in Ethiopia. Samples were initially shipped to South Africa as the Ethiopian Public Health Institute (EPHI) was in the process of putting infrastructure in place to do the testing in-country. Haramaya University laboratories have been and continue to be part of this effort. We present a summary of the establishment of testing platform, continuing testing, and challenges and lessons learned during the first 12 months of the continuing pandemic.

APPROACHES

Design

We conducted a desk review and discussions with individuals involved in the setup and conduct of the COVID-19 testing at the two laboratories in Haramaya University, Hararghe Health Research (HHR) Partnership and Campylobacter Genomics and Environmental Enteric Dysfunction (CAGED) laboratories under Haramaya University in Eastern Ethiopia. The summary focuses on the processes of start-up and implementation of COVID-19 testing between April 2020 and March 2021 in the two laboratories.

Setting

The HHR laboratory located in Harar campus in the premises of the College of Health and Medical Sciences, Haramaya University, is a collaborative effort between Haramaya University and London School of Hygiene and Tropical Medicine supported by the Bill & Melinda Gates Foundation as part of the Child Health and Mortality Prevention Surveillance effort to investigate the causes of stillbirth and death in children (9). The laboratory uses cutting-edge technologies to perform microbiological, molecular biology, and pathology tests (10). In addition to its regular research activities, the laboratory provides clinical diagnostic service support for cases requested by physicians from pediatric, gynecology, obstetric, and neonatal intensive care unit departments of Hiwot Fana Specialized University Teaching Hospital, Haramaya University, located in Harar (**Figure 1**). The other laboratory, CAGED, is a Bill & Melinda Gates Foundation–supported collaborative effort between University of Florida and Haramaya University set up to investigate the association of *Campylobacter* species exposure and childhood stunting in Haramaya district, Eastern Ethiopia (11, 12).

Data Management

We reviewed communications between EPHI officers in Addis Ababa, Capital of Ethiopia, and Haramaya University administration, leading to the start-up of the testing service in the two laboratories. Continuing discussions focused on the training, testing setup, process of laboratory approvals for testing, and challenges encountered were held with the people involved in the testing chain.

KEY SUMMARIES

Initial Communications and COVID Testing Intentions

As the COVID-19 pandemic rapidly spread worldwide and it became a matter of when and not if the first case would appear in Ethiopia, it became critical to start preparing for the testing. Discussions were held with the Centers for Disease Control and Prevention Atlanta on the possibility of providing Taqman Array Cards capable of detecting SARS-CoV-2 using the Quant Studio 7 platform that we had in both laboratories. Meanwhile, the EPHI facilitated the start of testing using RT-qPCR on the same platform.

Laboratory Assessment and Approval

The EPHI sent out prestart assessment forms to evaluate the laboratories for the presence of the required infrastructure. These included quality management systems, adequate and separate rooms for PCR and other laboratory work, reliable utilities, and ancillary PCR equipment and accessories. Other facilities assessed were sample storage and archiving capability, biosafety and biosecurity, and competent laboratory scientists. The HHR laboratory was deemed competent with no non-conformances and was designated by EPHI as one of the COVID-19 testing laboratories in Ethiopia. EPHI then assigned two senior laboratory experts to the HHR laboratory for 1 week to support with the setting up of the test workflow. This was also done for

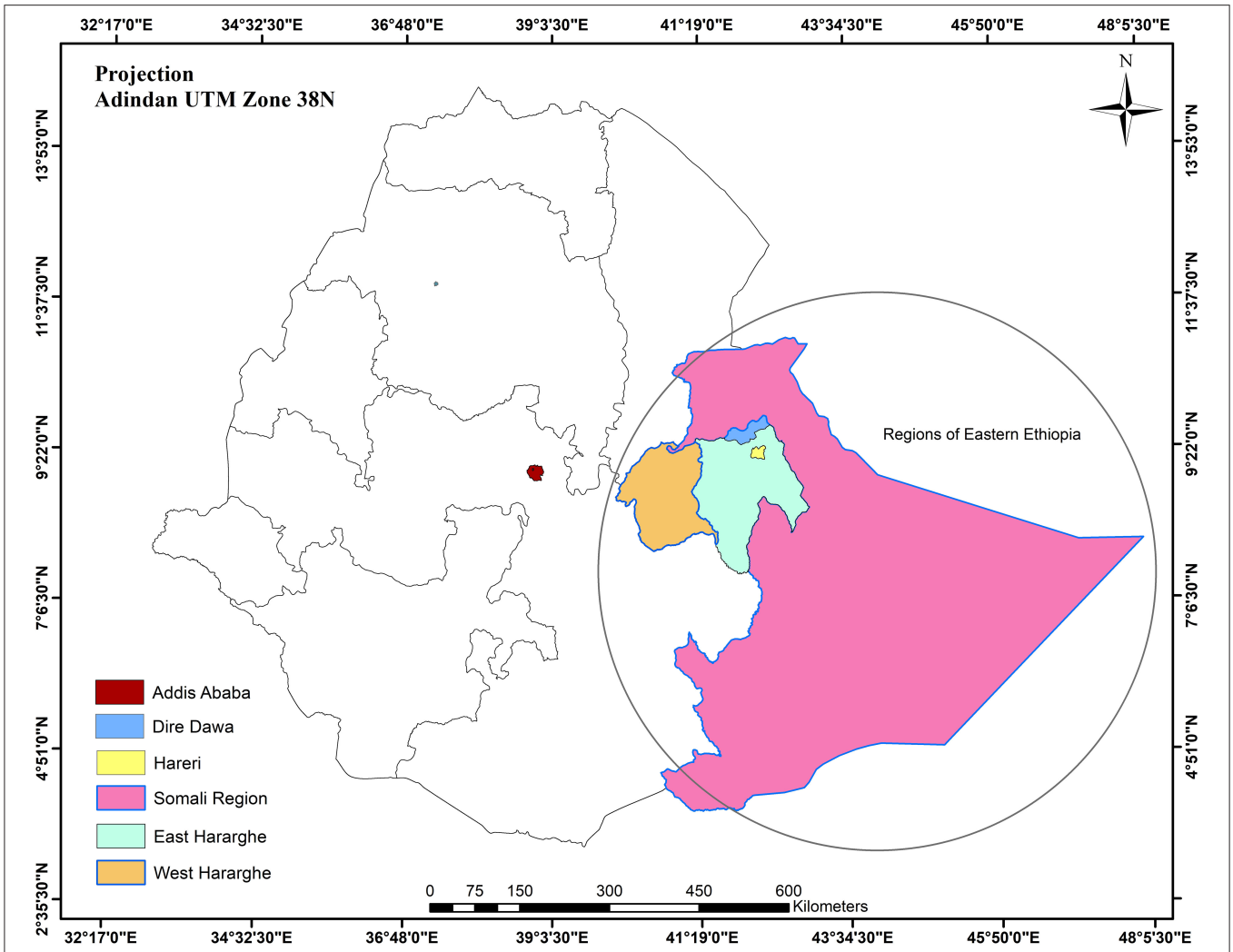

FIGURE 1 | Ethiopia regions, Eastern Ethiopia, including Somali, Dire Dawa, Harari, and Eastern and Western Hararghe areas.

the CAGED laboratory in anticipation of a surge in testing, which would mean sending samples to that laboratory.

Guidelines and Logistics

Standard protocol for specimen management, results communication, and the supply of test kits and other consumables were issued by the EPHI.

Staff Training and Supply of Logistics

A total of six HHR and two CAGED laboratory staff were trained on all the pre-analytical, analytical, and post-analytical processes of the COVID-19 testing. The CAGED laboratory staff were deployed to perform the tests in the main campus laboratory. An additional five staff were later trained and added to the testing team when there was a surge in the test numbers. Logistical support initially for 100 tests from EPHI started with supply of extraction kit, centrifuge tube, film gown, micropipette tips, masks (N95 and surgical masks), gloves, face shields, PCR plates, adhesive PCR cover, falcon tube, absolute ethanol, head and shoe cover, and plastic apron. As the sample we received increased, the supply of logistics also increased to support more tests, and these have progressively been increased for the laboratory to have been able to test the maximum of 768 samples in a day.

Start-Up of Tests

Initiation of sample testing was preceded by a letter from EPHI sent to health bureaus of Harari, Somali, and Oromia regions and Dire Dawa Administration toward the end of February, indicating that additional test facility has been established at Haramaya University and requesting these regions to send their samples to the said laboratory. The testing in the HHR laboratory was officially inaugurated by officials from Oromia and Harari region on April 12, 2020.

Sample Reception

The nasopharyngeal and oropharyngeal samples in viral transport medium and packaged using triple packaging to meet International Air Transport Authority (IATA) regulations were

TABLE 1 | COVID-19 tests conducted during April 2020–March 2021, HHR, and CAGED Laboratories, Haramaya University, Eastern Ethiopia.

Samples received from	No.	%
Harari region	5,025	16.6
Somali region	4,543	15.0
Dire Dawa Administration	3,295	10.9
East/West Hararghe zones	17,362	57.5
Total	30,225	100

transported to the laboratory and handled appropriately. Good laboratory practices were observed to ensure sample integrity and infection prevention and control for the people handling and testing the samples. The samples were logged into a laboratory samples register dedicated for COVID-19 and then received for processing by the laboratory staff.

Conducting the Test

The samples were labeled and processed following the standard operating procedure earlier provided by the EPHI while observing strict quality control measures already in place in the laboratory. The laboratory takes part and receives SARS-CoV-2 external quality assurance panels from the United Kingdom External Quality Assurance Scheme UK NEQAS, which are processed alongside the patient test samples, with result scores being consistently above acceptable lower limits of 80% successful performance.

Result Communication, Sample Archiving, and Disposal

The completed test results were reviewed by the tester, and one other reviewer for concurrence, and then the results were communicated to the Public Health Emergency Management sections of the respective regions from where the samples were collected and EPHI for the national daily COVID-19 updates. Aliquots of all samples testing positive for SARS-CoV-2 and 10% of negative tests are archived at negative 80°C freezers for further genomic analysis in the future. HHR laboratory is in the process of acquiring whole-genome sequencing capabilities to be able to perform this work in the future.

Support for Regional Laboratories

The Haramaya University laboratories, while receiving and testing samples from the designated regions, also provided support to the Somali, Harari, and Dire Dawa regional laboratories and Oromia regional state by building capacity for these laboratories to be able to also test these samples. The Haramaya University provided a PCR machine to the Dire Dawa regional laboratory to be able to set up its testing facility. Currently, all these regional laboratories are competently performing the SARS-CoV-2 testing for samples received from regions and zones. Staff have been deployed to help with knowledge and skill transfer to the personnel working in their respective places.

Tests Done so Far

Table 1 shows the areas Eastern Ethiopia and numbers of samples received from the respective places. There was a significant surge in the performance of the test during August 7 through September 8, 2020, due to ComBAT (Community-Based Testing) campaign set by the Ministry of Health and EPHI to evaluate the level of community level transmission.

LESSONS LEARNED

- The COVID-19 pandemic has created an opportunity for collaborative work and support mechanisms between researchers, public health authorities, and universities to overcome a public health emergency.
- The effort has created an opportunity for faster, easier, and quality test result in the management of suspected COVID-19 cases and those in quarantine and isolation.
- Even though most of the equipment needed for the testing is available in our facilities, they were not set up for quick and rapid emergency public health response. This is a good lesson for other diseases of both local and international health concerns.
- The setting up and running of advanced scientific laboratories in the universities require large initial resource outlay; however, it has been demonstrated that the collaborative effort to setup HHR and CAGED laboratories have been a good investment. This is a critical lesson for researchers, colleges, and universities to invest time and resource in cultivating the culture of creating collaborative platforms with various agencies in the advancement and practices of sciences and public health responses.
- Resource mapping is another area to focus on. At the start of the pandemic, Ethiopia was sending samples to South Africa, and the turnaround time for results reception was days. This was due to lack of a single laboratory in the country set to do the COVID test. Currently, the country has 54 such facilities in public and private health facilities, universities, and research laboratories located in different part of the country all performing the testing. This is due to scoping activities throughout the country to learn what the country has and the gaps. Similar actions should continue to support the research and diagnostic capacity of facilities.
- Using such an opportunity to train staff and students cannot be overemphasized. The emergency has created an option for training staff from the college, hospital, and clinic at the main campus within our facilities. It also provided opportunities for medical microbiology students at the college to get real-time experience at the facility.

DISCUSSION

Public health emergencies need concerted efforts by all stakeholders working together collaboratively to combat the problems for a good outcome. These situations are also good learning opportunities and facilitating new knowledge and advancement of sciences and medical knowledge. COVID-19

pandemic provide just such an opportunity, which caught the attention of not only the public but also decision-makers at all levels of governments and international agencies worldwide to work together for a good outcome (13, 14).

Alongside the preparations to test and treat patients, the pandemic also created an opportunity for the various actors in the different sectors to pull together in a concerted way and allocate resources to respond to the pandemic. This can be taken as a good lesson in the response against other disease including tuberculosis, malnutrition, mental health, and the rising chronic diseases (15–17).

The experience with this pandemic has exposed the basic nature of most of the laboratory facilities in the universities and health sectors in general, not only in Ethiopia but also in Africa. There is an acute lack of state-of-the-art laboratory facilities that are able to be put to emergency use to provide emergency support in outbreaks of public health significance such as the ongoing COVID-19 pandemic. As a result, this created a vacuum of professionals and equipment as special tests are required in certain disease conditions. This experience should act as an impetus for the government of Ethiopia to act in advancing teaching and research laboratories in universities and other research facilities. A lesson of cultivating a culture of collaborative platform with various agencies in good times in the advancement of sciences and medical practice is necessary to lend a hand at times of emergency (18). Although this is a public health emergency, opportunities have enabled the staff, and students learn new techniques of molecular testing (14). The how of vaccines and drug development demonstrated how challenges turn out to be opportunities for sciences and medicine (19, 20). The experiences shared in this report are those of the Haramaya University and do not necessarily demonstrate the experiences of other universities and institutions in Ethiopia.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: data related to this article can be sought from the first author. Requests to access these datasets should be directed to Nega Assefa, negaassefa@yahoo.com.

ETHICS STATEMENT

Ethical approval for this study and written informed consent from the participants of the study were not required in accordance with local legislation and national guidelines.

AUTHOR CONTRIBUTIONS

NA, JH, JO, MB, MD, ZT, and YD facilitated the setup of the lab. MB, MD, DM, ZT, and DA are involved in the testing process. NA and YD drafted the paper. NA, JH, ZT, JO, MB, MD, DM, DA, and YD edited and approved for submission. All authors contributed to the article and approved the submitted version.

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Estimating the Prevalence of Asymptomatic COVID-19 Cases and Their Contribution in Transmission - Using Henan Province, China, as an Example

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Background: Novel coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-COV-2), is now sweeping across the world. A substantial proportion of infections only lead to mild symptoms or are asymptomatic, but the proportion and infectivity of asymptomatic infections remains unknown. In this paper, we proposed a model to estimate the proportion and infectivity of asymptomatic cases, using COVID-19 in Henan Province, China, as an example.

Methods: We extended the conventional susceptible-exposed-infectious-recovered model by including asymptomatic, unconfirmed symptomatic, and quarantined cases. Based on this model, we used daily reported COVID-19 cases from January 21 to February 26, 2020, in Henan Province to estimate the proportion and infectivity of asymptomatic cases, as well as the change of effective reproductive number, R_t.

Results: The proportion of asymptomatic cases among COVID-19 infected individuals was 42% and the infectivity was 10% that of symptomatic ones. The basic reproductive number $R_0 = 2.73$, and R_t dropped below 1 on January 31 under a series of measures.

Conclusion: The spread of the COVID-19 epidemic was rapid in the early stage, with a large number of asymptomatic infected individuals having relatively low infectivity. However, it was quickly brought under control with national measures.

Keywords: COVID-19, asymptomatic cases, infectious dynamic model, the effective reproductive number, prevention and control measures

INTRODUCTION

In December 2019, cases of pneumonia with an unknown cause were reported. The disease was later named as novel coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus-2 (SARS-COV-2) (1, 2). The rapid increase in confirmed cases and subsequent secondary outbreaks in many countries caused concern on an international scale. As a result, the World Health Organization declared the COVID-19 outbreak a Public Health

Emergency of International Concern on January 31, 2020 and eventually classified it as a pandemic on March 11, 2020 (3). As of July 19, 2020, 14 million COVID-19 cases and 597,583 deaths have been confirmed globally, including 85,937 confirmed cases in China (4). Although the number of confirmed cases was staggering, only the sicker part of those infected were being reported. Li et al. used a metapopulation model to estimate that 86% of the infections (presumably of mild symptoms or asymptomatic) before January 23, 2020 were undetected in Wuhan, China (5); Chinazzi et al. used a GLEAM model to estimate that only one out of four cases were confirmed in Mainland China by February 1, 2020 (6, 7). Hao et al. used a SAPHIRE model to estimate that 87% of the infections before March 8, 2020 were unascertained in Wuhan, China (8). And some even suggested that most infections were caused by undetected cases (5, 9). A significant proportion of these undetected infected individuals were asymptomatic (8). In one documented case, a patient who disclaimed all symptoms and showed a normal chest radiography had multiple PCR cycle counts consistent with that of symptomatic patients (10), suggesting such patients are somewhat infectious (11).

The proportion of asymptomatic cases is a critical epidemiological characteristic that modulates the pandemic potential of the emergent respiratory virus, and is an important parameter in estimating the disease burden (5, 12–14). Estimating the proportion of asymptomatic cases will improve the understanding of COVID-19 transmission and spectrum of presentation, thereby providing insight into the spread of epidemics (14). But the estimated proportion of asymptomatic infected individuals varied widely from place to place. A recent analysis of 21 retrieved reports by the Centre for Evidence-Based Medicine in Oxford found that estimates of asymptomatic COVID-19 cases ranged from 5 to 80% (15). Meanwhile, most studies only showed that asymptomatic infected individuals are less contagious than symptomatic ones (16, 17). Only one previous study clearly showed that the asymptomatic cases could be one quarter as infectious as symptomatic cases in Ningbo, China (18). Therefore, it is important to estimate the proportion and infectivity of asymptomatic cases in various regions. Taking Henan Province as an example, we used a model-inference framework to explore the proportion and infectivity of asymptomatic cases, so as to estimate the prevalence of COVID-19.

METHODS

Study Area

The study area is located in east-central China (31°23′ to 36°22′ north latitude, 110°21′ to 116°39′ east longitude, **Figure 1**), with a population of more than 96 million and an area of 167,000 km^2. Most of Henan is located in the warm temperature zone and has the characteristics of climate transition from plains to hills and mountains from east to west.

Abbreviations: COVID-19, coronavirus disease 2019; R_0, the reproductive number; R_t, the effective reproductive number; SEIAUHR model, susceptible-exposed-asymptomatic-confirmed-unconfirmed symptomatic-hospitalized-removed model.

Source of Data

All data were obtained from the official websites of Provincial and Municipal Health Commissions (**Supplementary Table 1**), which published COVID-19 case data and information. The case data included the number of newly confirmed cases, cured cases, and deaths per day. The case information included age, gender, exposure history, date of symptom onset, and activity trajectory of confirmed cases. Identifiable personal information was removed for privacy protection.

Case Definition

Although the definition of COVID-19 cases has been changed several times, which has greatly affected the observed epidemic curve in Wuhan (19), the change of cases in Henan Province has been relatively stable, and the diagnosis of all cases in this study were based on the sixth edition of Diagnosis and Treatment Scheme for COVID-19 released by the National Health Commission of China (20). A laboratory-confirmed case was defined if the patient had a positive test of SARS-CoV-2 virus by real-time reverse-transcription-polymerase-chain-reaction (RT-PCR) assay or high-throughput sequencing of nasal and pharyngeal swab specimens. Only laboratory-confirmed cases were included in this study.

Modeling the Epidemic of COVID-19 in Henan Province

To consider asymptomatic infected individuals, we constructed the susceptible-exposed-asymptomatic-confirmed-unconfirmed symptomatic-hospitalized-removed (SEAIUHR) model by extending the classic susceptible-exposed-infectious-removed (SEIR) model to include asymptomatic cases, unconfirmed symptomatic cases who did not seek medical attention or get tested for mild symptoms, and quarantined confirmed cases. In this model, we divided the population into seven compartments: S (susceptible), E (latent), A (asymptomatic infectious), I (confirmed symptomatic infectious), U (unconfirmed symptomatic individuals), H (hospitalized), and R (removed). Susceptible individuals could acquire the virus after contact with infected cases (both symptomatic and asymptomatic) and became latent when they were infected but non-infectious. After a period of time, some of the latent individuals developed into symptomatic infections; some of these were confirmed and treated until they progressed into the removed stage and some went unconfirmed because they did not present themselves to healthcare facilities or get tested for mild symptoms. Others developed into asymptomatic infections and remained infectious until they progressed into the removed stage. Removed stage included individuals who were recovered or had died (**Figure 2**).

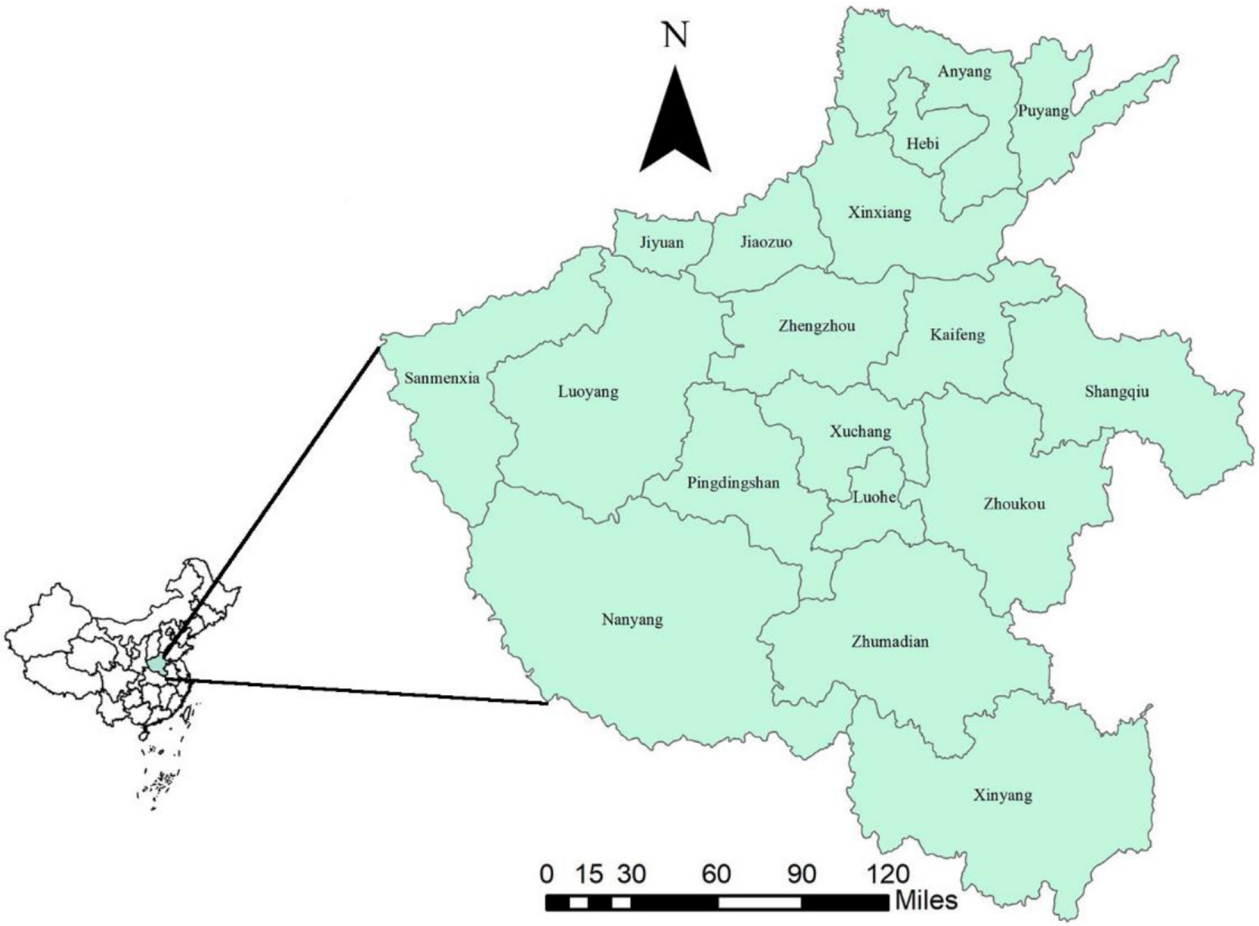

FIGURE 1 | Location of Henan Province (the map was created with ArcGIS software, 10.5).

Dynamics of these seven parts over time could be expressed by the following ordinary differential equation:

$$\begin{cases} \frac{dS}{dt} = -\frac{\beta_t S(I+U)}{N} - \frac{\beta_t \theta SA}{N} \\ \frac{dE}{dt} = \frac{\beta_t S(I+U)}{N} + \frac{\beta_t \theta SA}{N} - \frac{E}{z} \\ \frac{dI}{dt} = \frac{(1-\mu_1-\mu_2)E}{z} - \frac{I}{r_1} \\ \frac{dA}{dt} = \frac{\mu_1 E}{z} - \frac{A}{r_2} \\ \frac{dU}{dt} = \frac{\mu_2 E}{z} - \frac{U}{r_3} \\ \frac{dH}{dt} = \frac{I}{r_1} - \frac{H}{r} \\ \frac{dR}{dt} = \frac{H}{r} + \frac{A}{r_2} + \frac{U}{r_3} \end{cases}$$

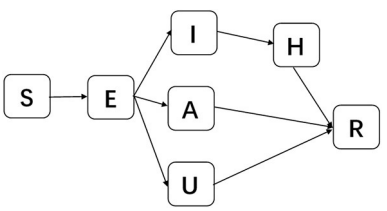

FIGURE 2 | Schematic diagram of SEAIUHR model propagation process. S, susceptible; E, latent; A, asymptomatic infectious; I, confirmed symptomatic infectious; U, unconfirmed symptomatic infectious; H, hospitalized; R, removed.

where β_t was the transmission rate due to symptomatic infected individuals at time t, defined as the proportion of cases from susceptible individuals to infected individuals, both asymptomatic and symptomatic, caused by symptomatic infected cases; θ was the ratio of the transmission rate due to asymptomatic over symptomatic cases; μ_1 and μ_2 were the proportion of the asymptomatic and unconfirmed symptomatic cases among infected individuals, respectively; z was the latent period; r_1, r_2, and r_3 were infectious periods of confirmed symptomatic, asymptomatic, and unconfirmed symptomatic cases, respectively; and r was the duration from hospitalization to recovery or death. Assume that $N = S + E + I + A + U + H + R$.

The differential equations in the model were numerically solved using a 4th order Runge-Kutta (RK4) method. Specifically, for each step of the algorithm, each term on the right side of the

equation was determined using a random sample of the Poisson distribution (5).

On January 25, 2020, Henan Province implemented a first-level public health emergency response to the epidemic and took a series of prevention and control measures, such as traffic restriction, quarantine, contact tracing, isolated treatment of confirmed cases, and so on (21, 22). We assumed that these major government measures caused the transmission rate to change from a constant rate to a time dependent exponentially decreasing rate (23).

Then, the formula of β_t could be expressed by the following step function:

$$\beta_t = \begin{cases} \beta_0 \,, t <= t_1 \\ \beta_0^* \exp(-a^*(t - t_1)) \,, t > t_1 \end{cases}$$

where β_0 was the transmission rate due to symptomatic infected individuals before implementing measures; a was the decreasing rate of transmission rate; and t_1 was the date to start implementing measures.

The effective reproductive number, R_t, could be computed as:

$$R_t = (1 - \mu_1 - \mu_2)\,\beta_t r_1 + \mu_1 \theta \beta_t r_2 + \mu_2 \beta_t r_3$$

In the initial state, namely, $t = 0$, $R_t = R_0$ is the basic reproductive number.

Estimation of Parameters in the Model

Initial states and parameter's setting in the model were presented in **Table 1**. We assumed that the initial latent population, asymptomatic infected population, and unconfirmed symptomatic cases were drawn from uniform distribution [0,10], the initial confirmed symptomatic infected population was 0, and the rest of Henan Province were susceptible. For parameters, we estimated β_0, μ_1, μ_2, θ, and α by assuming that the values of parameters z, r_1, r_2, r_3, and r were fixed throughout the process. We assumed that the initial values of each parameter to be estimated were drawn using Latin hypercube sampling in uniform distribution. The initial ranges of μ_1, μ_2, and θ were chosen to cover most possible values, i.e. [0,1]; the initial range of α was selected to more broadly cover what the previous research covered (23). The initial range of β was selected from the widest possible range of basic reproductive number (R_0).

We used the Ensemble Adjustment Kalman Filter (EAKF) to infer epidemiological parameters of the model based on the number of cases presenting symptoms per day in Henan Province (31–33). The EAKF is a data assimilation algorithm that only needs hundreds of ensembles to obtain good results, especially suitable for the estimation of high-dimensional parameters of the model (34, 35), and has been successfully applied to epidemics such as cholera and influenza (32, 35). In this study, we used 1,000 ensembles and 1,000 independent realizations to infer parameters and their corresponding 95% confidence intervals (Cls).

TABLE 1 | Initial states and parameter's setting in the model of the main analysis.

States or parameters	Values or prior distribution
States	
Susceptible (S_0)	96050000-E_0-I_0-A_0-U_0
Latent (E_0)	U(0, 10)
Confirmed symptomatic infectious (I_0)	0
Asymptomatic infectious (A_0)	U(0, 10)
Unconfirmed symptomatic infectious (U_0)	U(0, 10)
Parameters	
Latent period (z)	3 days (Fixed) (2, 24–27)
Infectious period of confirmed symptomatic cases (r_1)	3.5 days (Fixed) (5, 22, 28, 29)
Infectious period of asymptomatic cases (r_2)	5 days (Fixed) (17)
Infectious period of unconfirmed symptomatic cases (r_3)	5 days (Fixed) (17)
Duration removed from hospitalization (r)	10 days (Fixed) (30)
Transmission rate due to symptomatic cases (β_0)	U(0.8, 1.5) (*Estimated*)
Asymptomatic rate (μ_1)	U(0.02, 1) (*Estimated*)
Undetected rate (μ_2)	U(0.02, 1) (*Estimated*)
The ratio of transmission rate (θ)	U(0.02, 1) (*Estimated*)
The decreasing rate of transmission rate (α)	U(0.02, 0.3) (*Estimated*)

Sensitivity Analysis

Synthetic Testing

Before applying the model-inference framework to the number of daily incidence data, we tested the effect of model-inference framework with model-generated outbreak data. Specifically, we fixed the parameters of the model to specified values and used the model to generate synthetic outbreak data. We then applied the EAKF algorithm to the synthetic daily outbreak data and assessed the model-inference framework by analyzing whether the model could fit the synthetic outbreak data and estimate parameters.

Sensitivity of Parameters Estimation to the Range of Initial States and Values of Fixed Parameters

In initial states, the quantities of E_0, A_0, and U_0 were unknown, and our assumptions may affect the estimation of other parameters. Therefore, this study simultaneously investigated the results of parameter's estimation when shortening and expanding their ranges. At the same time, we changed values of fixed parameters, respectively, to test the robustness of our results.

RESULTS

As shown in **Figure 3**, our model could fit reported daily incidence data well and accurately capture the peak and tendency of the epidemic. The numbers of reported daily cases were within the confidence interval estimated by the model, except for a few days in the later stages of the outbreak.

The mean estimation of transmission rate due to symptomatic infected individuals was 1.14 (95% CI:1.07-1.23) at the beginning of the epidemic and the decreasing rate of transmission rate after implementing prevention and control measures was 0.16

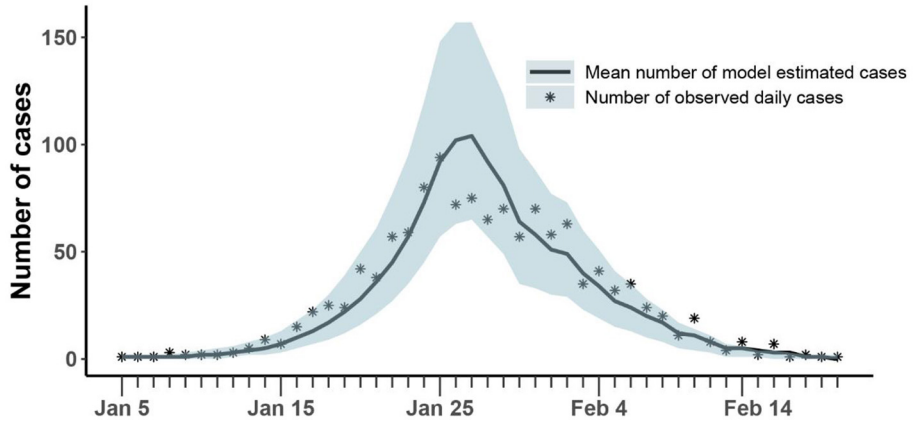

FIGURE 3 | Comparison of the number of cases estimated and observed. The asterisk represents the number of cases with symptoms observed on a daily basis; the curve shows the change in the average number of confirmed symptomatic cases per day estimated by the model. The light blue shade was the 95% confidence interval of the estimation.

TABLE 2 | Posterior estimates of key epidemiological parameters.

Parameter	Mean (95% CI)
Transmission rate due to symptomatic cases (β_0)	1.14 (1.07, 1.23)
Asymptomatic rate (μ_1)	0.42 (0.41, 0.47)
Undetected rate (μ_2)	0.11 (0.09, 0.22)
The ratio of transmission rate (θ)	0.10 (0.02, 0.11)
The decreasing rate of transmission rate (α)	0.16 (0.12, 0.19)

This table showed posterior estimates of key epidemiological parameters using proposed model-inference framework. In the second column, estimated mean and 95% confidence interval were outside and inside parentheses, respectively.

(95% CI: 0.12-0.19). Our model estimated that the asymptomatic rate among COVID-19 infected individuals was 42% (95% CI: 41-47%), and the mean ratio of the transmission rate of asymptomatic over symptomatic cases was 0.1 (95% CI: 0.02-0.11). At the same time, our model estimated that 11% (95% CI: 9-22%) of infected individuals were unconfirmed symptomatic cases who did not seek medical attention or get tested for mild symptoms (**Table 2**). Then, the fraction of undocumented infections in Henan Province was 53% (95% CI: 50-68%). Based on above parameters, we estimated the average effective reproduction number, R_t, to be 2.73(95% CI: 2.64-3.31) at the beginning of the epidemic, which was equal to the basic reproduction number (R_0). With the implementation of measures, R_t fell below 1 on January 31.

The results of the synthetic test were shown in **Figure 4** and **Table 3**. All generated values were within the confidence interval estimated by the model and values of all parameters were within the estimated 95% confidence interval, which demonstrated the ability of the model-inference-framework to fit the synthetic outbreak data and estimate all five target model parameters accurately.

Results of parameter estimation when changing the range of initial states and values of fixed parameters were shown in **Supplementary Table 2**. It could be seen that values of the resampled epidemiological parameters fall near the values estimated from the original data, with small fluctuations, indicating that the estimated results of our model are robust.

DISCUSSION

Taking Henan Province as an example, we constructed a SEAIUHR model to estimate the prevalence of asymptomatic COVID-19 cases and their contribution in transmission with EAKF algorithm. This model-inference framework is also applicable to studies of asymptomatic infected individuals in other regions.

Asymptomatic proportion, which is broadly defined as the proportion of asymptomatic infections among all infections of the disease, is important for estimating the true burden of disease and its transmission potential. At present, results of different studies on the asymptomatic proportion vary greatly (15). We estimated that the proportion of asymptomatic infections among infected individuals during the entire epidemic was 42% in Henan Province, within the confidence interval of the estimated asymptomatic rate of 13 cases imported from Wuhan to Japan (14). But it was higher than that of the Diamond Princess cruise ship, which showed that only 17.9% of those infected were asymptomatic (36). It could be that passengers and crew on the Diamond Princess were not drawn from a random sample of the general population, most of whom were older than 60 years and tended to have more severe symptoms after infection. Our model estimated that the mean ratio of transmission rate due to asymptomatic over symptomatic cases was 0.1, corresponding to a study showing that prolonged exposure to infected persons and short exposure to symptomatic persons (such as coughing) is associated with a higher risk of transmission, while short exposure to asymptomatic contacts is associated with a lower risk of transmission (24). The less contagious nature of asymptomatic individuals may be the result

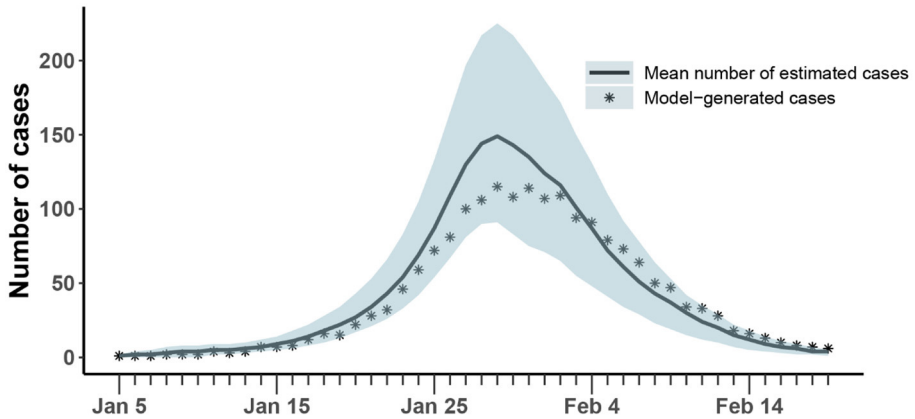

FIGURE 4 | Comparison of the number of cases estimated and generated. The asterisk represents the number of cases with symptoms observed on a daily basis; the curve shows the change in the average number of confirmed symptomatic cases per day estimated by the model. The light blue shade was the 95% confidence interval of the estimation.

TABLE 3 | Results of synthetic testing.

Parameters	True values	Estimated values[a]
β	1.1	1.14 (1.06, 1.22)
μ_1	0.4	0.34 (0.32, 0.45)
μ_2	0.1	0.09 (0.07, 0.23)
θ	0.2	0.18 (0.14, 0.22)
α	0.15	0.15 (0.12, 0.19)

[a] *The estimated mean and 95% confidence interval were outside and inside the parentheses.*

of a convolution of the shedding fraction of viable virus, the titer of viable virus in the primary/upstream case, and possibly behavioral factors.

The fraction of undocumented infections, including asymptomatic cases and unconfirmed symptomatic cases who did not seek medical attention or get tested for mild symptoms, was lower than that of Wuhan in the early stage of the epidemic (5, 6, 8), which may be caused by following reasons. Firstly, in the early stage, the medical configuration was not perfect and public awareness was still insufficient, while the undocumented rate gradually decreased with the development of the epidemic (5, 10, 37). Secondly, contact tracing measures implemented in China may become unfeasible when the number of cases in Wuhan rose sharply in the early stage (3). Finally, we need to point out that the differences in the estimated proportions of asymptomatic cases and unconfirmed symptomatic cases may be due to unidentifiability of parameters in epidemiological models. The theoretical analysis of identifiability of parameters in epidemiological models needs to be done in the future.

Basic reproductive number (R_0) is an important parameter to determine whether an infectious disease is prevalent or not. If $R_0 < 1$, infectious disease would gradually decline and die out without an epidemic; if $R_0 > 1$, an epidemic would break out. In this study, our estimation of $R_t = 2.73$ at the beginning of the epidemic measured the basic reproductive number R_0, that is, without intervention, each infected individual could infect an average of 2.73 susceptible individuals. This result was similar to some studies in other regions of China (28, 38, 39), although it was smaller than results from some other research (38). Combined with the latent period, the number of cases without intervention would increase exponentially (25, 29). However, Henan Province implemented a first level response on January 25, 2020, and adopted a series of prevention and control measures. The isolation treatment of confirmed cases and the testing of suspected cases aimed at removing infected individuals from the process of transmission. The closing of public places and the change of crowd behavior were to protect susceptible groups. Contact tracing, which identified possible chains of transmission between known infected persons and their close contacts, affected both susceptible and asymptomatic individuals and can effectively interrupt transmission. With the help of these measures, R_t dropped below 1 on January 31, 2020.

This study also has some limitations. Firstly, our estimation of the asymptomatic proportion and infectivity was obtained by a model, which could not be generalized because it has not been confirmed by serological investigation. Secondly, we only used data from Henan Province, which might limit the interpretation of our results, although our model-inference framework is also applicable to studies of asymptomatic infected individuals in other regions. Therefore, large-scale relevant studies are needed in the future. Thirdly, this study estimated the average asymptomatic infection rate in Henan Province over time, but the asymptomatic rate may vary in different periods of the epidemic.

CONCLUSION

The epidemic situation developed rapidly in Henan Province, and there were a large number of asymptomatic infected

individuals with relatively low infectivity. Our study further explored the prevalence of asymptomatic COVID-19 cases and their contribution to transmission so as to deepen people's understanding of asymptomatic cases and provide a reference for the prevention and control of COVID-19.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CL and XL conceived of and designed the research. CL, YZ, CQ, LL, DZ, XW, KS, YJ, and TL did the analyses. CL wrote and revised the paper. DH, MX, and XL contributed to the writing and revisions. All the authors have read and approved the submitted version. All the authors have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work are answered.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.591372/full#supplementary-material

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COMMUNITY CASE STUDY
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A Citywide Approach to SARS-CoV2 Testing

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The COVID-19 pandemic caused more than 30 million infections in the United States between March 2020 and April 2021. In response to systemic disparities in SARS-CoV2 testing and COVID-19 infections, health systems, city leaders and community stakeholders in Worcester, Massachusetts created a citywide Equity Task Force with a specific goal of making low-barrier testing available to individuals throughout our community. Within months, the state of Massachusetts announced the Stop the Spread campaign, a state-funded testing venture. With this funding, and through our community-based approach, our team tested more than 48,363 individuals between August 3, 2020 and February 28, 2021. Through multiple PDSA (Plan-Do-Study-Act) cycles, we optimized our process to test close to 300 individuals per hour. Our positivity rate ranged from 1.5% with our initial testing events to a high of 13.4% on January 6, 2021. During the challenges of providing traditional inpatient and ambulatory care during the pandemic, our health system, city leadership, and community advocacy groups united to broaden the scope of care to include widespread, population-based SARS-CoV2 testing. We anticipate that the lessons learned in conducting this testing campaign can be applied to further surges of SARS-CoV2, international environments, and future respiratory disease pandemics.

Keywords: COVID-19, SARS-CoV2, pandemic, testing, public health

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INTRODUCTION

The COVID-19 pandemic caused more than 30 million infections in the United States between March 2020 and April 2021 (1). Widespread testing remains one of the pillars of the COVID-19 pandemic public health response, as the probability of transmission can be limited by testing, quarantine and isolation (2–4). Testing is especially important given the risk of transmission during the pre-symptomatic period (5), and the need for contact tracing (4). In the early months of the COVID-19 pandemic, testing availability in the United States was limited by availability of test kits, insurance/financial considerations, and the need for symptoms and access to a health care provider to obtain an order for testing. This set of barriers created a significant lack of equity in access to testing; even egalitarian testing resources undersampled lower socioeconomic status populations at highest risk for COVID-19 infection (6).

In response to systemic disparities in SARS-CoV2 testing and COVID-19 infections, health systems, city leaders and community stakeholders in Worcester, Massachusetts created a citywide Equity Task Force with a specific goal of making low-barrier testing available to individuals

throughout our community. This task force, co-chaired by the University of Massachusetts (UMass) Memorial Health Care system and the City of Worcester Department of Health and Human Services, was comprised of more than 22 organizations and over 50 individuals.

Our city is located in Central Massachusetts, with a population of 191,575 people (7). In greater Worcester, 21% of the population is of Latino or Hispanic origin, and 13% of the population is Black or African-American. Thirty-four percent of Worcester adults speak another language besides English, with 15% of Worcester residents speaking Spanish (8).

Faced with the important challenge of providing testing to our diverse population of residents, our group began meeting in May 2020, with working groups to address (1) analytics regarding COVID-19 infection data analysis to identify local disease activity; (2) testing strategies; (3) education and outreach, and (4) equity. Each team met individually and together to review data on where outbreaks were occurring, populations at-risk, optimal testing approaches, preventive strategies, and barriers to access.

Within months, the state of Massachusetts announced the Stop the Spread campaign, a state-funded testing venture. With this funding, and through our community-based approach, our team tested more than 48,363 individuals between August, 3, 2020 and February 28, 2021.

Below, we describe the optimization of our community-based strategy to allow for low-barrier community access. We are hopeful that the advent of vaccination programs will slow the transmission of SARS-CoV2 and make the need for testing on this scale less acute. However, we predict that this low-barrier approach will be very helpful for the implementation of similar testing for other upper respiratory infection epidemics. We also are able to refocus our current process on vaccine distribution. And we further anticipate that this information will be useful in the historical perspective on the wide variety of public health responses to COVID-19.

CONTEXT

Through geospatial mapping analysis, our analytics team identified specific census tracts with the highest recent number of COVID-19 cases. Led by the Vice President of Health Policy and Public programs at UMass Memorial Medical Center (JS), this office produced reports of infections in Worcester and surrounding communities on a weekly basis with further breakdown by age, sex/gender, race/ethnicity, and census tract using data from the state of Massachusetts' MAVEN system (integrated case tracking system of record). We additionally obtained information from hospitalized patients, as well as available data from community testing. Based on these analyses, we identified several census tracts with the highest rates of infection in our community. Additionally, we identified the impact of COVID-19 in communities of color. For example, Hispanic/Latinx individuals made up 37% of persons with COVID-19 infections despite making up 21% of the population in February 2021. As such, any testing programs or prevention/outreach efforts would necessarily be provided in English and Spanish. Of note, the census tracts with the highest rates of COVID-19 infection ranked within the top 10% of communities based on the CDC Social Vulnerability Index; two of these census tracts are within the top 1% using this measure (9).

PROGRAMMATIC DETAILS

Utilizing the geospatial mapping, we were able to hone in on the census tracts with the highest number of COVID-19 infections at a street level view within census tracts (see **Figure 1**). We subsequently identified potential testing venues in high-traffic areas in each target census tract. Sites included churches, the grounds of city hall, local schools, large housing developments, and a community development center. Task force leaders traveled to each potential site, meeting with community stakeholders to discuss suitability and acceptance. We based decisions regarding suitability in part on accessibility to pedestrians and those using public transportation to mitigate this barrier. We selected sites based on accessibility of each site to facilitate walk-up (no appointment necessary) testing, distinguishing our approach from many public testing initiatives. Through outreach into BIPOC neighborhoods alongside community organizations, we were able to identify sites that promoted culturally appropriate services, fostered a sense of trust within their existing networks, and ultimately provided safe, accessible, and comfortable physical environments.

Prior to each testing date, project managers from the Center for Innovation and Transformational Change provided a site map (please see **Figure 2**). These site maps traced patient movement through either parking or a walk-up entrance (depending on the site), registration, swabbing, then public health/ public advocacy opportunities. A cellular engineer visited each site in advance to ensure that adequate bandwidth would be available to the Wi-Fi set-ups at each site. Dates and times of testing were disseminated via the local newspaper and multiple social media channels in English and Spanish to optimize engagement by community members. To address the digital divide, we additionally shared the location and timing information by newspaper, through community ambassadors, and via key stakeholders.

There were no requirements for testing with respect to either provider orders or symptoms. A blanket standing physician order allowed testing for all presenting patients. Infants, children of all ages and adults were tested at each site.

TESTING PROCEDURE

During the pandemic, the Ronald McDonald Care Mobile team that usually provides community outreach and dental care to underserved populations was redeployed from that primary mission due to school closures and infection control concerns. As a result, this team turned its operation into "Feet on the Street" in the earliest days of the pandemic, providing education and prevention information and materials in six different languages (33 languages available on our website), as well as hand sanitizer

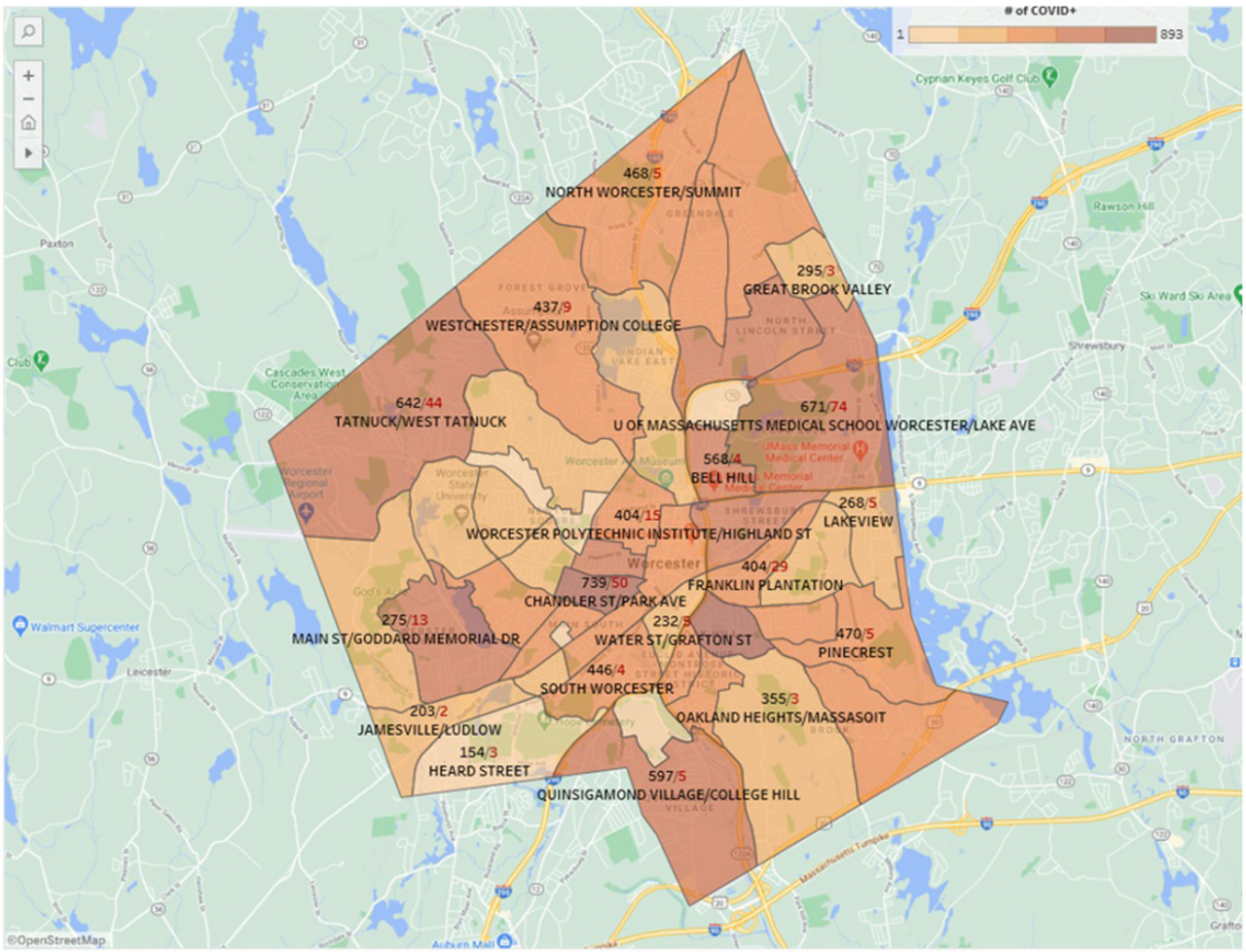


FIGURE 1 | Representative geospatial mapping of SARS-CoV2 positive cases in Worcester, MA from February, 2021. Black numbers represent infections, while red numbers denote deaths.

and masks. Once we began these community-wide testing initiatives, the Care Mobile team provided critical personnel, community recognition and trust, and clinical skills to anchor the mobile testing events.

Beyond the Care Mobile team, several groups of personnel were required for the deployment of each pop-up site. A facilities team provided person-power, transportation and equipment to set up tents, set up the wireless system and generator, and allot required PPE to each station. Registration personnel gathered demographic and contact information. Clinicians (typically nursing assistants, nurses, advanced practice providers, and/or physician staff) swabbed patients as they presented for testing. Additional staff members directed patients through the stations, provided language assistance and troubleshot problems as they arose. Registration personnel were hired or redeployed for this effort; volunteers from multiple community partners (including the Latino Education Institute) further supported the registration team. The clinician team was comprised of individuals redeployed for this effort, supplemented by physician and advanced practice provider volunteers, drawn largely from emergency medicine and primary care specialties.

All personnel were required to wear an N95 mask (or equivalent), as well as a surgical mask and eye protection. Clinical staff additionally wore gowns during swabbing; personnel protective equipment was provided on-site to staff.

The registration process was the most high-stakes, complex and time-consuming portion of our process. Data collected included name, date of birth, phone, email (when available), address, race, and ethnicity. Accurate data entry was critical to prevent difficulties reaching patients with results. Data were entered into the electronic platform used by receiving laboratory (Broad Institute/ELLKAY CareEvolve, Cambridge, MA). Each registrar required a laptop computer, power supply, cellular "air card" and wireless label printer. After entering all necessary demographic information, the registrar printed a patient-specific label and applied it to a specimen collection tube. Multiple bilingual staff (English/Spanish, English/Portuguese) facilitated this process. No identification or insurance information was

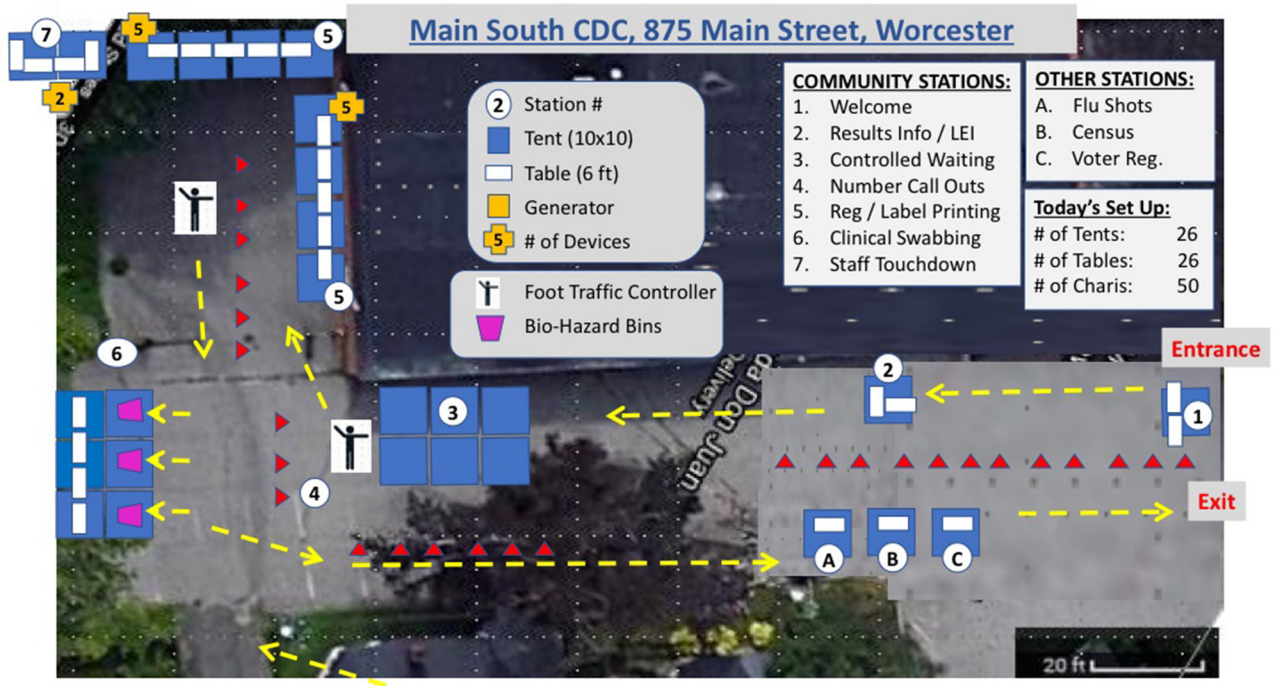


FIGURE 2 | Representative site map (Main South, Community Development Corporation) from August, 2020. LEI, Latino Education Institute.

FIGURE 3 | Photos of indoor and outdoor testing set-ups. **(A)** Clinician wearing PPE, swabbing walk-up community member; **(B)** Setting up outdoor registration area; **(C)** Indoor testing venue; **(D)** Indoor testing booth.

required to access testing. The patient was then directed to a swabbing station.

We chose clinician-administered mid-turbinate swabs based on efficiency. Using this approach, patients were asked to clear (blow) their noses prior to testing. After confirming patient information, clinicians would then swab the patient's anterior nares using a cotton-tipped swab for approximately 15 s on each side. The swabs were then placed in the previously labeled tubes. Samples were transported in batch to the Broad Institute by courier at the end of each testing session.

Several testing dates were canceled in October due to adverse weather. In November 2020, we moved our testing venue to an indoor location to prevent disruptions due to weather. A large commercial space was donated for this purpose. The location was ideal, located <10 walking minutes from Union Station, Worcester's transportation hub. Additionally, this testing center was located in a socially vulnerable neighborhood with high COVID-19 positivity. The tents used for the pop-up sites were deployed in this 22,000 square foot space, partitioning registration and swabbing staff (see **Figure 3** for pictures of the indoor and outdoor testing sites). Fit testing for N95 mask use was required prior to staff participation in these events. To further facilitate this requirement, some staff members were trained to perform fit testing on site for any volunteers or employees who had gone more than 1 year since formal fit testing.

The state-funded testing conferred two critical benefits. First, the turnaround time for testing was approximately 24 to 48 h; second, all negative tests were reported directly to the patient by email, obviating substantial callback burden. Team members shared the responsibility for callbacks to patients with positive results and appropriate counseling. We also created a small call center to provide additional assistance with calling back individuals who tested positive, and to respond to public inquiries regarding testing and results. We contacted all individuals with their results and were able to encourage household contacts to be tested during one of our subsequent

testing sessions. All positive SARS-CoV2 tests in MA were and are automatically reported to the MA Department of Public Health because COVID-19 is classified as a disease of high public health consequence. The state team separately followed-up with individuals who tested SARS-CoV2 positive to conduct contact tracing.

Additional resources were provided through the City of Worcester Division of Health and Human Services to address food insecurity for individuals requiring quarantine or isolation. The Department of Health and Human Services referred a total of 204 individuals from the months of August–November to the City's Hot Meal Program, which was coordinated by the Family Resource Center at YOU, Inc. These individuals received hot meal delivery from local restaurants for 2 weeks after their referral.

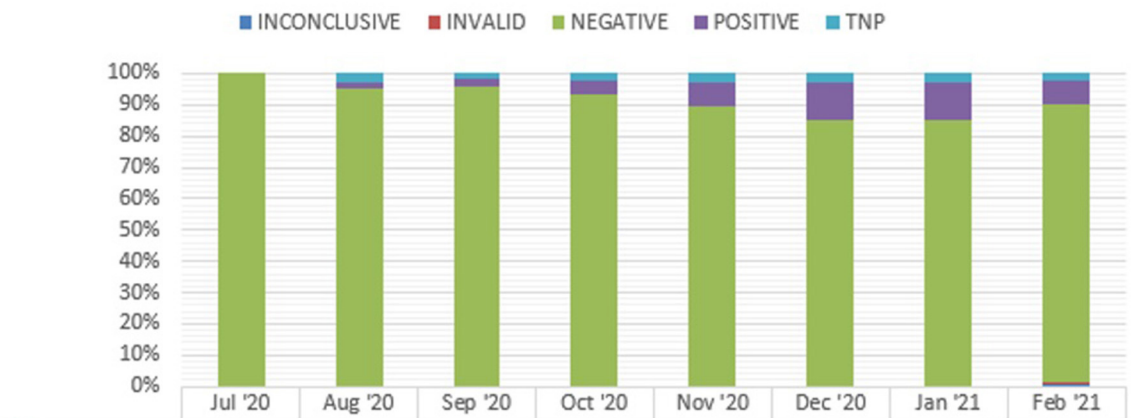
PREVENTION EDUCATION AND OUTREACH

Beyond community COVID-19 surveillance, the testing sites afforded an opportunity provide patients with other secondary benefits. Throughout our testing sessions, our team provided education and resources regarding COVID-19 infection prevention. Through outside grant funding, we were able to initially purchase thousands of masks and containers of hand sanitizer. Each individual presenting for testing (or their family members) received a packet containing: two surgical masks; a travel-sized container of hand sanitizer; instructions on avoiding COVID-19 infection and symptom recognition. On site, we partnered with community organizations, such as the Southeast Asian Coalition (SEAC), to provide PPE and other resources. Additionally, we worked with other private organizations and community partners to provide necessary services to our patients. A national retail pharmacy provided staff for the administration of influenza vaccines; over 1,100 vaccines were administered during sessions held in August 10 and September 17, 2020. Other groups, such as Worcester Interfaith, were also on-site to promote PPE and register community members for the United States Census. Through partnership with the League of Women Voters, we also had voter registration on site for several events. Community partner involvement was coordinated by the Department of Health and Human Services for the City of Worcester.

RESULTS

Between August 3, 2020 and February 28, 2021, our team performed 48,363 tests in community-based, non-medical locations. Our first nine-hour testing event was held on the evening of August 3, 2020, at the Community Development Corporation. We tested 680 individuals, at a rate of 75 patients per hour. Occasionally, tests could not be processed due to issues during the collection, transport, or analysis. During this first testing session, our "not processed" rate was 5%.

After this first pop-up testing event, our team made adjustments to subsequent site maps to facilitate testing (and minimal waiting) for individuals with decreased mobility. Our testing sessions averaged four hours in length. Through multiple PDSA (Plan-Do-Study-Act) cycles, we optimized our process to test close to 300 individuals per hour. We did try a patient-administered swabbing approach but found that it did not improve throughput. Additionally, with staff attention to specimen collection, specimen labeling and transportation, our "test not processed" rate rapidly fell to under 1% (see **Figure 4**).



	Jul '20	Aug '20	Sep '20	Oct '20	Nov '20	Dec '20	Jan '21	Feb '21
TNP	0.00%	2.82%	1.60%	2.07%	2.67%	2.68%	2.64%	2.41%
POSITIVE	0.00%	1.96%	2.74%	4.46%	7.46%	12.38%	11.97%	7.52%
NEGATIVE	100.00%	95.05%	95.27%	93.29%	89.65%	84.72%	85.08%	88.75%
INVALID	0.00%	0.17%	0.39%	0.18%	0.22%	0.22%	0.31%	0.85%
INCONCLUSIVE	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.47%

FIGURE 4 | SARS-CoV2 testing result percentages over time. TNP, test not processed.

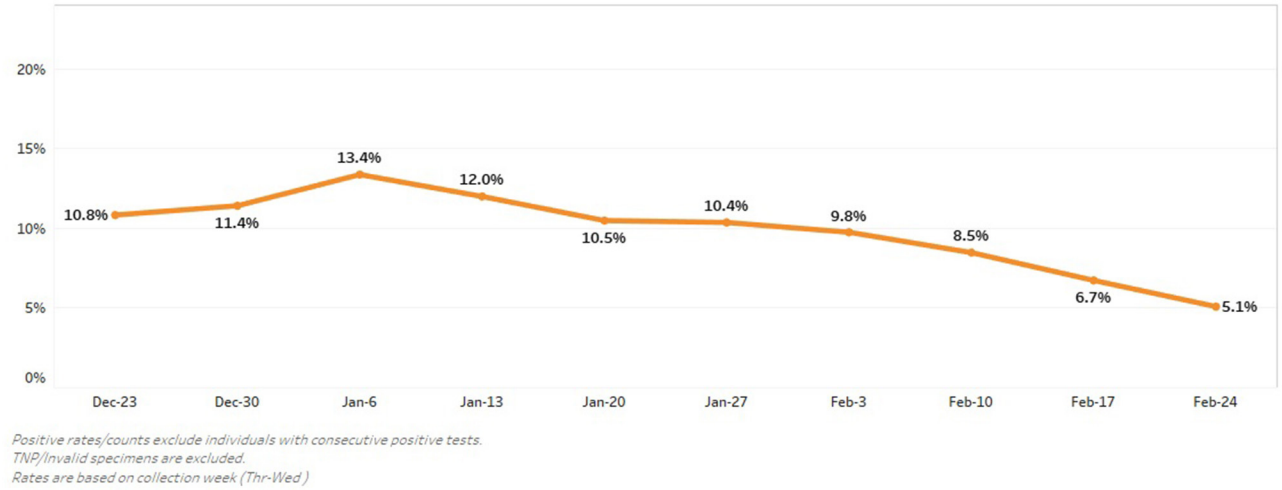


FIGURE 5 | Percent SARS-CoV2 positive tests over time.

From July 2020 to October 2020 (before cold weather led to relocation indoors), we conducted over 25 events in nine locations. We tested over 11,000 people and identified 342 positive community members within the City of Worcester during a window between the first and second COVID-19 infection surges in our community. Our positivity rate ranged from 1.5% with our initial testing events to a high of 13.4% on January 6, 2021 (see **Figure 5**). Engagement with community members also improved, leading to the testing of 1,388 patients in 4-h testing session on December 28, 2020.

DISCUSSION

The COVID-19 pandemic in our community led our team to rethink traditional models of public health strategy and delivery. Community public health interventions are usually the purview of cities, municipalities, community health centers and other government-funded organizations. The flagship health care system in our region, UMass Memorial Health Care, stepped in to drive and support this public testing initiative in Worcester, MA in support of a statewide and state-funded testing initiative made further possible by mass viral testing strategies of the Broad Institute.

Although the Care Mobile team functioned as an anchor, event staffing relied on the generosity of volunteers. These volunteers came from UMass Memorial Medical Center (Emergency and other physicians across our system, nurses, administrative assistants, executives, our Worcester EMS, CITC team, and many others), as well as from our community partners (e.g., City of Worcester, Latino Education Institute at Worcester State University, Worcester Interfaith and other organizations). Running the volunteer model required agility on the part of our core team—managing volunteer sign ups, adjusting to and training new volunteers at each event, and executing the testing under conditions of volunteer shortage. Our staff's agility allowed us to handle unexpected challenges, including high winds, changing foot traffic patterns, and community members' individual needs (e.g., mobility, apprehension).

Limitations of this work included lack of a comparison group to document the effectiveness of our intervention; further state-level data could provide additional insight comparing cities with and without Stop the Spread efforts. Additionally, we focused this review on process interventions to increase testing efficiency; we did not assess the role of communications through key community groups, traditional and digital media in creating awareness of our testing service and subsequent impact on volume.

During the challenges of providing traditional inpatient and ambulatory care during the pandemic, our health system, city leadership, and community advocacy groups united to broaden the scope of care to include widespread, population-based SARS-CoV2 testing. We anticipate that the lessons learned in conducting this testing campaign can be applied to further surges of SARS-CoV2, international environments, and future respiratory disease pandemics.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because our data are part of the larger MA Stop the Spread testing effort. We cannot share raw data but can offer aggregate statistics for our site. Requests to access the datasets should be directed to Olga Brown, olga.brown@umassmemorial.org.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the

local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Material preparation, data collection and analysis were performed by OB, WS, CM, and KB. The first draft of the manuscript was written by ML, OB, JB, and KB. All authors contributed substantially to the design, implementation and analysis of this work, commented on previous versions of the manuscript, read, and approved the final manuscript.

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Epidemiological Trends and Hotspots of Other Infectious Diarrhea *(OID)* in Mainland China: A Population-Based Surveillance Study From 2004 to 2017

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Background: The incidence of other infectious diarrhea *(OID)* ranked second in class C notifiable disease in China. It has posed a great threat to public health of all age groups. The aim of this study was to investigate the epidemiological trends and hotspots of OID in mainland China.

Materials and Methods: Incidence and mortality data for OID stratified by date, age and region from 2004 to 2017 was extracted from the data-center of China public health science. Joinpoint regression and space-time analyses were performed to explore the epidemiological trends and hotspots of OID.

Results: The average annual incidence of OID was 60.64/100,000 and it showed an increased trend in the mainland China especially after 2006 (APC = 4.12, 95 CI%: 2.06–6.21). Children of 0–4 year age group accounts for 60.00% (5,820,897/11,414,247) of all cases and its incidence continuously increased though 2004–2017 (APC = 6.65, 95 CI%: 4.39–8.96). The first-level spatial and temporal aggregation areas were located in Beijing and Tianjin, with the gathering time from 2005/1/1 to 2011/12/31 (RR = 5.52, LLR = 572893.59, $P < 0.001$). The secondary spatial and temporal aggregation areas covered Guangdong, Guangxi, Hainan and Guizhou from 2011/1/1 to 2017/12/31 (RR = 1.98, LLR = 242292.72, $P < 0.001$). OID of Tianjin and Beijing presented a decreased trend since 2006. However, the incidence of OID in Guangdong, Guangxi, Hainan and Guizhou showed increased trends through 2004–2017.

Conclusion: Our study showed that OID showed a constantly increasing trend and brought considerable burden in China especially in the 0–4 age group. The high-risk periods and clusters of regions for OID were identified, which will help government develop disease-specific and location-specific interventive measures.

Keywords: other infectious diarrhea, epidemiological trends, hotspots, joinpoint regression, space-time analyses

INTRODUCTION

Infectious diarrhea is one of the most common infectious diseases around the world and acts as an important indicator to regional hygiene, food safety and public health (1). In 2010, the diarrhoeal disease ranked second in the global burden of infectious diseases and in 2015, about 2.3 billion people had experienced diarrhea worldwide (2). It was also a major cause of malnutrition and mortality among children under 5 in developing countries (3). China is one of 15 countries with a high disease burden of pneumonia and diarrhea (4). In China, other infectious diarrhea *(OID)* was defined as infectious diarrhea other than cholera, dysentery, typhoid/paratyphoid fever (5). Due to the wide spectrum of pathogens and lack of effective vaccine protection, many regions presented high incidences (6). In 2017, the incidence of OID ranked second in class C notifiable diseases in China (7). It has posed a great threat to public health of all age groups, especially to infants and young children, which therefore caused a heavy economic burden in China.

Although many researches has pictured the epidemiological features of OID in China, the majority of these studies were based on the region-specific level (8, 9). Therefore, in order to further describe the overall epidemic characteristics and trends of OID, we systematically analyzed the reported cases of OID from 31 provinces in China. Meanwhile, we then performed the space-time analyses to identify the hotspots of OID. The results of this study would provide a theoretical basis for OID prevention and control in China.

MATERIALS AND METHODS

Data Sources

The incidence and mortality data for infectious diarrhea from 2004 to 2017 were obtained from the data-center of China public health science, which is the main data center of the National Population Health Science data sharing platform in China and covered a population of about 1.3 billion people from 31 provinces and regions in mainland China (10).

Data Collection

The definition of OID cases in our study followed the criteria issued by the Ministry of Health of the People's Republic of China. In each level medical institution, once a OID case identified, clinicians complete a standard case report card for infectious diseases. The field investigations were performed using a standardized form.

To assess the epidemiological trends and hotspots of OID in mainland China, the data of OID including the number of cases

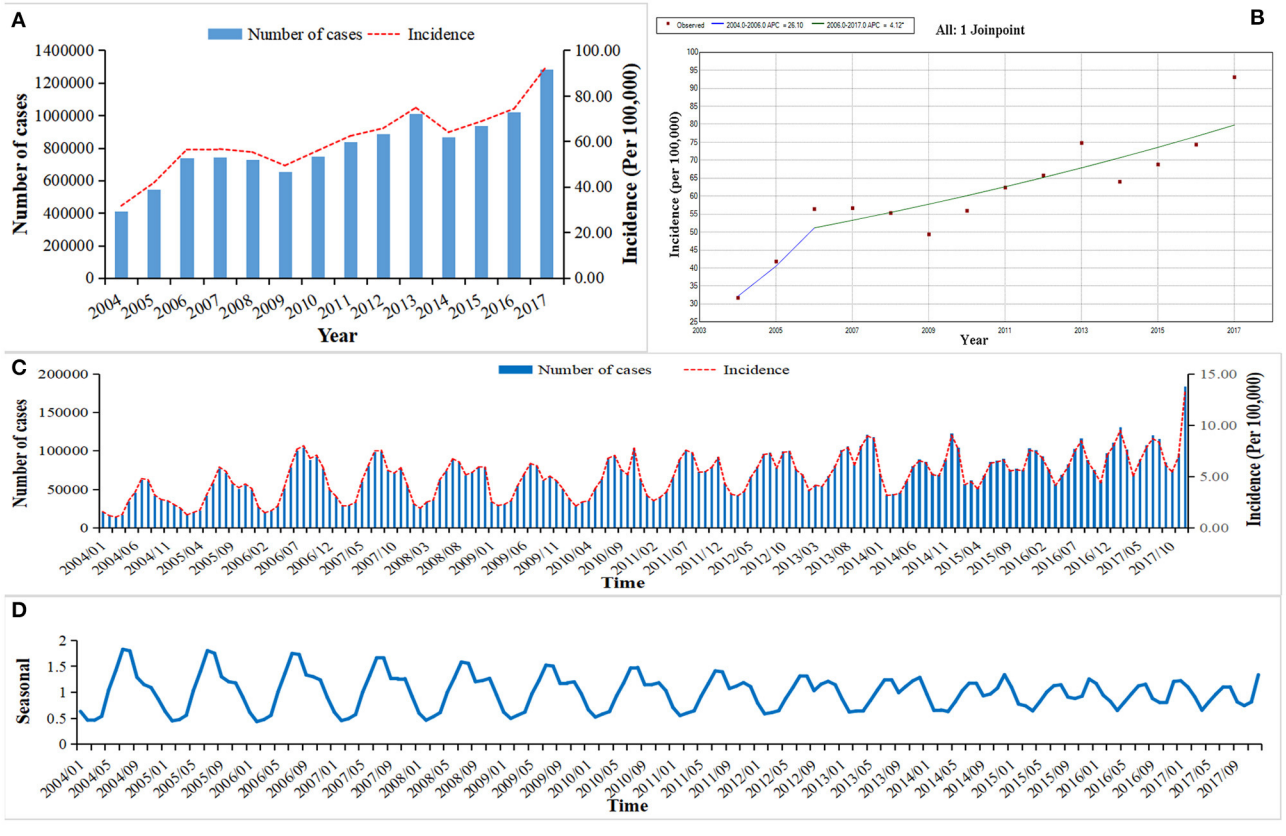

FIGURE 1 | The incidence and trend of other infectious diarrhea in mainland China from 2004 to 2017. **(A)** The annual incidence of OID from 2004 to 2017. **(B)** The Joinpoint analysis of OID from 2004 to 2017. **(C)** The monthly incidence of OID from 2004 to 2017. **(D)** Seasonal decomposition analysis of OID from 2004 to 2017.

and deaths, the incidence and mortality were stratified by date (month and year), age and region.

STATISTICAL ANALYSIS

Epidemiological Trends Analysis

We used joinpoint regression model to examine incidence trends of OID from 2004 to 2017. The annual percentage changes *(APCs)* with their 95% confidence interval *(CI)* were obtained for each trend segment (11). We used Z test to assess whether APCs was significant ($P < 0.05$), and the trends were further described as increased or decreased when the APCs were positive or negative, respectively. While, the trends were considered as stable when the APCs values were not significant ($P \geq 0.05$).

Seasonal Decomposition Analysis

The seasonal decomposition analysis was based on the seasonal trend decomposition using SEATS (Seasonal Extraction in ARIMA Time Series) (12), which filters the trend and seasonal component from the time series data and decomposes into three components: trend (T), seasonal (S), and remainder or random (R). The equation can be described as follows. $Yt = Tt + St + Rt$ (12). In this study, Yt is the number of OID cases. t is time in the unit of month.

Spatial Autocorrelation Analysis

Spatial autocorrelation refers to the potential interdependence of some variables between observed data in the same distribution area. The global Moran's I and local Moran's I were used

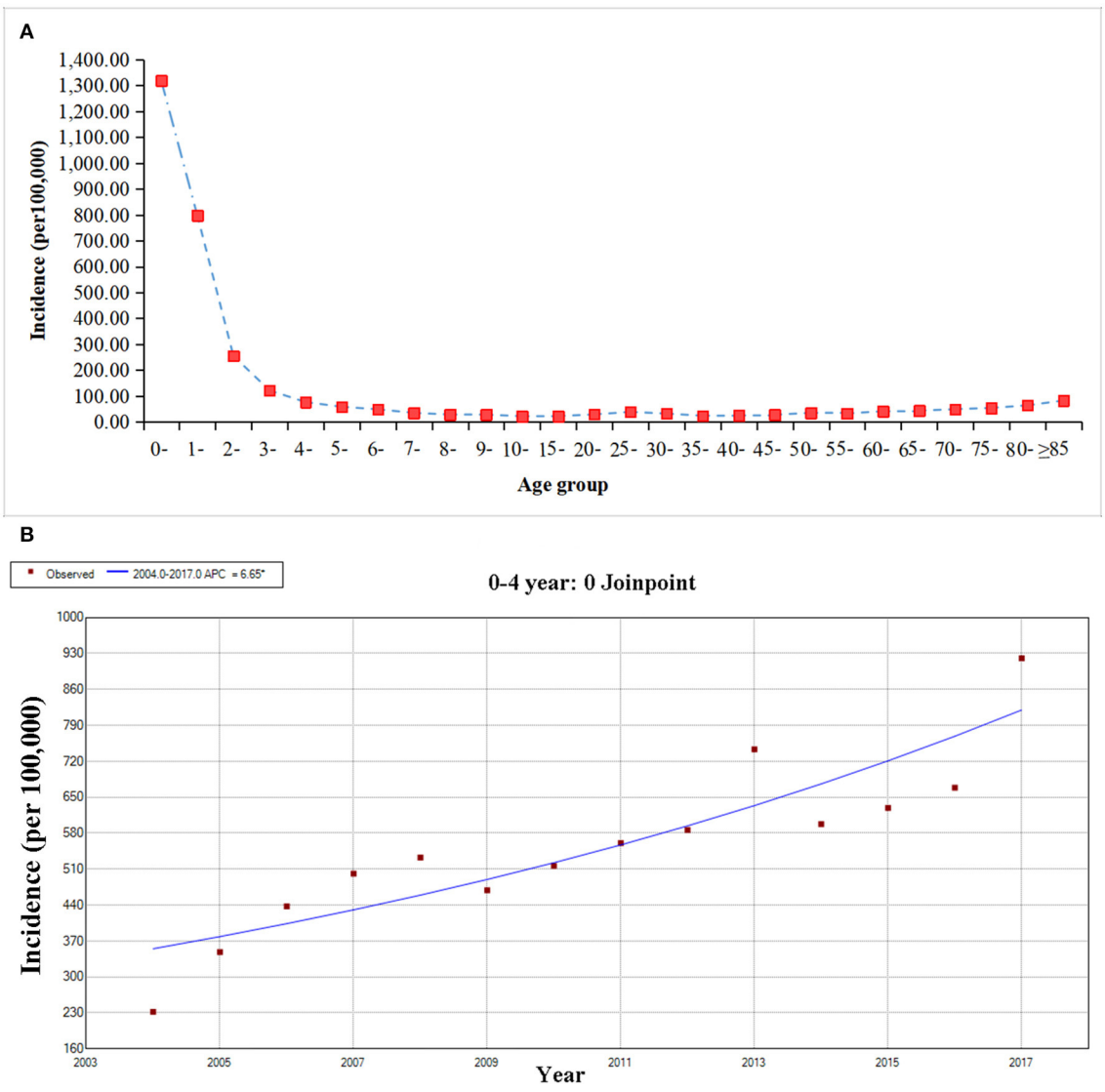

FIGURE 2 | The age group distribution and trend of other infectious diarrhea from 2004 to 2017. **(A)** The age group distribution of OID. **(B)** The Joinpoint analysis of OID in the age of 0–4 year.

to measure spatial autocorrelation (13). The global Moran's I ranging from −1 to 1 was used to detect the degree of spatial autocorrelation of research object from the whole region (14). The local Moran's I was used to explore the spatial position of clustering. According to the results of local Moran's I, it presented four categories results including high-high cluster, low-low cluster, high-low cluster, and low-high cluster (15). Z test was used to assess the significant difference.

Spatial and Temporal Aggregation Analysis

The retrospective spatiotemporal scan statistic based on the discrete Poisson model was applied to detect the space-time cluster of OID in China (16). The dynamic space-time two-dimensional cylinder scanning window was constructed to scan geographic units and time within the study area. The null hypothesis presumed that the window area and outside areas have the same relative risk *(RR)* of incidence. The actual and

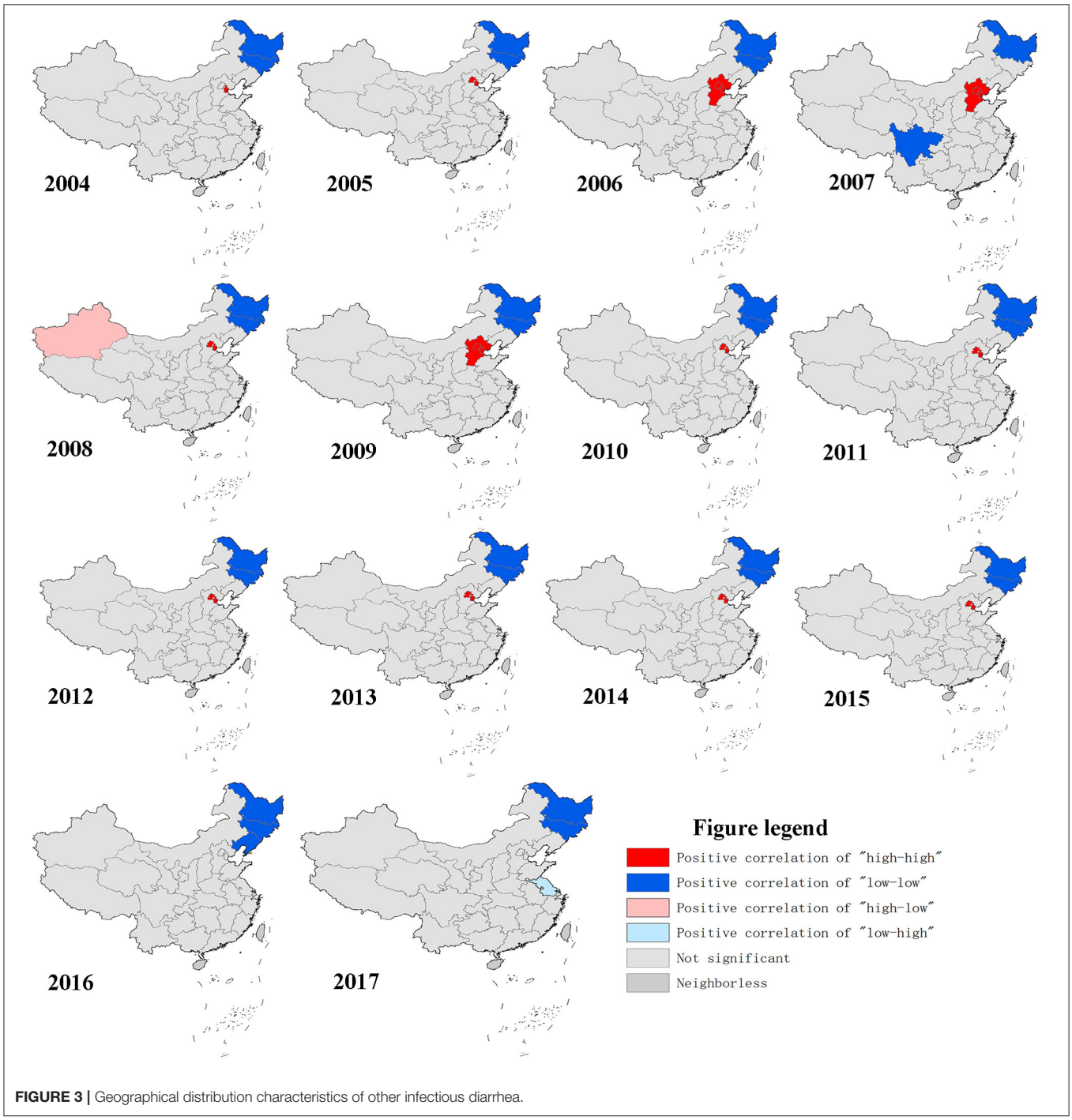

FIGURE 3 | Geographical distribution characteristics of other infectious diarrhea.

theoretical incidence numbers inside and outside the scanning window were used to calculate the log likelihood ratio *(LLR)*. The cluster was classified according to the LLR value (e.g. secondary cluster 1, 2) (17). Monte Carlo simulation was used to evaluate statistical significance. We used Microsoft Excel 2016 for data extraction, sorting, and cleaning, and R (version 3.2.3), and SatScan (version 9.5), Joinpoint (version 4.8.0.1) for further data analysis.

RESULTS

The Incidence and Trend of Other Infectious Diarrhea in Mainland China From 2004 to 2017

A total of 11,414,247 OID cases were reported in mainland China from Jan 1, 2004 to Dec 31, 2017. The average annual incidence was 60.64/100,000, from 2004 (31.70/100,000) to the 2017 (93.10/100,000), (**Figure 1A**). The incidence of OID presented an increased trend in mainland China especially after 2006 (APC = 4.12, 95CI%: 2.06–6.21, $P < 0.05$), (**Figure 1B**). Before 2013, two peaks of OID showed up during June to August and September to November, but it turned to the June to August and December to February (**Figures 1C,D**).

The Age Group Distribution and Trend of Other Infectious Diarrhea From 2004 to 2017

The age group of 0- years showed the highest average annual incidence of 1318.10/100,000. Children in 0–4 years group were most at-risk group infected with OID, accounting for 60.00% (5,820,897/11,414,247) of all cases. With the increase of age, the incidences showed a downward trend, and after the four-year age group, the incidences were relatively stable (**Figure 2A**). The results of Joinpoint analysis showed that the incidence of 0–4 age group continuously increased through 2004–2017 (APC = 6.65, 95CI%: 4.39–8.96, $P < 0.05$) (**Figure 2B**).

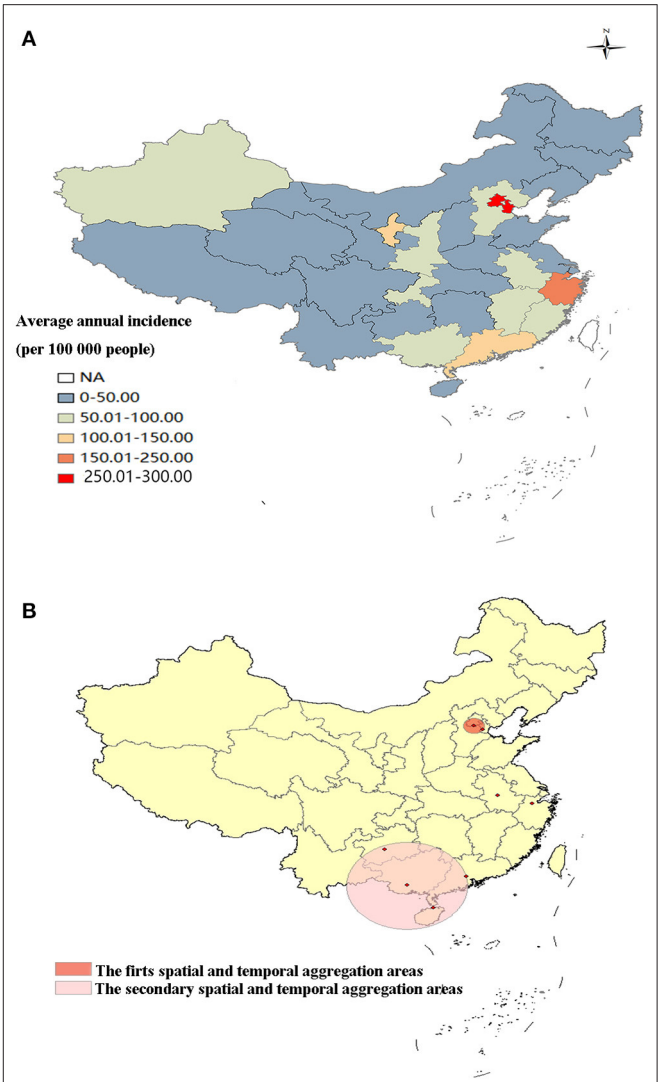

FIGURE 4 | Spatial and temporal aggregation of other infectious diarrhea. **(A)** Geographical distribution of OID in mainland China. **(B)** Spatial and temporal aggregation of OID from 2004 to 2017.

Geographical Distribution Characteristics of Other Infectious Diarrhea

During 2004–2017, The top three incidence regions of OID were Tianjin (289.33/100,000), Beijing (253.67/100,000) and Zhejiang province (200.34/100,000). The OID was higher in the eastern China (83.65/100,000) than central (43.79/100,000) and western China (47.95/100,000), (**Figure 4A**, **Supplementary Table 1**). The global spatial autocorrelation analysis results demonstrated a positive correlation during the 2004-2017 (**Supplementary Table 2**). Then, local spatial autocorrelation at the provincial and autonomous levels were further analyzed. The high-high aggregation areas of OID were found in Tianjin and Beijing from 2004–2015. The low-low aggregation areas were found in Heilongjiang and Jilin from 2004 to 2017 (**Figure 3**).

Spatial and Temporal Aggregation Analysis of Other Infectious Diarrhea

Two spatial and temporal aggregation areas were revealed according to spatial and temporal aggregation analyses. The first-level spatial and temporal aggregation areas were distributed in Beijing and Tianjin, with the gathering time in 2005/1/1 to 2011/12/31. The actual number of cases reported in the regions was 658,479, which was much higher than that of the number of expected cases, that is, 125,134 (RR = 5.52, LLR = 572893.59, $P < 0.001$). The secondary spatial and temporal aggregation areas covered four provinces from 2011/1/1 to 2017/12/31. The areas included Guangdong, Guangxi, Hainan and Guizhou. The actual number of cases reported in the region was 1,396,809, but the number of expected cases

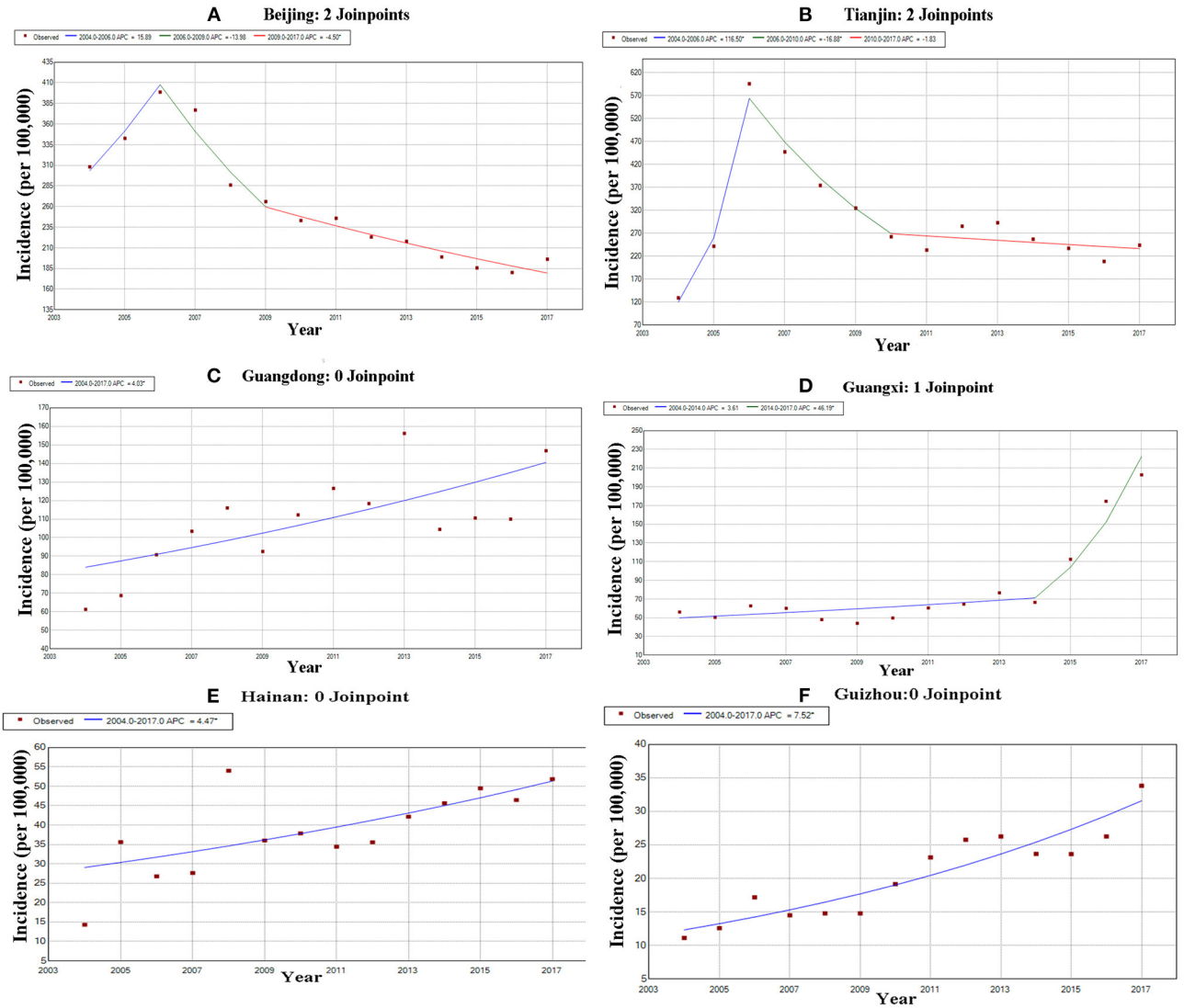

FIGURE 5 | The trends of other infectious diarrhea in hotspots. **(A)** The Joinpoint analysis of OID in Beijing from 2004 to 2017. **(B)** The Joinpoint analysis of OID in Tianjin from 2004 to 2017. **(C)** The Joinpoint analysis of OID in Guangdong from 2004 to 2017. **(D)** The Joinpoint analysis of OID in Guangxi from 2004 to 2017. **(E)** The Joinpoint analysis of OID in Hainan from 2004 to 2017. **(F)** The Joinpoint analysis of OID in Guizhou from 2004 to 2017.

was 749,523 (RR = 1.98, LLR = 242,292.72, $P < 0.001$), (**Figure 4B**).

The results of Joinpoint analysis by areas showed that OID showed an increasing trend in most provinces (21/31) (**Supplementary Table 1**). In Tianjin and Beijing, the OID presented a decreasing trend since 2006. However, the incidence of OID in Guangdong, Guangxi, Hainan and Guizhou showed an increased trend throughout 2004–2017 (**Figure 5**).

DISCUSSION

In this study, we investigated the epidemic trend and distribution characteristics of 11,414,247 OID cases in mainland China during a 14-year time period. Our results demonstrated that OID presented a constantly increased trend in mainland China and in 0–4 age group from 2004 to 2017. Two levels hotspots were found. Our findings will provide scientific evidence to policy maker to better prevent and control OID in the following stage.

The expanding of pathogenic spectrum, especially viral pathogens, has promoted the prevalence of OID and caused increasing outbreaks and public health emergencies which usually brought a large number of cases (18). In recent years, as the development of infectious disease surveillance and reporting system, the OID cases were more likely to be detected and reported. The prevalence of OID shifted from summer and autumn peaks to summer and winter peaks since 2013. This might attribute to the increased incidence of viral infectious

diarrhea such as rotavirus and norovirus which clustered in winter (19). In 2005–2019, the four most common pathogens of OID in China were rotavirus (85.74%), adenovirus (4.28%), salmonella (3.58%) and norovirus (2.82%) (20). Since 2013, norovirus was reported as the dominant pathogeny in OID outbreaks in China, and rotavirus also increased rapidly in children under 5 years of age (21, 22). The OID incidence of 0–4 years age group showed the increased trends in China. On the one hand, once children are infected, the symptoms are relatively severe due to immature immune system (23). Additionally, parents are more likely to seek medical treatments for their children and then the cases were more likely to be reported. On the other hand, as proportion of viral pathogens in OID increased, children under 5 years old were more vulnerable (24).

In previous studies, diarrhea was found presenting a specific temporal and spatial distribution which usually clustered spatially in different geographical locations (25–27). Several factors including sociodemographic variables, personal hygiene, and environmental and climatic changing were considered to be associated with the incidence of diarrhea (28, 29). Spatiotemporal aggregation of OID in China could be divided into two stages. The first hotspots were detected in the Beijing-Tianjin through 2005–2011. The two sites are both developed areas in China. In the early phase of development and urbanization, it attracted larger floating population characterized by low immune systems, poor living environments and living conditions, and poor health and knowledge of epidemic prevention measures which might cause OID to spread easily (30, 31). As the improvement of the environment and the management of mobile population, risks of infection by various pathogens would reduce. In the Joinpoint regression, the incidence of OID in Beijing-Tianjin decreased since 2006. The second hotspots was identified in Guangdong, Guangxi, Hainan and Guizhou through 2011–2017. The OID in those region showed an increased trend through 2004–2017. In weng's study, public health emergencies caused by OID were found mainly clustered in the Guangdong, Guangxi and Fujian provinces (18). South China is closer to the equator with a subtropical monsoon and tropical monsoon climate, which are characterized by high humidity, temperature, rainfall, and wind speed. And this also facilitated OID transmission (31). Furthermore, residents of coastal areas usually have the habit of eating raw seafood, which also contains a wide variety of pathogens such as rotavirus, norovirus, and vibrio parahaemolyticus (32, 33). Our results show that the incidences of OID in China has a clear population, seasonal and regional distribution. The comprehensive prevention and control measures should be implemented to reduce the incidences of OID before the epidemic peaks. The specific hotspots, highly risk groups should be considered as the priority of prevention and control. At the same time, the monitoring of pathogens should be conducted to further clarify the epidemic characteristics of OID in each jurisdiction. For some pathogens such as rotavirus could be prevented by vaccines. The knowledge should be strengthened to improve the coverage of vaccines, especially in children (34).

CONCLUSIONS

Our study demonstrated that the incidence of OID continuously increased in mainland China especially in 0–4 years age group. The seasonal peak of OID prevalence shifted from summer and autumn to summer and winter since 2013. Currently, Guangdong, Guangxi, Hainan and Guizhou were identified as the hotspots of OID in mainland China. The high-risk periods and clusters of regions for the OID were identified which will help governments to develop disease-specific and location-specific intervention measures.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SY, LJL, and JW: designed the study. CC, ZG, CH, DJ, XL, YZ, DY, XZ, YZ, and YL: collected data. CC, ZG, and CH: analyzed data and interpreted data and wrote the report. CD and LL: checked the data and results. SY: revised the report from preliminary draft to submission. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.679853/full#supplementary-material

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Eight Years of Collaboration on Biosafety and Biosecurity Issues Between Kazakhstan and Germany as Part of the German Biosecurity Programme and the G7 Global Partnership Against the Spread of Weapons and Materials of Mass Destruction

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In 2013, the German Federal Foreign Office launched the German Biosecurity Programme with the aim to minimise risks associated with biological substances and pathogens. In this context, the German-Kazakh Network for Biosafety and Biosecurity was established in 2013 and constitutes a successful collaboration between Kazakh and German biomedical organisations, under the co-management of the Bundeswehr Institute of Microbiology (IMB), and the Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ) GmbH. Ever since then, a network of scientists, stake holders and policymakers has been established, aiming to work on highly pathogenic, potential biological warfare agents with the focus on biosafety and biosecurity, surveillance, detection and diagnostics, networking and awareness raising of these agents in Kazakhstan. Over the past 8 years, the project members trained four PhD candidates, organised over 30 workshops and trainings with more than 250 participants and conducted more than 5,000 PCR assays and 5,000 serological analyses for surveillance. A great success was the description of new endemic areas for *Orthohantaviruses*, the mixture of two *Crimean-Congo haemorrhagic fever virus* genetic clusters, new foci and genetic information on tick-borne encephalitis virus and *rickettsiae* in Kazakh oblasts. The latter even led to the description of two new genogroups. Furthermore, joint contributions to international conferences were made. In this report, we summarise the evolution

of the German-Kazakh Network for Biosafety and Biosecurity and critically reflect on the strengths and possible weaknesses. We were able to establish a viable network of biosafety and biosecurity shareholders and to accomplish the aims of the German Biosecurity Programme to lower biosecurity risks by increased awareness, improved detection and diagnostic methods and surveillance. Further, we reflect on forthcoming aspects to lead this interstate endeavour into a sustainable future.

Keywords: Germany, Kazakhstan, biosafety & biosecurity, surveillance, orthohantavirus, CCHFV, rickettiosis, TBEV

INTRODUCTION

The Republic of Kazakhstan is a landlocked country in Central Asia dominated by continental climate. Totalling up to 2.7 million km^2, the size of the country approximately is seven times the size of Germany and hosts a relatively small population of 18.6 million inhabitants. Historically, Kazakhstan served as the cradle of many consequential pathogens. Its vast steppes and the hot and dry climate fuelled the evolution of plague (*Yersinia pestis*). Furthermore, diseases like Anthrax, Tularaemia and Brucellosis, to name but a few, are endemic to the region (1–3). There exists a vast amount of under-investigated potential natural foci for the vectors or reservoirs of some of these diseases. Animal burial sites, for instance, may cause spontaneous outbreaks of anthrax after heavy rains or landslides or can be unearthed by digging activities.

Because of this high endemic burden, local authorities in different oblasts (=territories) of Kazakhstan started to establish local so-called Anti-Plague Stations (APS) between 1914 and 1949. At the beginning, the main responsibility of these APS was the epidemiological and epizootic surveillance of plague and of the infection rates with *Yersinia pestis* in their natural hosts, wild living gerbils (*Gerbillinae* spp.), and the vectors, which are fleas (genus *Xenopsylla*) (4). Quickly, the APSs evolved to regional centres for the epidemiological surveillance of other especially dangerous endemic pathogens such as the *tick-borne encephalitis virus* (TBEV), the *Crimean-Congo haemorrhagic fever virus* (CCHFV), *rickettsiae* and *Orthohantaviruses* (5).

During the Soviet time, Kazakhstan played a role in the research on biological warfare agents and countermeasures. However, after the dissolution of the Soviet Union, Kazakhstan proactively opened all facilities to the public. In 1995, it was confirmed by international observers that all military facilities were shut down and that contaminations were removed.

Furthermore, in 2004 Kazakhstan signed an agreement with the USA to reduce the biological weapons proliferation risk. It is an addendum to the 1995 Nunn-Lugar Cooperative Threat Reduction to prevent the proliferation of biological weapons technology, pathogens and expertise (6). Therefore, today the main biological risk comes from Kazakhstan's rich and diverse natural foci of extremely dangerous pathogens (EDP).

Nevertheless, there are blank spots regarding the spread and epidemiology of EDPs in some areas of Kazakhstan as only limited data is available. Many reports of infections with EDPs in Kazakhstan are based on the clinical presentation of infected patients rather than on contemporary molecular biological and serological diagnostic as part of all-over surveillance studies. In conclusion, frequent fevers of unknown origin in rural and urban residents indicate that more research on the spread of endemic pathogens needs to be conducted. Many major infections display a relatively unspecific clinical picture that often leads to the problem of misclassification of diseases. It is suspected that many cases of infectious diseases caused by EDPs, such as *Orthohantaviruses*, TBEV, and CCHFV, go unnoticed in endemic and so-far non-endemic areas in Kazakhstan.

The Republic of Kazakhstan recognised this issue and actively approached international governmental and non-governmental institutions to modernise its diagnostic capabilities. As part of the U.S. Defense Threat Reduction Agency (DTRA) biological threat reduction programme, the USA funded the improvement of infrastructure, including the construction of the Central Reference Laboratory (CRL) - a BSL3 laboratory in the city of Almaty (6).

There are numerous efforts to support the establishment of a modern biosafety and biosecurity landscape in Kazakhstan together with international partners. Therefore, institutions in Kazakhstan proved as ideal focus points for the new German Biosecurity Programme that was launched by the German Federal Foreign Office in 2013.

Abbreviations: APS, Anti-Plague Station; BACAC, Biosafety association for central Asia and the Caucasus; BMBF, German Ministry of Education and Research; BSL, Biosafety level (1–3); B(T)WC, Biological (and Toxins) Weapons Convention; CCHFV, *Crimean-Congo Haemorrhagic Fever Virus;* DOBV, *Dobrava-Belgrade orthohantavirus;* EDP, Extremely dangerous pathogens; GerKazNet, German-Kazakh Network for Biosafety and Biosecurity; FUO, Fever of unknown origin; HTNV, *Hantaan orthohantavirus;* HFRS, Hemorrhagic fever with renal syndrome; IgG, Immunoglobuline G; IgM, Immunoglobuline M; OHV, *Orthohantavirus*; PUUV, *Puumala orthohantavirus*; RT-PCR, Reverse transcription Polymerase chain reaction; SEOV, *Seoul orthohantavirus*; SES, Sanitary Epidemiology Station; TBEV, *Tick borne Encephalitis Virus*; WKR, West Kazakhstan Region; WMD, Weapons and Materials of Mass Destruction.

INITIATION OF THE GERMAN-KAZAKH NETWORK FOR BIOSAFETY AND BIOSECURITY

In early 2013, the German Foreign Office initiated the German Biosecurity Programme with the aim to foster biosafety and biosecurity for a safer world. This programme was launched within the framework of the German engagement in the G7 Global Partnership Against the Spread of Weapons and Materials

of Mass Destruction (WMD) which expanded its activities to questions regarding biological security in 2012 (7). The German Biosecurity Programme is part of the Federal Government's preventive security policy and has the goal to minimise the risks associated with biological substances and pathogens and, therefore, cooperates with selected partner countries worldwide (8). It aims to promote a responsible approach to research and research findings on dual-use pathogens, to strengthen local public health and to prevent a potential menace to Germany. To reach these goals, the project activities are based on six different columns: increasing awareness, networking, capacity development, detection, and diagnostics, surveillance as well as biosafety and biosecurity. The German Initiative of the Federal Foreign Office aims at raising awareness of and minimising the risks associated with highly pathogenic agents, including their potential abuse for the purposes of terrorism. It reached out to German federal laboratories working on different aspects of EDPs and invited them to submit project proposals.

The call reflects part of the mission of the Bundeswehr Institute of Microbiology (IMB), a military research facility of the German Armed Forces for medical biological defence based in Munich. Its task is to develop methods and measures to protect soldiers from diseases caused by biological warfare agents and other dangerous pathogens (9). The focus is on diagnostics, research, teaching, and biosecurity. Due to the institute's interest in especially dangerous zoonotic diseases with natural foci for uncommon outbreaks and their historical significance to Central Asia, Kazakhstan was identified as a partner country to jointly participate in the German Biosecurity Programme.

The landlocked country in Central Asia indeed has several active foci of endemic EDPs, as mentioned above. Furthermore, it has 13.364 km of borders with its neighbouring countries Russia, China, Kyrgyzstan, Uzbekistan, and Turkmenistan and many migratory animals, such as birds or bats, and associated ticks that seasonally can carry new species of pathogens across the country. The additional heavy transport of livestock over far distances further poses the risk of pathogen dispersion e.g., also along the new Chinese Belt and Road Initiative. This diverse and changing EDP landscape is monitored by public health institutions that often reside in aged buildings with equipment still remaining from the Soviet era. The quantity of biosurveillance has decreased in comparison to Soviet times, due to the lack of financial support and the slow establishment of state-of-the art diagnostic tools. The retirement of experienced researchers and the brain drain of younger scientists to more prosperous countries further weakened the input to the Kazakh public health monitoring (10).

In summary, Kazakhstan has all the prerequisites to meet the goals of the German Biosecurity Programme by the Federal Foreign Office. Establishing a network between Kazakh and German biosafety and biosecurity stakeholders will contribute toward raising awareness of and minimising the risks associated with highly pathogenic pathogens, including their potential abuse for the purposes of terrorism. In doing so, it contributes to the G7 Global Partnership Against the Spread of Weapons and Materials of Mass Destruction (WMD) by means of a preventive security-policy.

THE GERMAN-KAZAKH NETWORK FOR BIOSAFETY AND BIOSECURITY

The German Biosecurity Programme and the German-Kazakh Network for Biosafety and Biosecurity (GerKazNet) are now in its third funding phase (2013–2016, 2017–2019, 2020–2022). Starting in 2013, the "Asfendiyarov Kazakh National Medical University" (KazNMU) and the "Scientific Practical Center for Sanitary Epidemiological Expertise and Monitoring" (SPC SEEM, 2013) together with the IMB and GIZ were the founding partners of the GerKazNet and the collaboration lasted from 2013–2018. Over the years, the network expanded and welcomed other key players to the Kazakh biosafety and biosecurity framework, such as the "Masgut Aikimbayev's National Scientific Center for Especially Dangerous Infections" (NSCEDI, 2014). The most recent institution, which joined the network in 2019, is the Otar-based "Research Institute for Biosafety Problems" (RIBSP, 2019). It is planned that the "National Center for Biotechnology Branch Almaty" (NCB) will join the network in 2021 as listed in **Table 1**.

An efficient coordination of such a project demands a professional, well-connected agency that enables a transparent handling of personnel and finances. An important partner in this is the "Deutsche Gesellschaft für Internationale Zusammenarbeit GmbH" (German Corporation for International Cooperation GmbH, GIZ), a federal enterprise and globally active service provider in the field of international cooperation for sustainable development and international education work (11). The GIZ offers consulting and capacity building services in around 120 countries pursuing the goal of permanently improving the living conditions of people worldwide. With its offices in Almaty and Berlin, the GIZ has helped to establish the GerKazNet through legal, financial and consulting services. Furthermore, it has also allowed for a highly professional interaction with policymakers.

The GerKazNet has established a wide network of partners in Kazakhstan. During the project, several reorganisations in ministries and committees took place. Thus, the network regularly exchanged information with the former (2013–2019) Chief Sanitary Doctor of Kazakhstan and the Deputy Head of the former Committee of Quality Control and Goods and Services Security (Safety) at the Ministry of Health which was reorganised in 2020. The board, now called the "Committee of Sanitary and Epidemiological Control of the Ministry of Healthcare" is the national institution to supervise all areas of public health protection including sanitary and epidemiological welfare of the population, as well as the field of food safety, controlling the implementation of regulatory measures. Furthermore, the GerKazNet is in close contact with the Kazakh head of the Department for Coordination of Activities of Scientific Organisations, located at the Ministry of Education and Science.

In 2020, the GerKazNet became a partner of the International Science and Technology Center (ISTC), which is an intergovernmental organisation connecting scientists from several Central Asian countries with their peers and research organisations in the EU, Japan, Republic of Korea, Norway, and the United States. The ISTC facilitates international science

TABLE 1 | List of all participants at the German-Kazakh network for biosafety and biosecurity (GerKazNet).

Name	Abb.	Year joined GerKazNet	Location	Belongs to
Institut für Mikrobiologie der Bundeswehr	IMB	2013	Munich, DEU	MoD
Deutsche Gesellschaft für Internationale Zusammenarbeit GmbH	GIZ	2013	Berlin, DEU, Almaty/Nur-Sultan, KAZ	–
Kazakh National Medical University	KazNMU	2013–2018	Almaty, KAZ	MoH
Scientific Practical Center for Sanitary Epidemiological Expertise and Monitoring	SPC SEEM	2013–2018	Almaty, KAZ	MoH
M.Aikimbayev National Scientific Center for Especially Dangerous Infections*	NSCEDI	2014, 2017**	Almaty, KAZ	MoH
Scientific Research Institute of Biological Safety Problems	RIBSP	2019	Otar, KAZ	MoES
International Science and Technology Center	ISTC	2020	Nur-Sultan, KAZ	–
National Center for Biotechnology, Branch Almaty	NCB Branch Almaty	2021	Almaty/Nur-Sultan, KAZ	MoES

**Formerly known as M. Aikimbayev Kazakh Scientific Center of Quarantine and Zoonotic Diseases (KSCQZD).*
***Active collaborative work started in 2017 in the second project phase.*
MoD, Ministry of Defense; MoH, Ministry of Health; MoES, Ministry of Education and Science.

projects and assists the global scientific and business community to source and engage with the Commonwealth of Independent States (CIS) and Georgian institutes that develop or possess an excellence of scientific know-how (12).

The role of the KazNMU was to offer the academic surrounding for education in medicine and pharmacy. With regard to that, the molecular biological laboratories were a budding partner in the project during the first two phases of the GerKazNet. The KazNMU is the national medical university, located in Almaty and operated by the Ministry of Health (13). Up to 9,000 students, including 1,000 students from 15 different countries, are enrolled to train for the medical and pharmaceutical profession.

The Ministry of Health also operates the SPC SEEM whose main mission is the surveillance and control of infectious diseases, the detection of epidemics and the recommendation as well as implementation of appropriate countermeasures, as well as providing information to the Kazakh government. For this, the parasitological department collecting vectors throughout Kazakhstan was an important project partner during the first two phases of the GerKazNet.

During the second phase, a close and productive collaboration with the NSCEDI (formerly M. Aikimbayev Kazakh Scientific Center of Quarantine and Zoonotic Diseases, KSCQZD) has been established and is still ongoing. The NSCEDI originated from the "Central Asia Research Anti-Plague Institute" in Almaty, which was founded in 1949 and whose main mission was the epidemiological surveillance of highly pathogenic diseases (anthrax, plague, tularaemia, brucellosis, cholera, listeria, CCHF, and HRFS) and other zoonotic diseases (yersinioses, listerioses, leptospirosis, pasteurellosis, etc.,). The institute is now under the control of the Ministry of Health and is considered a scientific and methodological center for eight anti-plague stations and 15 district departments.

A more recent partner, who joined the network in 2020, is the RIBSP, based in Otar. It conducts research on highly pathogenic viruses and bacteria as well as exotic diseases of farm animals and plants and is subordinate to the Ministry of Science and Education. They establish and mass-produce diagnostic kits and vaccinations against EDPs and other zoonotic agents and animal diseases to support the Kazakh livestock industry. The RIBSP also plays an important role in biosafety, biosecurity, diagnostics and detection of EDPs mainly in animal hosts and vectors. In 2020, it featured prominently in the development of the national COVID-19 vaccine and first clinical tests were announced in August 2020.

To support the development of a biotechnological industry in Kazakhstan, the National Center for Biotechnology (NCB) was established. The NCB carries out government funded scientific and technical programmes in the areas of biotechnology, biosafety, and ecology and translates them into economic endeavours. It is a decentralised institution that operates facilities all over the country with the Branch Almaty focusing on the molecular biological characterisation of newly emerging pathogens.

Part of their research is conducted in the Central Reference Laboratory (CRL) in Almaty. This laboratory was built by the Americans as part of the DTRA activities and contains a

state-of-the-art BSL-3 facility. It was ceremonially handed over to the Kazakh government in September 2018 and is jointly operated by three ministries, namely the Ministry of Health *via* the NSCEDI, the Ministry of Education and Science *via* its NCB Branch Almaty and the Ministry of Agriculture with the latter not being a part of the GerKazNet. USA, Great Britain, Germany and Poland actively participated in the training of staff and personnel.

THE FOOTPRINT OF THE GERKAZNET

The purpose of the Global Partnership Against the Spread of Weapons and Materials of Mass Destruction (WMD) is defined by five deliverables that aim at reducing biological warfare threats: (i) control materials that represent biological proliferation risks, (ii) detect and disrupt misuse of biological agents, (iii) improve identification of biological attacks, (iv) reinforce and strengthen Biological and Toxins Weapons Convention (BTWC) practices and instruments, and (v) promote biosafety and biosecurity (7). To reach these goals, the German Biosecurity Programme, coordinated by the German Foreign Office, defined six modes of action: Increasing awareness, biosafety and biosecurity, detection and diagnostics, networking, surveillance, and capacity development. An accompanying toolbox approach containing 25 items was established to help the projects to successfully start their endeavour. The GerKazNet translated these modes of action into several study topics that were put into practice together with the partner institutes.

Increasing Awareness

To raise sensibility and awareness of issues regarding biosafety and biosecurity, each joint scientific meeting was started by a keynote lecture given by the programme manager or invited speakers on respective topics. On four occasions, scientific lectures on EDPs and on pathogens relevant for medical biodefense and their associated human diseases helped the project partners to identify contact points regarding biosafety and biosecurity. Intense discussions of these issues clarified open questions and set the ground for future projects. Partner institutes and their branches were also informed about the dual-use concept that is applicable to many of the pathogens endemic to Kazakhstan and the importance of blocking access to EDPs to unqualified personnel that could proliferate it to groups with bad intensions.

As part of the programme, an exchange on the importance to share scientific insights on EDPs with the international community was arranged with policy makers at the Ministry of Education and Ministry of Health, in order to enable a bioforensic characterisation that would help to localise the source of future potential illegal use of EDPs. Regular meetings with the former Committee of Quality control of Goods and Services of the Ministry of Health (2013–2019) were used to inform the committee members about the progress of the project and about scientific findings like raising awareness on possible new endemic areas of EDPs but also on legislation in work safety including biosafety and biosecurity. Another key ambition was to raise awareness of the health risks related to some non-endemic pathogens that may migrate to Kazakhstan through birds or enhanced trade resulting from the China's Belt and Road Initiative which could lead to diseases among the residents.

Biosafety and Biosecurity

A major focus of the project was to organise sustainable trainings on issues of biosafety and biosecurity for staff such as medical doctors, scientists, and lab technicians in small groups. In regular workshops, local staff learned about modern techniques and diagnostic tools with the focus on biosafety and biosecurity. In order to promote a new generation of scientists, four PhD candidates were selected to conduct doctoral studies under the scientific supervision of the IMB in cooperation with the Munich Ludwigs-Maximilians-University and the Center for International Health (CIH) PhD programme. This programme aims to train PhD students from low- and middle-income countries to become the new generation of health care developers in their countries. In this three-year programme, the first part of the education takes place in Munich and the second part in the student's country of origin. The courses in Munich cover topics such as epidemiology, research design and general health issues. Back home in their research laboratory, students are asked to perform scientific research on a selected topic (14). The GerKazNet supported the application of four candidates for this PhD graduate school. They were nominated by the partner institutes in Kazakhstan. All of them had profound medical training and intended to specialise in the area of infectious diseases. During their doctoral research, they implemented the above-mentioned scientific studies. Two of the PhD students successfully graduated in 2019, while the other two will defend their theses in reasonable time. After receiving their PhD degree, the junior scientists got permanent contracts in positions of responsibility at their home institutions. In this role, they contribute to the further improvement of the quality of research and diagnostic services at the institutes and pass on their acquired knowledge to a new generation of researchers following the train-the-trainer principle.

In addition to the complex PhD education, the project aims to give regular training to Kazakh diagnostic and research facilities' staff. In workshops at the partner institutes and anti-plague stations all over the country, small groups ranging from 10 to 12 participants were trained on topics such as hygiene, the safe handling of patient samples, molecular biology, cell culture, the trapping of animals, and next generation sequencing (**Table 2**). The selection of topics resulted from mutual identification of needs or emphasis by the Ministry of Health. In total 240 technicians and scientists were enrolled in different trainings. The workshops were held in English, Russian, and Kazakh and contained a mixture of theoretical, practical, and applied sessions to coach new methods, diagnostics, and quality assurance under the aspect of biosafety and biosecurity. Group sizes were deliberately kept small to ensure supervised hands-on training in step with actual practice. The quality of the workshops was assessed in pre- and post-tests conducted before and after the training. Due to the high appreciation of these workshops, the GerKazNet intends to continue and expand its training programme, also as online webinars. To this purpose, the GerKazNet hired a dedicated trainer in 2020 who will regularly

TABLE 2 | List of all Workshops held at the partner institutes of the GerKazNet.

#	Year	Topic of the Workshop	Location
1	2014	Hygiene as a basis of biosafety and biosecurity: disinfection, decontamination, and sterilisation	Almaty
2	2014	Pipetting in diagnostic, research, and regarding biosafety	Almaty
3	2015	International Workshop on molecular biology including next-generation sequencing	Almaty
4	2015	International Workshop on molecular biology	Almaty
5	2015	Modern aspects of parasitological and entomological works in the framework of epidemiological surveillance of tick-borne infections	Almaty
6	2016	International Workshop on molecular biology "Working on RNA"	Almaty
7	2017	Workshop on the safe handling of patient's samples and PCR diagnostics in the field of highly dangerous pathogens	Almaty
8	2018	Workshop on Biosafety and Biosecurity in field studies and highly pathogenic rodent-borne infections	Almaty
9	2018	Workshop on cell culture methods I	Almaty
10	2018	Workshop on cell culture methods II	Almaty
11	2018	Workshop on PCR diagnostics in the field of highly dangerous pathogens	Almaty
12	2019	Workshop on Biosafety and Biosecurity in field studies and highly pathogenic rodent-borne infections	Uralsk
13	2020	Workshops on personal protection in the biological laboratory I	Online
14	2020	Workshops on personal protection in the biological laboratory II	Online
15	2020	Self-learning modules on COVID19 diagnostics	Online

APS, Anti Plague station.

offer workshops and training units, in order to promote a lasting research culture in Kazakhstan.

Surveillance, Detection, and Diagnostics

As part of this mode of action, the GerKazNet encourages local scientists to introduce modern diagnostic and detection methods, to perform studies as well as surveillance approaches in their laboratories. In concert with scientists from Germany, they established a bundle of new molecular biologic methods on the prevailing EDPs in Kazakhstan and documented the methods in peer reviewed standard operating procedures (SOPs), that are now routinely used for diagnostic and research in the partner institutions (**Table 3**). Beyond the scientific output (see chapter below), surveillance data were shared with all partners including stake holders.

To ensure an efficient sharing of methods and protocols, and to offer a low-key scientist interaction, the GerKazNet

TABLE 3 | Established serological and molecular biological diagnostic methods by the GerKazNet at KazNMU, SPC SEEM (both partners from 2012 to 2018), and NSCEDI (active partner since 2017).

Pathogen	Method	Institute	Reference
Orthohantaviruses	Serological (Immunoblot, IIFT, ELISA); molecularbiological (RT-PCR, real-time PCR)	KazNMU SPC SEEM NSCEDI	(15)
CCHFV	Serological (ELISA); Molecularbiologcial (RT-PCR, real-time PCR)	KazNMU SPC SEEM NSCEDI	(16)
Rickettsia (Spotted fever group)	Serological (ELISA, IIFT); Molecularbiological (real-time PCR, PCR)	KazNMU SPC SEEM NSCEDI	(17)
Rickettsia (Typhus group)	Serological (ELISA, IIFT);	KazNMU SPC SEEM NSCEDI	(17)
TBEV	Serological (ELISA, IIFT); molecularbiological (RT-PCR, real-time PCR)	KazNMU SPC SEEM NSCEDI	(18)
Borrelia	Molecularbiological (real-time PCR)	KazNMU SPC SEEM NSCEDI	(19)

This methods are now routinely in use to facilitate a nationwide surveillance of extremely dangerous diseases in natural habitats.
CCHFV, Crimean-Congo Haemorrhagic Fever Virus; SFG, Spotted fever group, TBEV, Tick borne Encephalitis Virus; KazNMU, Kazakhstan National Medical University; NSCEDI, M.Aikimbayev National Scientific Center for Especially Dangerous Infections, here work is performed in the CRL branch of MoH, SPC SEEM, Scientific Practical Center for Sanitary Epidemiological Expertise and Monitoring.

became part of the "German Online Platform for Biosecurity and Biosafety" (GO4BSB), coordinated by the "Bernhard Nocht Institute for Tropical Medicine" (BNITM) in Hamburg, Germany. This platform is run within the framework of the German Biosecurity Programme, is open to all partners, and serves as a depository for methods, protocols, background information and training material. Incorporating the use of this platform into the workshops opened it up to a broad audience. It is the aim to establish the GO4BSB platform as the central hub of Kazakh zoonosis scientists when it comes to the local sharing of information, methods and ideas (20).

Networking

International perception of the GerKazNet-activities was successfully increased by jointly attending international and national conferences, in order to present the progress of individual scientific projects, but also the goals of the GerKazNet itself. Members of the project presented the progress of their research in 41 oral talks and 21 poster presentations (**Table 4**). Due to the continuous attendance of conferences, the German-Kazakh initiative became recognised as a reliable partner in the Central Asian area. This led to an active participation in the organisation of the "Biosafety Association for Central Asia and the Caucasus" (BACAC) conference in 2019.

The participation of Kazakh scientists at a series of travel conferences on the distribution, modern detection methods and aspects of biosecurity for highly pathogenic zoonotic agents

TABLE 4 | List of all conferences jointly visited by scientists of the GerKazNet.

Year	Name of Conference	Location	Participating partners of the GerKazNet	Co-organisation by the GerKazNet
2013	Medical Biodefense Conference	Munich, DEU	IMB KazNMU	No
2013	German Symposium on Zoonoses Research 2013	Berlin, DEU	IMB	No
2014	German Symposium on Zoonoses Research 2014 and International conference on Emerging Zoonoses	Berlin, DEU	IMB KazNMU	No
2014	Meeting of Parasitologists	Almaty, KAZ	IMB GIZ SPC SEEM	No
2014	The first international scientific-practical conference of the Agency of the Republic of Kazakhstan on the protection of consumer rights, dedicated to the 100 anniversary of the anti-plague service of the Republic of Kazakhstan and the Ural anti-plague station"	Uralsk, KAZ	IMB GIZ NSCEDI APS Uralsk	No
2014	ASM Biodefense and Emerging Diseases	Washington D.C., USA	IMB	No
2014	The Future of Biosafety and Biosecurity in Central Asia and further countries from the region	Bishkek, KGZ	IMB GIZ	Yes
2014	Kongress für Infektionskrankheiten und Tropenmedizin,	Cologne, DEU	IMB GIZ	No
2015	ASM Biodefense and Emerging Diseases	Washington D.C., USA	IMB	No
2015	National Symposium on Zoonoses Research 2015	Berlin, DEU	IMB	No
2015	Biostudy Tour	Munich, Berlin, DEU	IMB GIZ MoES MoH SPC SEEM	Yes
2016	ASM Biodefense and Emerging Diseases	Washington D.C., USA	IMB	No
2016	Roundtable MRI-Global, DTRA, ASM Biodefense	Washington D.C., USA	IMB	No
2016	National Symposium on Zoonoses Research 2016	Berlin, DEU	IMB KazNMU	No
2016	Congress "Diagnosis and prevention of infectious diseases at the present stage"	Novosibirsk, RUS	IMB KazNMU	No
2016	Actual Problems of epidemiology, microbiology, and natural foci of human diseases	Omsk, KAZ	IMB KazMNU	No
2016	Medical Biodefense Conference	Munich, DEU	IMB GIZ MoH NSCEDI SPC SEEM	Yes (session)
2016	Symposium des deutsch-kasachischen Netzwerkes zur Diagnostik von gefährlichen Infektionskrankheiten	Almaty, KAZ	IMB GIZ MoH KazNMU SPC SEEM NSCEDI SES DTRA CDC	Yes

(Continued)

TABLE 4 | Continued

Year	Name of Conference	Location	Participating partners of the GerKazNet	Co-organisation by the GerKazNet
2016	Kazakh Youth Forum, Presidents Roundtable at Presidents Day - 25 years of Kazakhstan's Independence	Almaty, KAZ	IMB KazNMU	No
2017	ASM Biothreats, Research, Response and Policy	Washington D.C., USA	IMB	No
2017	International Conference "Current Issues on Zoonotic Diseases"	Ulan-Bataar, MNG	KazMNU	No
2017	Scientific Practical Conference	Nur-Sultan, KAZ	IMB GIZ KazNMU	Yes
2017	National Symposium on Zoonoses Research 2017	Berlin, DEU	IMB GIZ KazNMU	No
2017	Meeting of the State Parties to the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction	Geneva, CHE	IMB	No
2018	ASM Biothreats	Washington D.C., USA	IMB	No
2018	The third Baikal International Scientific Conference on vector-borne natural foci infections dedicated to the 100th anniversary of the Irkutsk State Medical University Foundation	Irkutsk, RUS	IMB KazNMU	No
2018	National Symposium on Zoonoses Research 2018	Berlin, DEU	IMB	No
2018	Regional Workshop for Central Asian States Parties to the Biological Weapons Convention on scientific and practical implementation issues	Almaty, KAZ	IMB CRL/NSCEDI	Yes
2018	International Scientific Symposium "Emerging infections: Increasing preparedness by networking"	Berlin, DEU	IMB	No
2018	Medical Biodefense Conference	Munich, DEU	IMB GIZ MoH MoES NSCEDI KazNMU	Yes
2019	EU CBRN Centres of Excellence - Biosafety Association of Central Asia and the Caucasus Conference BACAC: Bridging the Gaps	Tashkent, UZB	IMB GIZ	Yes
2019	International Conference "Current Issues on Zoonotic Diseases"	Ulaanbaatar, MNG	IMB	No
2019	LMU, Center for International Health: Occupational Safety & Health Symposium 2019	Munich, DEU	IMB KazNMU	No
2019	International Scientific Conference "Dangerous infections: new solutions – a look into the future"	Almaty, KAZ	IMB NSCEDI GIZ	Yes
2019	Travelling conferences on actual distribution, modern detection methods and aspects of biosecurity for highly pathogenic zoonotic agents in Central Asia and Mongolia	Almaty, KAZ/ Duschanbe, TJK	IMB NSCEDI 16 APS	Yes

(Continued)

TABLE 4 | Continued

Year	Name of Conference	Location	Participating partners of the GerKazNet	Co-organisation by the GerKazNet
2021	Online Symposium "COVID-19 and other emerging zoonoses"	Online	IMB GIZ NSCEDI RISBP NCB	Yes

IMB, Bundeswehr Institute of Microbiology; KazNMU, Kazakhstan National Medical University; NSCEDI, M.Aikimbayev National Scientific Center for Especially Dangerous Infections, SPC SEEM, Scientific Practical Center for Sanitary Epidemiological Expertise and Monitoring; GIZ, Gesellschaft für Internationale Zusammenarbeit GmbH; APS, Anti Plague Station; MoH, Ministry of Health; MoES, Ministry of Education and Science; CRL, Central Reference Laboratory Almaty; SES, Sanitary Epidemiology Stations; DTRA, Defense Threat Reduction Agency; CDC, Centers of Disease Control and Prevention.

was also highly valued. These events were co-funded by the BMBF and the German Research Platform for Zoonoses and were held four times in four different cities - Dushanbe (Tajikistan), Ulaanbaatar (Mongolia), Munich (Germany), and Almaty (Kazakhstan) - to discuss actual issues of biosafety and biosecurity (21, 22).

SCIENTIFIC OUTPUT OF THE PROJECT

Peer Reviewed Publications

The characterisation of new and emerging diseases caused by highly pathogenic agents or biological warfare agents in Kazakhstan and the publication of these scientific findings in peer-reviewed journals constitutes great publicity for the GerKazNet and also increases the sustainability its achievements. As described above, the GerKazNet employed four postgraduate students to explore so far under-investigated foci for *Tick Borne Encephalitis Virus* (TBEV), *Crimean-Congo haemorrhagic fever virus* (CCHFV), *Orthohantaviruses* (OHV), and *Rickettsia* in Kazakhstan (**Figure 1**).

In order to get an impression of the distribution of these four selected tick- and rodent-borne highly pathogenic agents in natural foci in Kazakhstan, several surveillance studies were conducted. This was done with the aim to prevent infections and to improve countermeasures against the possible natural spread of zoonotic - but also biowarfare - agents always including the aspects of biosafety and biosecurity.

The tick borne encephalitis virus (TBEV) causes a devastating disease in humans and is transmitted *via* the bite of ticks. TBEV cases are described in Kazakhstan. However, it is still a matter of debate as to how this virus migrated to and spreads in Kazakhstan. To determine the prevalence and circulating subtypes of TBEV in the oblasts of Almaty and Kyzylorda, more than 2.300 ticks were screened for the virus. The sequencing of E-genes of the virus revealed that Kazakh TBEV samples belong to two different clades of the Siberian subtype (**Figure 1D**). Three endemic tick species are responsible for the transmission of the virus (18). The results of these investigations were recognised by the Ministry of Health in Nur-Sultan. As a consequence, the ministry also commissioned a survey of new suspected foci of TBEV, e.g., in the Akmola oblast. These investigations are still in progress and are in accordance with the goals of the GerKazNet.

A second study on vectors focused on the prevalence of *Rickettsia* species in the above-mentioned ticks in two pilot regions in Kazakhstan. More than 2.300 ticks were screened by RT-PCR for DNA of spotted-fever group *Rickettsia* (17). This screen reported the prevalence of four Rickettsia species: *R. raoultii, R. slovaca, R. yenbekshikazahkensis,* and *R. talgarensis*. In the course of this, two new Rickettsia genotypes *R. yenbekshikazahkensis* and *R. talgarensis* were found that were new to taxonomists. This study also confirmed *Rickettsia*-positive ticks in the endemic region of Kyzylorda and highlights the prevalence of rickettsiae in the non-endemic area of Almaty (**Figure 1A**). In the last 25 years, over 4,000 persons suffered from rickettsioses in Kazakhstan, but no cases are reported from the region of Almaty (23). Furthermore, this new insight into the spread of *Rickettsia* in ticks highlights how incomplete the knowledge of the spread of *Rickettsia* in Kazakhstan currently is, and that there is a demand for a closely monitoring for *Rickettsia,* not only in Kazakhstan but the entire Central-Asian region.

Small mammals such as rodents, insectivores and bats are the reservoir for *Orthohantaviruses*. There is only limited data available from *Orthohantaviruses* in small mammals from Uralsk (**Figure 1C**). A still ongoing surveillance study in the GerKazNet is the investigation of wild rodents, such as voles, mice and rats, as vector or reservoir for *Orthohantaviruses* and also *Rickettsia*. The aim of this study is to investigate the *Orthohantavirus* diversity in rodents in three areas: in West Kazakhstan, Almaty Oblast and Almaty city. This study will - for the first time - reveal the occurrence and prevalence of *Orthohantaviruses* in wild living rodents.

Small mammals were also found to harbour rickettsial DNA in ear tissue. The analysis of a broad species panel of small mammals will show which *Rickettsia* occur in the area of western and south-eastern Kazakhstan. These studies also help to further coordinate attempts of timely anti-epidemic measures in Kazakhstan.

In addition to these surveillance studies in vectors and reservoirs, several studies in human patients were conducted. The first of these took place in 2014–15 on patients with fever of unknown origin in 13 hospitals in Almaty and Kyzylorda oblast. Fevers of unknown origin (FUO) are frequently occurring in low and middle income countries and are often a result of incomplete diagnostic setups (19, 24).

CCHFV causes severe forms of haemorrhagic fevers in humans. First documents of CCHFV exist since 1948 in

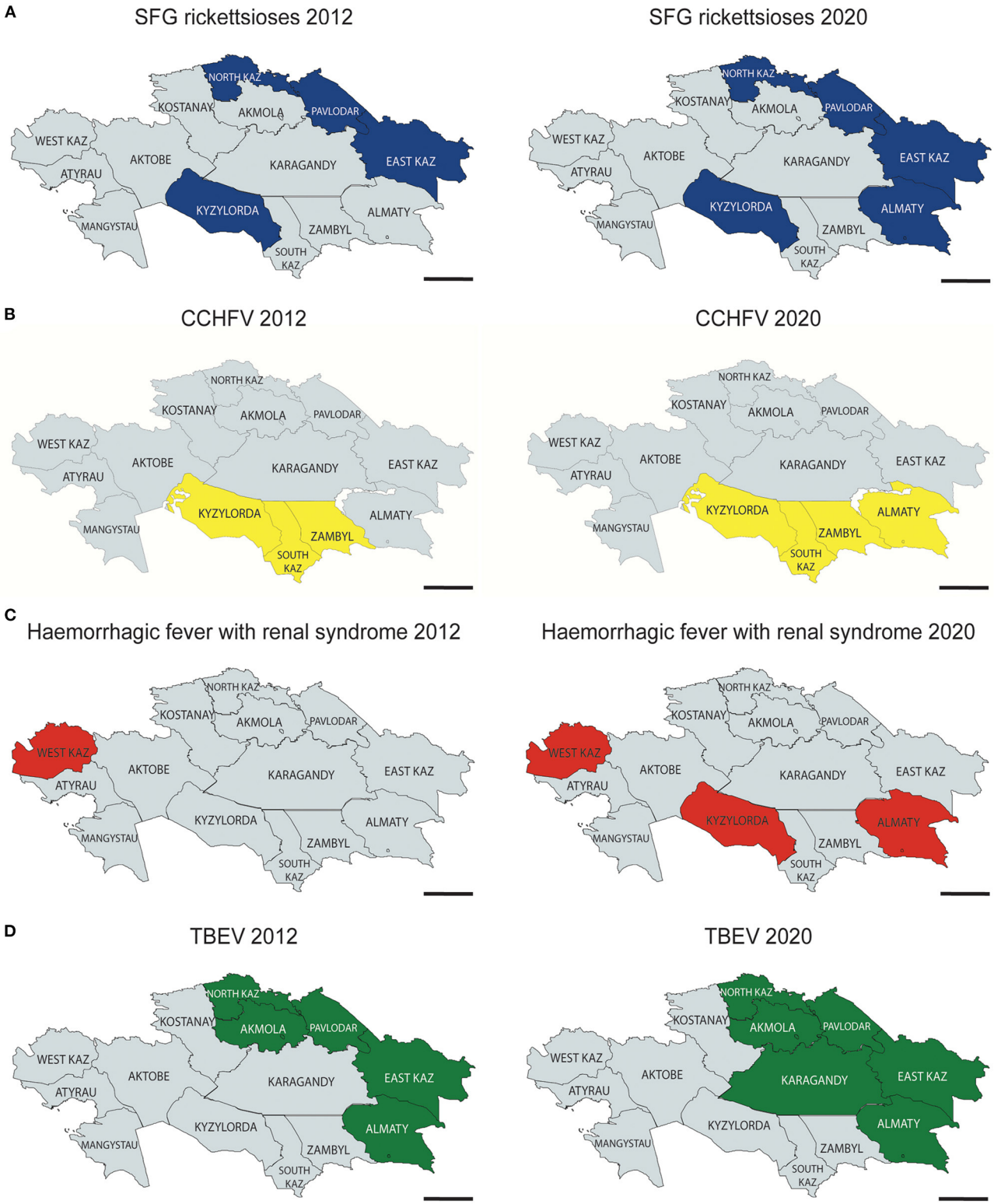

FIGURE 1 | Geographical overview of Kazakhstan and pathogens known to be endemic. Maps summarising the distribution of endemic areas for extremely dangerous pathogens (EDP) in Kazakhstan as known by 2012 (left panel) and 2020 (right panel). Pathogens investigated in the project were Rickettsia (Spotted Fever Group, SFG) **(A)**, *Crimean-Congo Haemorrhagic Fever Virus* (CCHFV) **(B)**, *Orthohantavirus* (Haemorrhagic fever with renal syndrome) **(C)**, and *Tick Borne Encephalitis Virus* (TBEV) **(D)**. Scale = 500 km.

Kazakhstan and it had an average lethality rate of 15%. So far, however, the description was limited to Kyzylorda, Zhambyl and South-Kazakhstan oblast (25). To get a contemporary picture of the situation in Kazakhtsan, a study was carried out which compared FUO patients in the non-endemic area Kyzylorda with patients from the endemic area Almaty. This investigation proved that CCHFV is much more distributed than previously assumed (16). About 13% of sera from 802 patients with FUO contained IgG antibodies against CCHFV that gives evidence about a previous CCHFV infection. 0.87% of the patients had an acute infection. Importantly, this rate was also found in the non-endemic area of Almaty (**Figure 1B**). Molecular biological analysis of viral RNA in the serum samples was successful and specified as Asia 1/2 subtypes of CCHFV. The explanation for the expansion of endemic regions for CCHFV to the south-western oblasts of Kazakhstan might rest in the high density of the population in this area and the high migratory potential of the *Hyalomma* ticks that serve as a vector for CCHFV. These ticks migrate on birds and animals in and out of neighbouring endemic areas such as Uzbekistan and Tajikistan. Endemic regions in Kazakhstan recorded 119 cases of CCHF in the years 2000–2013. This number ignores all FUO events in Almaty Oblast. Future FUO patients of the Almaty region will now be systematically screened for CCHFV infections (16).

Furthermore, the sera from FUO patients described above were examined for the presence of *Orthohantavirus* (OHV)-specific antibodies. HFRS caused by *Orthohantavirus* infection is frequently registered in the endemic areas of West Kazakhstan, but may also occur in non-endemic areas in Kazakhstan. A similar conclusion could be drawn from this OHV study. In more than 800 FUO patients sera, about a fifth had antibodies against *Orthohantaviruses* (15). Further, serotyping characterised the genotypes *Puumala orthohantavirus* (PUUV), *Hantaan orthohantavirus* (HTNV) and *Dobrava-Belgrade Orthohantavirus* (DOBV) for the Almaty region, and PUUV and DOBV for the Kyzylorda region – both regions that were previously considered non-endemic for *Orthohantaviruses* (26). Currently, only West Kazakhstan Oblast is declared an endemic area for *Orthohantaviruses* and so only there do practitioners have access to diagnostic tools that enable a proper detection of the viral infection (Wagner and Tukhanova et al., in revision). Doctors and patients in other oblasts do not have access to *Orthohantavirus*-laboratory diagnostics and have to rely on differential diagnosis. Therefore, this demands the building of awareness of this disease among doctors and the public (**Figure 1C**).

Thirdly, the serum samples from the FUO patients from Almaty and Kyzylorda region were examined for the occurrence and prevalence of spotted-fever group and typhus group *Rickettsia* antibodies (27). So far, Rickettsioses are not on the routine check lists for patients with FUO in Kazakhstan. The data reveal that about 30% of all tested sera of patients showed antibodies against spotted fever group and typhus group *rickettsiae*. This is the first time that past and acute rickettsia infections were diagnosed in Kazakh residents and it highlights the need to further investigate the distribution of *Rickettsia* in Kazakhstan.

In the next phase of the project, two studies on acute infections with some of the agents were initiated. To visualise TBEV infections of humans with tick bites, a study in hospitals in Almaty and Kyzylorda will be performed. Sera and spinal fluid of patients developing a meningitis or meningoencephalitis will be screened for TBEV antibodies. This project will help to establish a reliable TBEV diagnostic in Kazakh laboratories by implementing further, more modern serological and molecular biological assays.

A collection of samples for the examination of *Orthohantaviruses* with regard to their role concerning infections in acute patients was performed in eight hospitals in 2018 and 2019. Included in the study were patients presenting with either fever and/or abdominal pain and/or feeling seriously ill and/or with renal insufficiency (19). The results of this study will be obtained in the following phase of the project.

Textbooks and Monographies

Besides making possible basic science investigations, the German-Kazakh Network also has seen to the realisation of a textbook by Alim Masgutovich Aikimbayev, MD (28). In this textbook titled "The biological safety system in Kazakhstan," the Almaty based scholar summarises the current knowledge on endemic infectious diseases in Kazakhstan, possible medical, ecological and socio-economic consequences of this diseases and gives an in-depth insight into the Kazakh biosafety and biosecurity landscape. This book was published in two languages, Russian and English, to bring the information to a broad national and international audience.

THE GERKAZNET – A SUCCESS STORY?

Since 2013, in its eight years of existence, the GerKazNet was able to set a prominent mark in the biosafety and biosecurity landscape of Kazakhstan. Constant efforts in raising awareness on topics regarding biosecurity, biosafety, dual-use and EDPs established a lasting expertise at Kazakh public health institutions. It was possible to translate this expertise into a notable scientific output. In at least five publications and several more to come, many white spots of pathogen diversity in Kazakhstan were erased. Surveillance studies were initiated and carried out to investigate tick-borne encephalitis, haemorrhagic fevers and rickettsioses in natural foci. Furthermore, patients with FUO were examined and the causative agents of their illness was identified in many cases. The studies on acute patients were important for the establishment of modern techniques for the diagnostics of the respective disease. Regular reporting of the scientific insights to policy makers kept the issue of bio surveillance on the top of the political agenda. This may have a direct impact on the treatment quality of infected patients and increase the life quality of the local population in the long term. The results from our investigations also highlighted the need for further surveillance studies - supported also by the MoH - to investigate the spread of diseases such as tick-borne encephalitis, haemorrhagic fevers and rickettsioses in natural foci in other areas of Kazakhstan.

The high quality publications were achieved by the excellent cooperation of all the stakeholders of the GerKazNet. In addition to providing state of the art BSL-2 laboratory equipment, a massive effort was put into educating and training staff and scientists on state-of-the-art diagnostic procedures and new means of pathogen detection methods in the context of biosafety and biosecurity. This training campaign was well-received and highly demanded and all participants showed effective gain in knowledge. For all milestones, a strict project monitoring is done by the GIZ in cooperation with the Federal Foreign Office. Internal monitoring is performed by weekly short reports of the PhD students, by presentations at least four times a year at consultant meetings and by annual reports.

Top-class scientific output of the four PhD-graduates, who received excellent training at the CIH-LMU international graduate programme, will also be maintained in the future. Their experience will have a lasting impact on the surveillance, detection, and diagnostic capacities of pathogens and further raise awareness of issues of biosafety and biosecurity in the country. Furthermore, they are now familiar with applying for international scientific funding and the proper handling of the publishing process, which will help the Kazakh scientific community to further thrive and become more visible internationally.

A bilingual approach has been constantly chosen for all of the above mentioned activities. In some cases, when requested, trainings were also conducted in the Kazakh language. The employment of simultaneous translation brought about fruitful discussions across language barriers and so further promoted the shaping of international collaborations.

CHALLENGES IN THE PAST

Operating such a global network of scientists and stakeholders naturally poses several challenges that need to be addressed in order to establish a smooth progress.

Scientists in Kazakhstan are used to cooperate with laboratories from all over the world. In the last 30 years, many collaboration projects were put in place with either Russia or the United States of America. The US American way of collaboration focussed on the development of infrastructure (such as the construction of the BSL-3 laboratory in Almaty in 2015) and large-scale training (6). In comparison, the German scientific interaction focuses on the personal development of human resources. Financial aid is always coupled with the accomplishment of clearly defined goals, such as publications or obtained degrees, and measurable changes in the work routine at the laboratories according to biosafety and biosecurity principles.

Besides the challenges of establishing cross-border joint scientific projects, the GerKazNet also made a great effort to make the best out of the intercultural challenges. With the employment of key management personnel that understands both mentalities, an efficient communication was established. Designated consultants at each partner institute maintained an efficient and direct communication. Over the years, the network grew considerably and reliable communication was the basis to build trust. This communication was eminently professionalised by the involvement of the "Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ) GmbH" and its country office in Almaty in the GerKazNet. The GIZ staff operates a well-established network and is familiar with Kazakh routines and traditions. Thus, a local project coordinator ensured clear, effective and coordinated communication that is critical to the successful implementation of the project. As a result, the GerKazNet has built and maintained good rapport with all target groups, including its strategic partners in Kazakhstan such as the scientific institutions, but also the involved Kazakh ministries, the German embassy and the German General Consulate, the project consultants and the project team.

Communication was also the key to lead the organised events to success. All the joint conferences, workshops, field trips, trainings and participations in events of the partner institutions were only possible through regular concertation on ideas and needs. Hence, due to the nature of work at the partner institutions, all events organised by the programme needed to be coordinated much in advance.

It is also worth mentioning that the scientific results published in the programme's framework also started a political debate. Identifying new areas of highly infectious pathogens naturally triggers a reaction of the local authorities (**Figure 1**). Before Kazakh regional medical persons in charge commissioned any changes in their surveillance policy, they intensely scrutinised the publications of the GerKazNet. This step was very legitimate, since changes in healthcare policy are cost intensive. However, since all the published results reached high levels in quality and since all the necessary controls and a gapless documentation of all the cases existed, policymakers trusted the results of the project and initiated appropriate steps. This acceptance even led to the request by the Ministry of Health to, e.g., further, investigate the distribution of TBEV in the area of Pavlodar.

Despite all the efforts to maintain a transparent bilingual communication, the language barrier remained a constant challenge. In general, the English language is taught at schools. Still, not every young Kazakh citizen speaks or understands English, let alone persons who received their education in the former USSR. It was utterly important for the project that the recruited PhD students for the International PhD programme were fluent in English as this was a prerequisite to attend the classes. In addition, scientists in advanced positions often were not fluent in English. This was especially challenging when it came to summarise research results in order to publish them in international peer-reviewed journals. For joint meetings, the network maintained the service of real time translation between German and Russian by an interpreter to avoid potential pitfalls and misunderstandings. A thorough knowledge management in the form of clear communication and documentation of all decisions reached jointly was also important, since there was a frequent fluctuation of personnel in the German and the Kazakh delegation. Clearly defined project goals and written roadmaps helped new members of the team to quickly grasp the concept and proceed with the project.

Nevertheless, the efficient management of scientific analysis was very extensive since there are strict Kazakh governmental

regulations on the transport, export and import of biological specimens. According to Kazakh law, biological samples generated in Kazakhstan are not allowed to leave the country. This drawback actually turned in a gain for the project: since it promoted the exchange of methods and diagnostic expertise between Kazakhstan and Germany, the motivation of local scientists was increased, since they were able to completely conduct the pipeline of research in their home institution from collecting the specimens, to isolation and analysis of the results. However, it was not possible to use the equipment of the IMB as a backup reference laboratory in cases of non-specific diagnostic results obtained in a Kazakh laboratory. Transportation of supply material from Germany to Kazakhstan was solved by the full support of the ISTC gained by the new partnership.

OUTLOOK

Over the last 8 years, the GerKazNet, run by the German Biosecurity Programme as part of the G7 Global Partnership group, established new means of scientific and personal interaction in both Kazakhstan and Germany, to promote issues of biosafety and biosecurity. The close cooperation has led to a synergy effect. While learning to trust each other's capabilities and recognising each other as equivalent partners, it was possible to gain new scientific insights on the spread of highly infectious diseases. By implementing the capacity development approach by the German Biosecurity Programme, international safety standards on biosafety and biosecurity were adopted in all the partner institutes and risk identification and risk management were put to a contemporary level. Furthermore, by fully exploiting its toolbox, the German Biosecurity Programme contributed to the reinforcement and strengthening of biological non-proliferation principles and practices, and to the reduction of the risk of proliferation through the advancement and promotion of safe and responsible conduct in the biological sciences.

The unanticipated outbreak of SARS-CoV-2 starting in early 2020 brought topics such as biosafety and biosecurity into the minds of the general public. In his speech to the 75th General Debate of the UN General Assembly in September 2020, the president of Kazakhstan, Kassym-Jomart Tokayev, proposed the establishment of a biological weapons control system in light of the global COVID-19 pandemic. The proposition entailed the establishment of a special multilateral body – the International Agency for Biological Safety – based on the 1972 Biological Weapons Convention and accountable to the UN Security Council. Furthermore, he suggested to closely examine the idea of a network of Regional Centers for Disease Control and Biosafety under the UN auspices and expressed Kazakhstan's readiness to host such a regional center (29). This initiative reflects Kazakhstan's commitment to the field of biosecurity and may give new momentum to the stalled negotiations on the implementation of the BWC goals. Furthermore, it would sustain and accelerate the knowledge of biosafety, biosecurity and dual-use that was initiated by the GerKazNet among others.

Eight years of GerKazNet brought together scientists and other stakeholders from all over the country. Scientists from different institutions became partners and ultimately trusted friends and started projects outside of the GerKazNet. This establishment of a stable network nourishes the hope that a close interaction between the scientists and institutes will have a long lasting impact on the Kazakh scientific landscape stretching far beyond the third project phase in 2022.

AUTHOR CONTRIBUTIONS

LP, EW, and SE conceived the layout of the project. LP and EW wrote the manuscript. EW and LP created the figures and tables. AS, NTuk, NTur, KA, OS, YS, ET, AD, AZ, and SF contributed additional information and reviewed the manuscript. SE and LP supervised the project and were in charge of the revision process. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: Hyperinflammatory Status in an Immunocompromised Child With a Highly Sustained Viral Load of SARS-CoV-2

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Coronavirus disease 2019 (COVID-19) is spreading throughout the world. Limited data are available for the dynamics of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral load (VL) in immunocompromised pediatric patients. Here, we report the clinical characteristics and the dynamics of SARS-CoV-2 VL of a pediatric patient with acute myeloid leukemia who developed a hyperinflammatory status mimicked MIS-C. The clinical course was characterized by the late onset of fever, GI symptoms, rash, and respiratory distress, including oxygen requirement with sustained VL of SARS-CoV-2 around 7 $\log_{10}$ RNA copies/mL for 6 weeks. It is important to note that the hyperinflammatory status developed early at the third week of hospitalization—in a context of high VL and immunocompromised status. All these characteristics make this clinical case unique. On the other hand, while many reports have characterized the dynamics of SARS-CoV-2 VL in adults and immunocompetent hosts, it remains unreported in pediatrics—even less in immunosuppressed children.

Keywords: SARS-CoV-2, COVID-19, viral load, immunocompromised state, hyperinflammatory status, pediatrics

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INTRODUCTION

In April 2020, serious cases of children with systemic hyperinflammatory status with a temporary association with SARS-CoV-2 infection were reported in the United Kingdom (UK) (1). Since then, pediatric cases with a similar condition have been reported in Europe, South Africa, and America (2–4). This entity identified as multisystem inflammatory syndrome in children (MIS-C) has a spectrum of manifestations similar to Kawasaki disease, toxic shock syndrome, sepsis, and macrophage activation syndrome. The World Health Organization (WHO), the Center for Disease Prevention and Control (CDC), and the Royal College of Pediatrics and Child Health (RCPCH) in the UK issued definitions for case identification (5–7). Although MIS-Cs were reported in

immunocompetent patients, so far there are no records in the literature about this condition in immunosuppressed children.

Here, we report a case of an immunosuppressed child who developed a hyperinflammatory status temporally associated with SARS-CoV-2 infection in the presence of sustained high viral load (VL).

CASE

A 3-year-old male patient diagnosed with M7 acute myeloid leukemia in January 2020 in Paraguay was admitted to the Hospital de Pediatria Garrahan (Buenos Aires, Argentina) in February 2020 for treatment and follow-up. By the end of July, the child had completed the first and second intensification block of chemotherapy with cytarabine and etoposide to keep the patient in complete remission, awaiting a bone marrow transplant. On August 4th, due to a close contact with a person with COVID-19, the child and his mother were tested. A reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay for SARS-CoV-2 in nasopharyngeal swab specimen was performed with detectable results in both cases. The child was asymptomatic for COVID-19, but he was immediately hospitalized for isolation following national health policies at that time.

The child remained asymptomatic for 28 days, when beginning with febrile neutropenia. Peripheral blood and urine cultures were negative for bacterial and fungal infection. Galactomannan antigen tests in serum were negative. Other respiratory viruses including influenza A and B, adenovirus, parainfluenza 1, 2, 3, and 4, respiratory syncytial virus, metapneumovirus, rhinovirus, enterovirus, and pan-coronavirus (NL63, 229E, OC43, and HKU1) were also negative by molecular tests. He started broad-spectrum antibiotic treatment with piperacillin tazobactam associated with amikacin. On day 31, the child started coughing and had dysphonia. Twenty-four hours later, he started getting abdominal pain, diarrhea, and a maculopapular rash affecting the face and high upper thoracic area without compromised hand and feet. Laboratory tests showed that pancytopenia (white blood cells 100/mm^3, hemoglobin 6.2 g/dl, platelets 8,000/mm^3), fibrinogen 526 mg/dl, ferritin 4489.3 ng/ml, CPK (creatine phosphokinase) 14 UI/l, NT-PROBNP (N-terminal portion of pro-B-type natriuretic peptide) 226 pg/ml, C-reactive protein 136.14 mg/l, procalcitonin 1.22 ng/ml, normal hepatogram, and anti SARS-CoV-2 antibodies were negative. Detailed laboratory markers are shown in **Table 1**. No evidence of coagulopathy was shown. Myocardial dysfunction, pericarditis, valvulitis, or coronary abnormalities were discarded by echocardiography. In the presence of high inflammatory laboratory markers, acute SARS-CoV-2 severe infection or MIS-C was suspected. Gamma globulin treatment with a dose of 2 g/kg was immediately administered.

On day 33, febrile neutropenia persisted, and the child started bronchospasms and hypoxemia requiring oxygen therapy by nasal cannula. Blood cultures were performed, and antibiotics were switched to meropenem, vancomycin, and lipid formulation of amphotericin B. A chest CT scan showed (i) patched areas of airspace occupation with confluent sectors and ground glass opacity in both upper lobes and (ii) extensive areas of consolidation with bronchogram in both lower lobes. A second echocardiogram showed no abnormal findings. On day 34, the patient remained feverish with diarrhea and abdominal pain and started with hypoxemia and vomits.

At day 35, the patient had a normal neutrophil count but remained lymphopenic. Due to worsening rash and respiratory symptoms, the child required a non-rebreathing mask and was admitted to the pediatric intensive unit (PICU). During the PICU stay, the patient did not require inotropic drugs or mechanical respiratory support. A 10-day treatment with dexamethasone 0.6 mg/kg per day was started, and convalescent plasma at a dose of 5 ml/kg was administered with good tolerance. The administration of convalescent plasma was in the context of institutional approved protocol. On day 38, the child was without fever and decreased oxygen requirements, going from PICU to an intermediate care unit. At day 53, the child was discharged with normalized laboratory markers, except for lymphopenia and high ferritin (**Table 1**).

SARS-CoV-2 VL monitoring was performed in nasopharyngeal swabs using an *in-house* quantitative RT-PCR, which was validated according to the guidelines proposed by Burd et al. (8) and Bustin et al. (9). It is important to mention that our assay included the measure of a housekeeping gene (RNase P) cycle threshold (Ct) to correct the specific-SARS-CoV-2 Ct—before its extrapolation from the standard curve—according to the number of cells in the samples and as recommended by Han et al. (10). The dynamic of SARS-CoV-2 VL was reconstructed in six samples along the 64 days of hospitalization (**Figure 1**). In summary, the child had a VL of 8.34 $\log_{10}$ copies per milliliter on the first day of symptom onset and remained above 8 $\log_{10}$ copies per milliliter and close to 7 $\log_{10}$ copies per milliliter until days 29 and 42 from admission, respectively. Then, VL decreased, reaching a value of 3.29 $\log_{10}$ copies per milliliter at day 49, and remained detectable but below the limit of quantification at day 64.

During the whole hospitalization, the patient did not show signs of hemodynamic instability or shock. After 3 months from hospital discharge, the patient received unrelated hematopoietic stem cells transplantation with normal clinical and laboratory markers. At present (June 2021), 6 months after transplantation, the child was in remission of his underlying disease with good clinical evolution.

The study was approved by the Institutional Review Board, and written informed consent was obtained from the parents.

DISCUSSION

Recently, a case report showed persistent infection of SARS-CoV-2 in an immunocompromised adult with confirmed infectious virus in nasopharyngeal samples from days 75 and 143 (11). Our case adds new evidence about persistently high VL of SARS-CoV-2 in an immunocompromised child, with diagnosis of acute severe infection of SARS-CoV-2 with hyperinflammatory status mimicking MIS-C. In acute severe infection and MIS-C, common features are gastrointestinal

TABLE 1 | Laboratory characteristics along SARS-CoV-2 infection.

	Days post-infection								
Laboratory markers	**28**	**31**	**32**	**33**	**34**	**35**	**38**	**49**	**64**
Absolute neutrophil count ($\times 10^9$/l)	0	0	0	0	0	1,107	750	4,515	1,496
Absolute lymphocyte count ($\times 10^9$/l)	0	0	0	0	0	305	64	531	528
Platelet count ($\times 10^9$/l)	47,000	8,000	37,000	18,000	29,000	27,000	23,000	112,000	136,000
C-reactive protein (mg/l)	-	136.14	152.09	181.51	250.05	223.45	77.52	1.38	2.36
VSG (mm/h)	-	104	93	85	110	115	78	10	25
Ferritin (ng/ml)	-	4,489.30	-	-	16,955.38	23,899.44	16,394.78	5,351.16	3430.68
Procalcitonin (ng/ml)	-	0.63	0.77	-	1.22	0.60	0.16	-	-
PT (%)/PTT (s)	-	-	68/35	81/38	79/46	78/49	74/49	97/25	94/33
Fibrinogen (mg/dl)	-	526	571	513	530	486	447	158	274
Troponin (ng/ml)	-	5.0	5.0	-	6.8	6.8	3.5	-	8.6
ProBNP (pg/ml)	-	226	1,893	1,098	835	512	1,088	135	8.4
Urea (mg/dl)	-	13	18	17	10	14	16	39	20
Creatinine (mg/dl)	-	0.37	0.37	0.34	0.36	0.34	0.30	0.33	0.34
AST/ALT (IU/ml)	-	13/10	18/8	19/9	29/13	43/25	26/22	31/74	22/27
Albumin (g/dl)	-	3.64	-	-	2.70	3.10	3.16	4.10	-
Triglycerides (mg/dl)	-	54	67	-	105	99	-	88	61
CPK (IU/l)	-	-	19	-	14	13	-	-	-

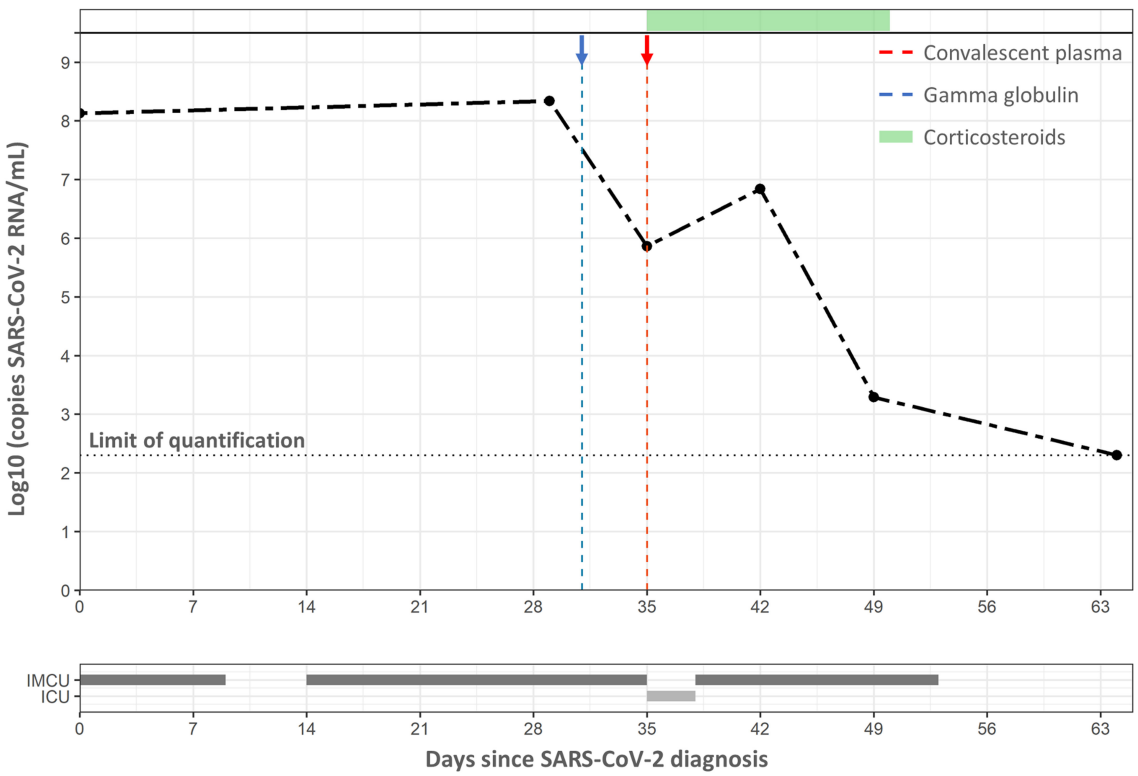

FIGURE 1 | Dynamic of SARS-CoV-2 since admission. Dashed lines in gray, blue, and red represent the limit of quantification value, gamma globulin treatment, and convalescent plasma treatment, respectively, whereas the green bar represents the days under corticosteroids therapy. The bars below the VL curve show the period in which the child stayed in the intermediate care unit (IMCU) and intensive care unit (ICU).

manifestations (abdominal pain, diarrhea), cutaneous signs (rash), inflammatory markers, and lymphopenia. However, children with severe COVID-19 mimicking MIS-C present more frequently with respiratory symptoms (e.g., cough, respiratory distress) while gastrointestinal symptoms are less common. Also, comorbidities (e.g., malignancy, chronic lung diseases, neurological disorders) are frequently associated with severe forms of disease as in the case reporting here (12). On the other hand, the clinical presentation of the inflammatory status in this patient appeared at the same time that MIS-C were usually diagnosed (3rd/4th week since SARS-CoV-2 diagnosis) (5–7). The majority of MIS cases—in children and adults—were diagnosed with negative SARS-CoV-2 RT-PCR, and detectable antibodies. It is important to highlight that in our case, the specific SARS-CoV-2 antibodies were negative, but it is an immunocompromised host (13). Up to our knowledge, this is the first immunocompromised child, with persistent high VL who developed a hyperinflammatory status mimicked MIS-C. Further studies are needed to unravel the host–virus interaction that led to hyperinflammatory status in an immunocompromised host.

DATA AVAILABILITY STATEMENT

There are no restrictions to apply to the data generated in this article. Requests to access the datasets should be directed to andreammangano@gmail.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité Revisor y de Ética en la Investigación, Hospital de Pediatría Garrahan. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AM, DB, and MM conceived the study. SG, MP, DB, SR, LF, AB, and RB collected and analyzed the clinical data. MM, MF, and MG developed and performed the VL of SARS-CoV-2. MM, MF, MG, DB, and AM drafted the manuscript. All authors contributed and approved the final manuscript.

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SYSTEMATIC REVIEW
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Chronic Diseases as a Predictor for Severity and Mortality of COVID-19: A Systematic Review With Cumulative Meta-Analysis

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Introduction: Given the ongoing coronavirus disease 2019 (COVID-19) pandemic and the consequent global healthcare crisis, there is an urgent need to better understand risk factors for symptom deterioration and mortality among patients with COVID-19. This systematic review aimed to meet the need by determining the predictive value of chronic diseases for COVID-19 severity and mortality.

Methods: We searched PubMed, Embase, Web of Science, and Cumulative Index to Nursing and Allied Health Complete to identify studies published between December 1, 2019, and December 31, 2020. Two hundred and seventeen observational studies from 26 countries involving 624,986 patients were included. We assessed the risk of bias of the included studies and performed a cumulative meta-analysis.

Results: We found that among COVID-19 patients, hypertension was a very common condition and was associated with higher severity, intensive care unit (ICU) admission, acute respiratory distress syndrome, and mortality. Chronic obstructive pulmonary disease was the strongest predictor for COVID-19 severity, admission to ICU, and mortality, while asthma was associated with a reduced risk of COVID-19 mortality. Patients with obesity were at a higher risk of experiencing severe symptoms of COVID-19 rather than mortality. Patients with cerebrovascular disease, chronic liver disease, chronic renal disease, or cancer were more likely to become severe COVID-19 cases and had a greater probability of mortality.

Conclusions: COVID-19 patients with chronic diseases were more likely to experience severe symptoms and ICU admission and faced a higher risk of mortality. Aggressive strategies to combat the COVID-19 pandemic should target patients with chronic diseases as a priority.

Keywords: chronic diseases, COVID-19, systematic review, cumulative meta-analysis, severity, mortality

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The COVID-19 outbreak was declared as a public health emergency of international concern by the World Health Organization (WHO) on January 30, 2020 (1). Since then, the disease has been spreading quickly around the world, reaching 9.296 million cases and 479,133 deaths as of June 25, 2020 (2). The ongoing COVID-19 pandemic has led to a rapidly growing demand for healthcare facilities and healthcare workers, leaving healthcare systems in many countries overstretched and unable to perform effectively (3).

The COVID-19 symptoms range from very mild to severe problems. While it was reported that the majority of COVID-19 cases were mild and required limited treatment (4), those patients with severe COVID-19 might need hospitalization or intensive care and have worse outcomes, such as death. Identifying risk factors for serious cases and mortality can be helpful in guiding public health interventions for protecting the most vulnerable groups of the population from COVID-19. For example, the risk factor information can be used to design risk stratification tools and clinical pathways, thus establishing more effective early intervention strategies and resource allocation policies.

COVID-19 is a serious global health threat, with more than 99% of confirmed cases currently coming from countries outside China. However, the vast majority of the published review articles relied almost exclusively on the studies conducted in China (5–11). In fact, several published reviews included data from only few countries outside China (12–14). Consequently, the limited information prevented decision-makers and patients from better recognizing the global evidence about risk factors for adverse COVID-19 outcomes. In addition, there is a serious concern about the validity and generalizability of the evidence on risk factors in COVID-19 patients generated by the published review articles, which failed to address the clinical heterogeneity of patients with COVID-19 among the observational studies. For instance, a meta-analysis (11) combined data from intensive care unit (ICU) admission and mortality into a single effect measure to find risk factors for progression of COVID-19, while another meta-analysis (8) pooled data from patients with severe COVID-19 symptoms and those who were admitted to the ICU into one group. Further systematic reviews are needed to address this issue of patient heterogeneity to improve the validity and generalizability of the evidence.

This paper aimed to fill the gap by conducting a systematic review with meta-analysis to determine the predictive value of chronic diseases for the severity and mortality of COVID-19. Our analysis examined global evidence to generate systematic and robust findings. To our knowledge, this study represented the most comprehensive meta-analysis of COVID-19 severity, mortality, ICU admission, and acute respiratory distress syndrome (ARDS). Furthermore, it was the first study to determine the associations between several chronic conditions, including obesity, asthma, and hyperlipidemia, with clinical outcomes of COVID-19 patients. We also included only studies from the peer-reviewed journals to ensure the validity of conclusions, while some meta-analyses used manuscripts in preprint servers to increase the sample size (9, 15–17).

METHODS

Methods for this systematic review were developed according to the recommendations from the MOOSE statement (18) and PRISMA statement (19) for reporting of systematic review and meta-analysis.

Criteria for Considering Studies for This Review

Observational studies that focused on adult patients (aged over 16 years) with COVID-19 and investigated the association between chronic diseases and severity, ICU admission, mortality, and ARDS of COVID-19 were included.

The following types of studies were excluded: (1) studies that only included infants, children, and pregnant women; (2) studies that only included decedents (only death patients were enrolled in each group); (3) studies that did not classify patients into different groups by severity, type of hospital wards (i.e., general wards, ICU), mortality, or ARDS; (4) studies that did not have enough statistical information to be extracted from each group of patients; (5) duplicated publication of the same research results, i.e., data from the same hospitals within the same period; and (6) descriptive reviews, systematic review, meta-analysis, opinion, editorial, comments, and conference abstracts without full article publication.

Study Outcomes

The primary outcome measure was the association between chronic diseases and the severity of COVID-19 patients. Secondary outcomes included the association between chronic diseases and mortality, ICU admission, and ARDS of COVID-19 hospitalized patients. The chronic diseases in our review were hypertension, diabetes, pulmonary disease [chronic obstructive pulmonary disease (COPD), asthma, and unspecified type], cardiovascular disease (coronary heart disease, heart failure, and unspecified type), cerebrovascular disease, hyperlipidemia, obesity, chronic liver disease, chronic renal disease, cerebrovascular disease, and cancer. The association between Charlson comorbidity index and the clinical outcomes of COVID-19 patients was also analyzed.

Search Strategy

Studies were identified by searching PubMed, Embase, Science Citation Index Expanded (Web of Science), and Cumulative Index to Nursing and Allied Health (CINAHL) Complete. Our search strategy is listed in **Appendix 1** in Supplementary Material. References from the retrieved papers were also searched. Studies published between December 1, 2019, and December 31, 2020 were included.

Study Selection and Data Extraction

In accordance with the defined inclusion criteria, two reviewers independently read the title and abstract of each study retrieved by the search. The reviewers excluded studies that did not meet

the inclusion criteria. After screening the title and abstract of each study, the full texts of eligible citations were then assessed by the two reviewers independently.

A third reviewer was consulted when the two reviewers could not agree on selecting a study. The reviewers developed a data extraction form and used it to extract data to reflect the characteristics of each included study. If the data from the same hospitals in the same period were published several times, only the paper with the largest sample size was included.

The included studies varied in their classification of disease severity, ranging from mild, moderate, severe, to critical severe. We categorized mild and moderate cases into the non-severe group and severe and critical severe cases into the severe group. We considered the following cases as the ICU groups—ICU admission and requiring invasive mechanical ventilation, and critical cases of illness that were admitted to the ICU.

Risk of Bias Assessment

We used the tools developed by the Joanna Briggs Institute (JBI) (20–23) to assess the risk of bias of the included studies. The JBI critical appraisal tools for cohort studies, case series, case–control studies, and cross-sectional studies included 11, 10, 10, and 8 items, respectively. The appraisal tools addressed the internal validity and risk of bias of the study design, particularly confounding, selection, and information bias, in addition to the importance of clear reporting.

Statistical Analysis

We conducted a meta-analysis when data from more than one study could be combined. We calculated pooled estimates of odds ratio (OR) and 95% confidence interval (CI) by the generic inverse variance method using STATA 14.2 (STATA Corporation, College Station, TX, USA). We tested the heterogeneity of effective measures using the I^2 statistic. We defined I^2 values greater than 50% as considerable or substantial heterogeneity (24). For data with substantial heterogeneity, a random-effects model using the method developed by DerSimonian and Laird (25) was specified to address heterogeneity among the studies. For data with unsubstantial heterogeneity, a fixed-effects model with the inverse variance method was used to synthesize the data.

For the most prevalent chronic diseases including hypertension, diabetes, COPD, coronary heart disease, cerebrovascular disease, and cancer, we conducted the cumulative meta-analysis according to the season of admission of the patients and the increasing sample size of the included studies within each season. A cumulative meta-analysis is helpful to assess the dynamics of how the summary results change with a newly added study (26). R 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria) was used to conduct a cumulative meta-analysis.

RESULTS

Literature Search and Study Selection

Two hundred and seventeen observational studies (27–243) with 624,986 patients met the inclusion criteria in our systematic review. A PRISMA flowchart summarized our search results and study selection procedure (**Figure 1**).

Characteristics and Quality of the Included Studies

Table 1 presents the characteristics of the 217 included studies. The studies were carried out in 26 countries, 83 (38.25%) of them were performed in multicenters, 123 (56.68%) were case series, and 82 (37.79%) were cohort studies. The date of admission of the patients was from December 11, 2019, to August 1, 2020. Most of the outcome variables were about mortality (48.39%) and severity (34.10%). Details of the characteristics are shown in **Appendix 2** in Supplementary Material.

The quality assessment score for the case series ranged from 6 to 10 (9.13 $\pm$ 0.84) with 102 of them (82.93%) having a score higher than 8 (10 indicates the best quality). Among the case series, 48 studies (39.02%) did not indicate a consecutive inclusion of patients, and 45 studies (36.59%) did not have a complete inclusion of patients.

The score of cohort studies ranged from 6 to 11 (10.04 $\pm$ 0.92) with 62 cohort studies (75.61%) having a score more than 9 (11 indicates the best quality). Length of follow-up was not mentioned in 34 cohort studies, and the reasons for losses to follow-up were not described in 6 studies. In addition, we were not sure whether patients across different centers had similar characteristics in relation to exposure (27 studies). **Appendix 3** in Supplementary Material presents details of the risk of bias assessment.

Association Between Chronic Diseases and Severity of COVID-19

We identified 74 studies of COVID-19 severity, involving a total of 32,213 patients and 8,433 cases of severe COVID-19. Among these studies, 65 of them were performed in China, 3 in the USA, and 1 in Bulgaria, Congo, Kuwait, Saudi Arabia, South Korea, and Spain, respectively.

As shown in **Table 2**, the prevalence of patients with any type of chronic disease in the group of severe COVID-19 was substantially higher than that of the non-severe group (60.71 vs. 31.81%; OR 3.70, 95% CI 2.98–4.61). Hypertension (OR 3.05, 95% 2.60–3.59), diabetes (OR 2.55, 95% CI 2.14–3.03), COPD (OR 3.91, 95% CI 3.05–5.02), asthma (OR 1.93, 95% CI 1.53–2.42), unspecified type of pulmonary disease (OR 2.48, 95% CI 2.03–3.03), coronary heart disease (OR 2.04, 95% CI 1.72–2.42), unspecified type of cardiovascular disease (OR 3.01, 95% CI 2.64–3.43), cerebrovascular disease (OR 2.32, 95% CI 1.83–2.94), obesity (OR 2.63, 95% CI 1.70–4.07), chronic liver disease (OR 1.96, 95% CI 1.64–2.35), chronic renal disease (OR 2.09, 95% 1.52–2.87), and cancer (OR 2.33, 95% 1.90–2.87) were all associated with significantly higher risk of severity among COVID-19 patients. There were only two types of the study chronic diseases (i.e., heart failure and hyperlipidemia) that were not significantly associated with the severity of COVID-19 ($P >$ 0.05). However, the prevalence of heart failure or hyperlipidemia was only reported in three studies.

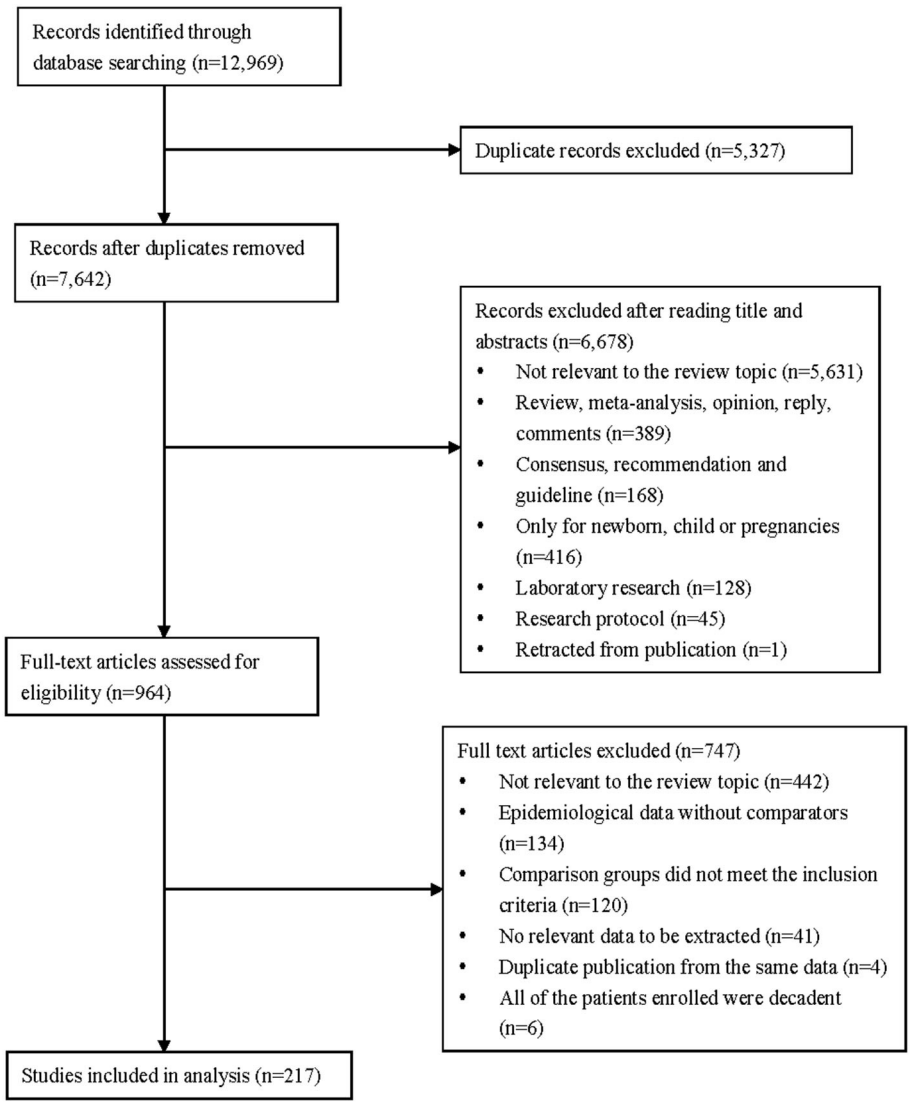

FIGURE 1 | Flow diagram of the study selection for inclusion in the systematic review.

Appendix 4 in Supplementary Material presents the forest plots of cumulative meta-analysis for major types of chronic diseases. Subsequent studies increased the precision of the point estimate, and no change occurred in the direction of the effect size.

Association Between Chronic Diseases and Mortality of COVID-19

We found that 105 studies with a total of 350,522 patients and 68,157 deaths presented data on mortality. Among the studies, 40 of them were carried out in China, 12 in Italy, 11 in the USA, 6 in Iran, 6 in Spain, 4 in India, 4 in the UK, 3 in Brazil, 3 in South Korea, 2 in France, 2 in Mexico, and 1 in Bangladesh, Bolivia, Congo, German, Greece, Ireland, Kuwait, Saudi Arabia, Switzerland, and Turkey, respectively. We also found two studies that were conducted in multicountries.

As shown in **Table 3**, 36.49% of patients who died had at least one type of chronic disease. Hyperlipidemia (52.80%) was the most common chronic condition among patients who died, followed by hypertension (37.53%) and unspecified type of cardiovascular disease (28.56%). Hypertension (OR 2.31, 95% CI 2.04–2.61), diabetes (OR 1.99, 95% CI 1.82–2.18), COPD (OR 2.95, 95% CI 2.48–3.50), unspecified type of pulmonary disease (OR 2.05, 95% CI 1.83–2.31), coronary heart disease (OR 2.46, 95% CI 2.14–2.82), heart failure (OR 2.74, 95% CI 2.21–3.40), unspecified type of cardiovascular disease (OR 2.59, 95% CI 2.24–3.00), cerebrovascular disease (OR 2.46, 95% CI 2.08–2.91), hyperlipidemia (OR 1.72, 95% CI 1.07–2.77), chronic liver disease (OR 1.52, 95% CI 1.30–1.77), chronic

TABLE 1 | Characteristics of the included studies.

Characteristics	Number of studies	Percentage of the included studies (%)
Country		
China	111	51.15
USA	21	9.68
Italy	20	9.22
South Korea	7	3.23
Spain	7	3.23
Iran	6	2.76
Germany	5	2.30
France	5	2.30
Mexico	5	2.30
India	4	1.84
UK	4	1.84
Brazil	3	1.38
Congo	2	0.92
Kuwait	2	0.92
Saudi Arabia	2	0.92
Bangladesh	1	0.46
Bolivia	1	0.46
Bulgaria	1	0.46
Denmark	1	0.46
Greece	1	0.46
Ireland	1	0.46
Oman	1	0.46
Poland	1	0.46
Qatar	1	0.46
Switzerland	1	0.46
Turkey	1	0.46
Multicountries[a]	2	0.92
Data source		
Multicenters	83	38.25
Single center	134	61.75
Type of study		
Case series	123	56.68
Retrospective cohort study	71	32.72
Prospective cohort study	10	4.61
Ambispective cohort study	1	0.46
Case–control study	4	1.84
Cross-sectional study	8	3.69
Type of comparisons[b]		
Severe vs. non-severe	74	34.10
Death vs. survival	105	48.39
ICU vs. non-ICU	53	24.42
ARDS vs. non-ARDS	6	2.76

[a] *One was carried out in Europe, and the other was conducted in China, Europe, and North America.*
[b] *Seventeen studies had two types of comparisons. Two studies had three types of comparisons.*

renal disease (OR 2.85, 95% CI 2.44–3.33), and cancer (OR 2.11, 95% CI 1.85–2.42) were associated with a higher risk of mortality.

We found no significant correlation between obesity (OR 1.19, 95% CI 0.94–1.51) and death. We also did the subgroup analysis for morbid obesity (BMI $\geq$ 40 kg/m^2), and the results were not statistically significant (OR 0.98, 95% CI 0.80–1.20). Our meta-analysis showed that asthma was associated with a reduced risk of mortality (OR 0.74, 95% CI 0.68–0.80). The Charlson index score equals to 0 seemed to be a protective factor for mortality (OR 0.31, 95% CI 0.18–0.51), while a score $\geq$2 might be consistent with the higher likelihood of death (OR 4.22, 95% CI 2.56–6.96).

The cumulative meta-analysis showed that the sample size increased; the CI for hypertension, diabetes, COPD, coronary heart disease, and cancer became increasingly narrower; and statistical significance was more common (**Appendix 4** in Supplementary Material). However, the subgroup of cerebrovascular disease with the admission date of patients in summer was not significant ($P > 0.05$), probably due to only two studies were included.

Association Between Chronic Diseases and ICU Admission of COVID-19

Fifty-three studies involving a total of 260,465 patients and 12,233 cases of ICU admission were included. Of these studies, 14 were conducted in China; 9 in the USA; 6 in Italy; 4 in South Korea; 3 in France, Germany, and Mexico, respectively; 2 in Kuwait; and 1 in Denmark, India, Iran, Oman, Poland, Qatar, Saudi Arabia, Spain, and Turkey, respectively.

We found that 73.62% of the ICU patients had at least one type of chronic disease, which was significantly higher than that in the non-ICU group (OR 2.82, 95% CI 2.23–3.56) (**Table 4**). Hypertension (OR 2.24, 95% CI 1.90–2.63), diabetes (OR 2.50, 95% CI 2.18–2.87), COPD (OR 2.76, 95% CI 1.99–3.82), unspecified type of pulmonary disease (OR 1.40, 95% CI 1.26–1.56), coronary heart disease (OR 2.16, 95% CI 1.56–2.99), heart failure (OR 1.80, 95% CI 1.44–2.55), unspecified type of cardiovascular disease (OR 2.38, 95% CI 1.92–2.96), hyperlipidemia (OR 1.53, 95% CI 1.22–1.93), obesity (OR 1.86, 95% CI 1.49–2.31), chronic renal disease (OR 2.25, 95% CI 1.73–2.94), and cancer (OR 1.57, 95% CI 1.39–1.77) were significant predictive factors for admission to ICU. On the other hand, asthma and chronic liver disease were not significantly associated with ICU admission ($P > 0.05$). The association between cerebrovascular disease and ICU admission was not very obvious ($P = 0.048$).

The cumulative meta-analysis showed that the statistical significance of hypertension, diabetes, COPD, coronary heart disease, and cancer had the tendency of becoming evident with increasing sample size (**Appendix 4** in Supplementary Material).

Association Between Chronic Diseases and ARDS of COVID-19

Six studies involving a total of 2,128 patients and 635 cases of ARDS admission were included. Two of them were conducted in China, two in Italy, and 1 in Germany and the USA, respectively.

We found a significant association between hypertension (OR 2.17, 95% CI 1.78–2.66), diabetes (OR 2.32, 95% CI 1.70–3.17), coronary heart disease (OR 1.96, 95% CI 1.32–2.92),

TABLE 2 | Associations between chronic diseases and severity of COVID-19.

Chronic diseases	Number of included studies	Number of severe COVID-19 patients	Number of non-severe COVID-19 patients	Prevalence in the severe group (%)	Prevalence in the non-severe group (%)	I^2	Pooled OR (95% CI)	*P*-value
Hypertension	67	6,453	18,352	42.97	20.04	76.7	3.05 (2.60–3.59)	0.000
Diabetes	70	7,184	21,106	20.98	10.78	67.7	2.55 (2.14–3.03)	0.000
Pulmonary diseases								
COPD	33	4,571	11,949	4.11	1.13	0.0	3.91 (3.05–5.02)	0.000
Asthma (conclusion changed)	9	997	3,671	14.24	13.54	0.0	1.93 (1.53–2.42)	0.000
Unspecified type[a]	20	1,720	5,917	18.84	16.48	5.7	2.48 (2.03–3.03)	0.000
Cardiovascular diseases								
Coronary heart disease	20	3,184	9,133	9.58	4.03	34.7	2.04 (1.72–2.42)	0.000
Heart failure	3	83	100	14.46	14.00	57.6	1.74 (0.26–11.45)	0.567
Unspecified type[a]	32	3,052	9,158	21.04	8.83	45.9	3.01 (2.64–3.43)	0.000
Cerebrovascular disease	25	3,085	10,306	4.64	1.85	19.5	2.32 (1.83–2.94)	0.000
Hyperlipidemia	3	222	315	2.70	2.54	0.0	1.01 (0.34–3.02)	0.985
Obesity	9	1,075	2,025	18.14	8.74	63.1	2.63 (1.70–4.07)	0.000
Chronic liver disease	27	4,176	12,622	8.19	9.55	26.4	1.96 (1.64–2.35)	0.000
Chronic renal disease	33	5,198	15,388	4.66	2.56	50.2	2.09 (1.52–2.87)	0.000
Cancer	40	3,507	10,917	5.47	2.56	6.6	2.33 (1.90–2.87)	0.000
Any types of chronic disease[a]	33	3,118	8,206	60.71	31.81	71.4	3.70 (2.98–4.61)	0.000

[a] *The included studies did not mention the specific types of disease in this category.*

TABLE 3 | Associations between chronic diseases and mortality of COVID-19.

Chronic diseases	Number of included studies	Number of COVID-19 deaths	Number of COVID-19 survivors	Prevalence in the death group (%)	Prevalence in the survival group (%)	I^2	Pooled OR (95% CI)	*P*-value
Hypertension	96	49,072	205,854	37.53	26.86	92.2	2.31 (2.04–2.61)	0.000
Diabetes	105	57,121	250,467	22.34	15.29	84.8	1.99 (1.82–2.18)	0.000
Pulmonary diseases								
COPD	54	13,013	52,915	13.18	6.93	73.0	2.95 (2.48–3.50)	0.000
Asthma	16	11,026	36,444	8.51	8.50	0.0	0.74 (0.68–0.80)	0.000
Unspecified type[a]	29	19,961	120,999	21.91	13.72	63.1	2.05 (1.83–2.31)	0.000
Cardiovascular diseases								
Coronary heart disease	35	9,533	36,597	22.36	11.73	63.1	2.46 (2.14–2.82)	0.000
Heart failure	24	18,504	125,276	21.17	6.99	92.9	2.74 (2.21–3.40)	0.000
Unspecified type[a]	52	16,043	52,536	28.56	12.15	79.3	2.59 (2.24–3.00)	0.000
Cerebrovascular disease	40	16,889	122,332	11.82	4.23	69.7	2.46 (2.08–2.91)	0.000
Hyperlipidemia	6	9,875	69,330	52.80	29.94	97.8	1.72 (1.07–2.77)	0.000
Obesity	27	35,778	119,672	7.00	8.77	93.0	1.19 (0.94–1.51)	0.147
Morbid obesity	3	1,556	3,975	9.51	10.06	0.0	0.98 (0.80–1.20)	0.858
Chronic liver disease	27	9,988	26,493	2.93	2.30	32.3	1.52 (1.30–1.77)	0.000
Chronic renal disease	60	23,024	82,836	14.11	5.68	78.3	2.85 (2.44–3.33)	0.000
Cancer	65	26,704	142,413	11.58	5.66	70.5	2.11 (1.85–2.42)	0.000
Charlson index								
0	3	34,308	129,484	78.54	83.17	99.3	0.31 (0.18–0.51)	0.000
1	2	23,103	97,763	4.79	7.06	98.1	1.25 (0.67–2.33)	0.481
≥2	3	34,308	129,484	20.29	10.95	98.7	4.22 (2.56–6.96)	0.000
Any type of chronic diseases[a]	43	35,905	113,002	36.49	24.88	88.8	3.11 (2.64–3.65)	0.000

[a] *The included studies did not mention the specific type of disease in this category.*

TABLE 4 | Associations between chronic diseases and ICU admission of COVID-19.

Chronic diseases	Number of included studies	Number of ICU COVID-19 patients	Number of non-ICU COVID-19 patients	Prevalence in the ICU group (%)	Prevalence in the non-ICU group (%)	I^2	Pooled OR (95% CI)	*P*-value
Hypertension	50	12,062	247,158	47.78	24.72	87.1	2.24 (1.90–2.63)	0.000
Diabetes	51	12,188	247,334	37.27	17.28	79.6	2.50 (2.18–2.87)	0.000
Pulmonary disease								
COPD	25	7,648	223,993	6.85	2.43	69.7	2.76 (1.99–3.82)	0.000
Asthma	11	7,070	220,430	3.92	3.02	26.1	1.07 (0.94–1.22)	0.280
Unspecified type[a]	14	2,690	18,052	20.86	16.62	0.0	1.40 (1.26–1.56)	0.000
Cardiovascular disease								
Coronary heart disease	20	2,743	19,716	24.32	14.09	79.9	2.16 (1.56–2.99)	0.000
Heart failure	11	3,969	24,536	20.58	11.81	62.7	1.80 (1.44–2.25)	0.000
Unspecified type[a]	28	7,801	212,550	17.13	3.00	54.8	2.38 (1.92–2.96)	0.000
Cerebrovascular disease	19	2,587	20,957	10.86	4.67	80.7	1.66 (1.00–2.75)	0.048
Hyperlipidemia	4	1,713	11,354	54.64	51.77	53.2	1.53 (1.22–1.93)	0.000
Obesity	19	10,016	232,568	30.65	20.52	89.4	1.86 (1.49–2.31)	0.000
Chronic liver disease	16	889	6,699	2.92	1.97	0.0	1.48 (0.95–2.29)	0.082
Chronic renal disease	31	10,550	237,131	11.54	3.63	82.8	2.25 (1.73–2.94)	0.000
Cancer	30	3,286	21,949	14.39	11.93	34.2	1.57 (1.39–1.77)	0.000
Any type of chronic disease[a]	22	8,329	223,276	73.62	47.79	81.0	2.82 (2.23–3.56)	0.000

[a] *The included studies did not mention the specific type of disease in this category.*

TABLE 5 | Associations between chronic diseases and ARDS of COVID-19.

Chronic diseases[a]	Number of included studies	Number of COVID-19 patients with ARDS	Number of COVID-19 patients without ARDS	Prevalence in the ARDS group (%)	Prevalence in the non-ARDS group (%)	I^2	Pooled OR (95% CI)	*P*-value
Hypertension	6	635	1,493	58.11	38.71	0.0	2.17 (1.78–2.66)	0.000
Diabetes	6	635	1,493	16.54	9.58	21.5	2.32 (1.70–3.17)	0.000
COPD	4	499	986	15.23	8.01	68.4	1.35 (0.54–3.37)	0.516
Cardiovascular disease								
Coronary heart disease	2	449	856	12.03	6.43	0.0	1.96 (1.32–2.92)	0.001
Heart failure	2	449	856	10.69	5.26	58.4	1.85 (0.834–4.11)	0.130
Unspecified type[b]	3	162	611	10.49	7.20	0.0	2.35 (1.24–4.47)	0.009
Cerebrovascular disease	3	473	882	12.69	6.35	71.5	1.41 (0.46–4.30)	0.546
Obesity	4	525	1,272	28.95	12.42	70.7	2.25 (1.18–4.28)	0.014
Chronic renal disease	4	499	986	12.63	7.71	14.1	1.63 (1.14–2.33)	0.008

[a] *Only data for these chronic diseases were available to conduct meta-analyses.* [b] *The included studies did not mention the specific type of disease in this category.*

unspecified type of cardiovascular disease (OR 2.35, 95% CI 1.24–4.47), obesity (OR 2.25, 95% CI 1.18–4.28), chronic renal disease (OR 1.63, 95% CI 1.14–2.33), and occurrence of ARDS (**Table 5**). COPD, heart failure, and cerebrovascular disease were not significantly correlated with the risk of ARDS ($P > 0.05$).

DISCUSSION

Given the ongoing COVID-19 pandemic and the consequent global healthcare crisis, there is an urgent need to better understand risk factors for symptom deterioration and identify the vulnerable populations at higher risk for COVID-19 mortality. Our meta-analysis aimed to meet the need by examining global evidence, including 217 studies from 26 countries with 624,986 COVID-19 patients. Compared with a prior meta-analysis that showed that among COVID-19 patients, 20.3% required ICU admission and 32.8% had ARDS (244), our analysis provided further data on the association between chronic diseases and the different clinical prognoses of COVID-19 patients. According to our findings, COVID-19

patients with chronic diseases were more likely to have severe symptoms, ICU admissions, and an increased risk of mortality.

On the contrary, a meta-analysis found that pre-existing chronic conditions were not correlated with COVID-19 mortality (OR 2.09, 95% CI 0.26 to 16.67) (12). However, the conclusion of that meta-analysis was not reliable due to the fact that it only included three studies with a small sample size (453). In comparison, our meta-analysis had a much large sample size and identified the significant associations between a variety of chronic conditions and COVID-19 mortality, such as hypertension, diabetes, COPD, unspecified type of pulmonary disease, coronary heart disease, heart failure, unspecified type of cardiovascular disease, cerebrovascular disease, hyperlipidemia, chronic liver disease, chronic renal disease, and cancer.

We found that among COVID-19 patients, hypertension was a common comorbidity and was associated with COVID-19 severity, ICU admission, ARDS, and mortality. We found that COPD was the strongest predictive comorbidity for COVID-19 severity, ICU admission, and mortality, a finding that is consistent with prior research results, confirming that COPD patients are particularly vulnerable for very severe or critical COVID-19 cases (6).

Whereas, the published COVID-19 systematic reviews used the term "cardiovascular disease" generally (11, 13, 245), merely merged different types of cardiovascular diseases into a single measure, or only used the data from "unspecified type of cardiovascular diseases" as the outcome (6, 11, 13), we categorized cardiovascular diseases into three groups—coronary heart disease, heart failure, and unspecified type of cardiovascular diseases—to provide specific evidence for decision-makers. We found that coronary heart disease was a potential risk factor for the severity, ICU admission, mortality, and ARDS of COVID-19, while heart failure could increase the probability of ICU admission and mortality.

According to our results, cerebrovascular disease was an important comorbidity for COVID-19 mortality. We also identified cerebrovascular disease as a risk factor for severity of COVID-19 patients, which was consistent with other meta-analyses (14, 245). However, we found that there was a weak association between cerebrovascular disease and the risk of ICU admission, a finding that was different from a meta-analysis showing that cardio-cerebrovascular diseases were about 3-fold higher in ICU patients than in their non-ICU counterparts (17). However, only six studies were included in that meta-analysis, and its method was problematic as it calculated relative risk despite the fact that it included retrospective studies. Furthermore, cardiovascular disease and cerebrovascular disease were combined into a single outcome measure in the previous meta-analysis.

Asthma is a chronic disease of the air passages of the lungs which inflames and narrows them. Both its prevalence and mortality increased in recent decades, accounting for 272.68 million cases (3.57%) and 0.49 million deaths (0.006%) in the year 2017 (246). A multicenter retrospective study in 10 US hospitals found that asthma did not lead to an increased risk of hospitalization (RR 0.96, 95% CI 0.77–1.19) for COVID-19 patients after adjusting for age, sex, gender, and comorbidities (247). However, we found that asthma was associated with the severity of COVID-19 but tended to become a protective factor to reduce mortality risk. On the other hand, a published meta-analysis demonstrated that asthma patients were not predisposed to severe COVID-19 infections (248). It should be noted that the meta-analysis searched for articles published from January 1, 2020, to August 28, 2020, which was even shorter than our study, and only five studies were included in that meta-analysis.

The prevalence of obesity in many countries has been increasing rapidly in recent decades. We found that obese patients were at a higher risk of developing severe COVID-19 symptoms. However, the association between obesity (BMI $\geq$ 28 or 30 kg/m^2) and mortality was not statistically significant. We did not find a significant relationship between morbid obesity (BMI $\geq$ 40 kg/m^2) and mortality.

Hyperlipidemia involves an imbalance of cholesterol levels, including low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) in the blood. It has become common in many countries (249), especially in the USA where low HDL-C among adults aged 20 and over was 17.2% (250) and roughly 53% of adults had elevated LDL-C levels (251). Our results showed that hyperlipidemia was associated with increased ICU admission and mortality of COVID-19 patients.

A meta-analysis revealed an insignificant correlation between the increased risk of severe COVID-19 and liver disease, cancer, or renal disease (245). The insignificant results were probably due to the small number of studies included in the analysis—only five studies were included and all of them were from China. In contrast, our large sample size from multiple countries enabled us to find that those COVID-19 patients with chronic liver disease, cancer, or chronic renal disease were more likely to become severe cases and had a higher risk of mortality.

Our results emphasize the need for enhanced vigilance, priority for detection and testing, and aggressive COVID-19 therapy for patients with chronic diseases. Given our findings that COVID-19 patients with various chronic diseases were more likely to experience severe symptoms and ICU admissions and faced a higher risk of mortality, policymakers across different countries need to target patients with chronic diseases as a priority of their strategies to combat the COVID-19 pandemic. In particular, measures should be taken to protect the vulnerable groups with specific types of chronic disease, such as hypertension, diabetes, cardiovascular disease, and hyperlipidemia, each of which has a high prevalence in the general population.

For some of the less common chronic conditions, a targeted and intensive health protection strategy is also warranted. For example, although COPD is a less common condition among the general population, our analysis indicated that it is strongly associated with COVID-19 severity, ICU admission, and mortality. We also found that cerebrovascular disease, a less common condition that is the leading cause of serious long-term disability, was a significant comorbidity predicting mortality in COVID-19 patients. Thus, patients with COPD and/or cerebrovascular diseases

should receive special attention from both policymakers and healthcare professionals.

Finally, our analysis suggested that more adequately powered studies should be conducted to investigate how the severity and mortality of COVID-19 are associated with morbid obesity and hyperlipidemia, and a composite measure of comorbidity such as the Charlson comorbidity index must be utilized. The risk factors for ARDS in patients with severe COVID-19 are also worthy of further analysis in the future.

The results of our systematic review should be interpreted in the context of its limitations. First, we did not include studies that only analyzed children, pregnancies, and healthcare professionals in order to ensure the homogeneity and representativeness of the general population. The existing systematic reviews found that children seemed to have a milder disease course and better prognosis than adults (252) and that vertical transmission of COVID-19 from pregnancies to newborns could not be ruled out (253). Second, there was a limited sample size on risk factors for ARDS, and future observational studies are still needed on this topic. Third, the predictive value of concurrent multiple chronic diseases for the prognosis of COVID-19 patients remains unclear. Fourth, we were unable to conduct subgroup analysis according to community dwellings and institutionalized individuals due to a lack of data from the included studies. The association between chronic diseases and severity of COVID-19 should be further analyzed in community care and institutional care, respectively. Finally, further observational studies and meta-analyses are still needed to explore the impacts of chronic diseases on the severity and mortality in later waves of the COVID-19 pandemic.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

HY and JG designed the protocol. JG, XC, and CL performed the literature search and screening. XYu, HB, ZF, JZ, and XC extracted the data and did the quality assessment. JG and YC checked the data. XYuan took part in the interpretation of the data. JG and HY contributed to the meta-analysis and interpretation of the results and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.588013/full#supplementary-material

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Operationalizing Cooperative Research for Infectious Disease Surveillance: Lessons Learned and Ways Forward

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The current COVID-19 pandemic demonstrates the need for urgent and on-demand solutions to provide diagnostics, treatment and preventative measures for infectious disease outbreaks. Once solutions are developed, meeting capacities depends on the ability to mitigate technical, logistical and production issues. While it is difficult to predict the next outbreak, augmenting investments in preparedness, such as infectious disease surveillance, is far more effective than mustering last-minute response funds. Bringing research outputs into practice sooner rather than later is part of an agile approach to pivot and deliver solutions. Cooperative multi- country research programs, especially those funded by global biosecurity programs, develop capacity that can be applied to infectious disease surveillance and research that enhances detection, identification, and response to emerging and re-emerging pathogens with epidemic or pandemic potential. Moreover, these programs enhance trust building among partners, which is essential because setting expectation and commitment are required for successful research and training. Measuring research outputs, evaluating outcomes and justifying continual investments are essential but not straightforward. Lessons learned include those related to reducing biological threats and maturing capabilities for national laboratory diagnostics strategy and related health systems. Challenges, such as growing networks, promoting scientific transparency, data and material sharing, sustaining funds and developing research strategies remain to be fully resolved. Here, experiences from several programs highlight successful partnerships that provide ways forward to address the next outbreak.

Keywords: cooperative research, global health security, infectious disease surveillance, capacity building, Central Asia, COVID-19

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BACKGROUND

The COVID-19 pandemic exemplifies the importance of infectious disease surveillance in various aspects of preparedness, response, hypothesis generation for research purposes, implementation of interventions like mask-wearing and development of therapeutic and vaccine products. In addition to enabling early detection, the ongoing and systematic monitoring of infectious disease surveillance data allows us to assess the transmission dynamics of disease, which can then help develop predictive models with a higher level of accuracy. These predictive models, in turn, can help inform the development of preparedness and response policies that can then curb transmission. Moreover, effective infectious disease surveillance allows us to understand the clinical presentation of disease, the pathogen, the detection of the pathogen in natural foci, host risk factors associated with severity or protection, changes in these risk factors and which populations are most at risk. This type of information is critical to the development of effective interventions, prophylaxis, therapeutics and vaccines against infectious diseases (**Figure 1**). For example, if we are able to assess the reason behind asymptomatic or mild cases of disease, we may have a better understanding of the immune correlates of protection in those individuals, which then can inform effective vaccine development.

The COVID-19 pandemic has also created a global "all hands-on deck" effect where national governments recognize that they must collaborate internationally across sectors and among communities and individuals to achieve containment (1). Industry needs assistance to accelerate funding opportunities, as exemplified by the US Food and Drug Administration Emergency Use Authorization (FDA EUA), which was essential in efforts to make COVID diagnostic tests and treatments more available, by streamlining the regulatory process. Another important challenge is the ability to operationalize research outputs and increase success rates for products in the development pipeline. Well-known examples exist where opportunities arose during crises and those who collaborated effectively were better prepared to excel (2). Similarly, collaboration during an outbreak enhances communication and coordination, and the numerous resulting R&D outputs are additional beneficial by-products. While cooperative research can take place in many forms among public and private partnerships within a country, as well as in collaborations among different countries, we focus on those international programs aimed at biological threat reduction and enhancing biosecurity engagement (3, 4).

Cooperative research programs develop capacity that can be applied to infectious disease surveillance and research that enhances detection, identification, and response to emerging and re-emerging pathogens with epidemic or pandemic potential. In this paper, we describe work done in three Central Asia countries: Kazakhstan, Kyrgyzstan, and Tajikistan. It quickly became apparent that program funding from Germany, UK and US programs had overlapping research programs and activities. Recognizing that these similar efforts led to opportunities to reinforce cooperation, we describe research that has been operationalized in each country: Kazakhstan (Germany, UK, and US), Kyrgyzstan (UK, US, Canada, China, Russia and WHO), and Tajikistan (UK). Ideally, cooperative research continues peer mentorships that first promote international norms and best practices to encourage hypothesis-based studies and scientific transparency. Successful mentorships form collaborations that create greater networks for participants, furthering scientific knowledge, infrastructure and related capabilities. Other benefits include the return on relationships (e.g., joint publications) and construction of sustainable networks that arise as a product of collaboration (5).

Robust networks, based on the above approaches, offer agility, creativity, and trust which can accelerate engagements through familiarity and rapport among colleagues and peers. The elements of further multi-disciplinary and multi-sectoral collaborations are key to furthering outputs that can be operationalized. However, a major challenge is the often-different set of expectations among program funders and partner country recipients. Getting these aspects resolved is essential to building goodwill and trust, as well as promoting mutually-beneficial good practices in partner countries.

Our three case histories are from countries of the Former Soviet Union, which is a common area of interest for biosecurity-based engagement programs. These countries have histories of state programs for biological weapons development (6), thus appealing to agencies interested in countering biological weapons, preventing use and reducing threats, in line with the Geneva Convention of 1975. Our three examples illustrate outputs from cooperative research that point to its value in real-life public health situations.

COOPERATIVE BIOLOGICAL RESEARCH PROGRAMS IN THE REPUBLIC OF KAZAKHSTAN

The US Defense Threat Reduction Agency (DTRA) has implemented its Biological Threat Reduction Program (BTRP) with Kazakhstan for over 20 years (4). DTRA funded the construction and commissioning in 2018 of the Central Reference Laboratory (CRL) in Almaty, Kazakhstan (**Figure 2**), which is now operational and serves as national level reference laboratory. During the CRL's commissioning, DTRA also supported several research studies intended to bridge activities that the CRL would eventually house. The CRL involves cooperation among three Kazakh ministries, DTRA and their collaborators which included scientists from the US and UK, and contractors who implemented much of the work. As a result of this activity, substantial scientific and medical progress has been made, both at the practical and personal level and Kazakh scientists who have taken part in DTRA programs are now publishing independent work in the international press (7).

The CRL has recently supported research by Kazakhstani scientists for COVID-19 research that includes animal biosafety laboratory studies for a national vaccine. Kazakh government, universities and commercial companies fund additional research.

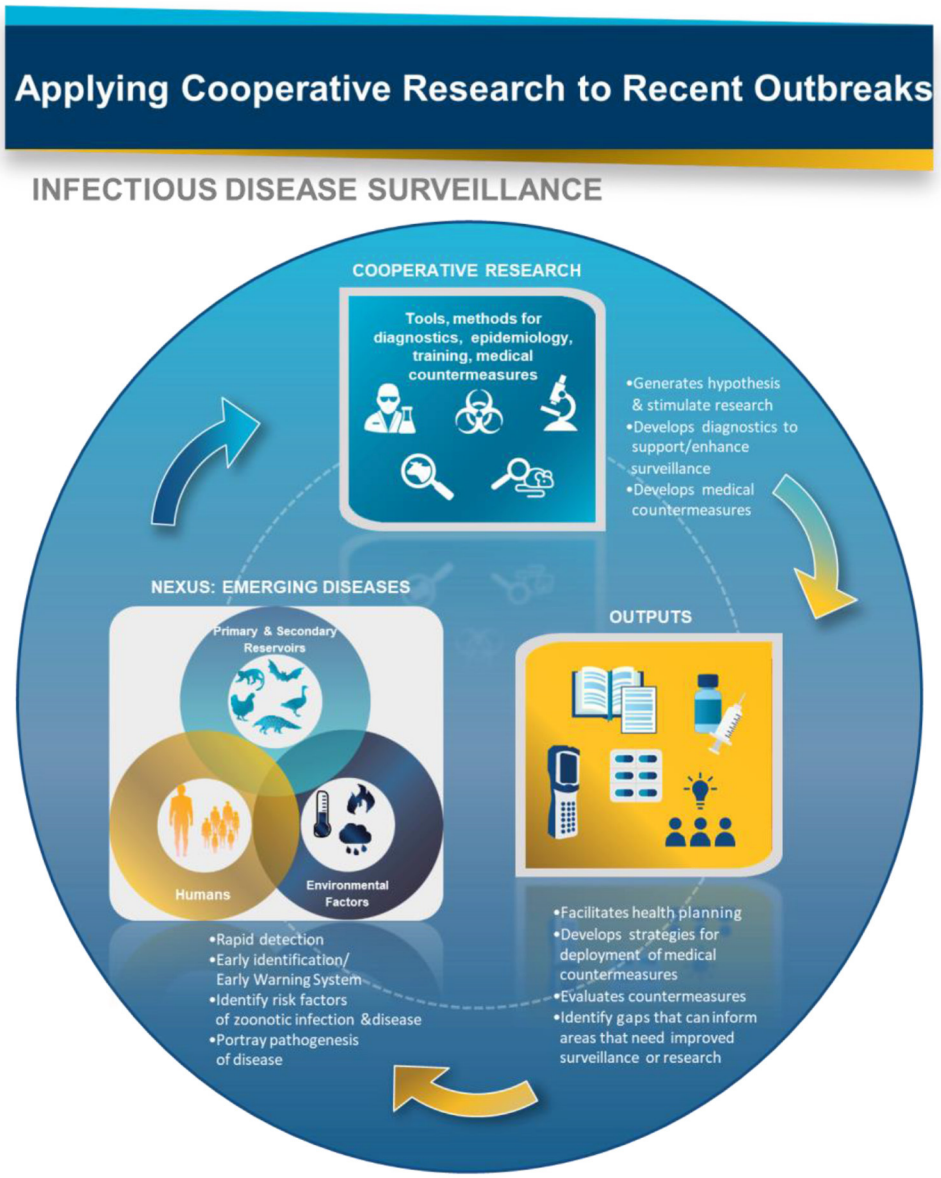

FIGURE 1 | Infectious disease surveillance is a vital component in the response to disease outbreaks and the subsequent development of effective countermeasures. COVID-19 demonstrates the need for faster turnaround to meet technical, logistical, and production demands.

The German Federal Foreign office in 2013 launched the German Biosecurity Program (GBP) in order to implement sustainable biosafety and biosecurity projects in various countries. The current program phase runs from 2020 until 2022 and is currently active in nine countries including two supraregional projects (8). Through the German Federal Foreign Office's German Biosecurity Program, the Bundeswehr Institute of Microbiology (BIM) and the Deutsche Gesellschaft für Internationale Zusammenarbeit GmbH (GIZ) have managed a project in Kazakhstan for the past seven years in collaboration with key Kazakh partners including the aforementioned NSCEDI at the CRL and the Research Institute for Biosafety Problems (RIBSP). Under the auspices of the G7 Global Partnership against the Spread of Weapons and Materials of Mass Destruction, the GBP focuses on surveillance, detection and diagnostics, biosafety and biosecurity including work published on Crimean Congo haemorrhagic fever, tick-borne encephalitis virus, *Rickettsia* and orthohantaviruses (9–12).

The GBP also finances a e-learning platform (German Online Platform for Biosecurity & Biosafety (GO4BSB), which contributes to development of a sustainable network in Kazakhstan which includes COVID-19 training and information, available in Russian language. The initiative is a collaborative effort by the Bernhard Nocht Institute for

FIGURE 2 | Political map of Kyrgyzstan, showing its proximity to Kazakhstan and Tajikistan. The major cities mentioned in the text are shown (Almaty, Bishkek, Osh and Dushanbe). Map courtesy of the University of Texas Libraries (lib.utexas.edu).

Tropical Medicine, BIM, Friedrich Loeffler Institut, Federal Research Institute for Animal Health, Robert Koch Institute and GIZ. Informal interactions among collaborators of the US and German cooperative programs in Kazakhstan also enhanced collaboration.

The cooperative research outputs, namely the CRL infrastructure, capacity building through workforce training, and the established multi-national collaboration and networks, have all been leveraged in response to the COVID-19 pandemic. Examples include publishing the genetic sequence of spike protein, development of a COVID-19 subunit vaccine, and preclinical testing of this subunit vaccine. Through the e-learning platform COVID-19 training and information, available in Russian language, was also deployed.

COVID-19 RESPONSES IN THE KYRGYZ REPUBLIC

For the past 15 years or so, Kyrgyzstan has been the recipient of multinational cooperative research assistance, aimed at resolution of health problems. Entities that have worked there include ISTC (International Science and Technology Committee; based in Moscow but funded by a consortium of countries), CRDF (Civilian Research and Development Foundation; US), Dstl (Defence science and technology laboratories; UK) and the Canadian Weapons Threat Reduction Program. As a result of these joint efforts, the Republic has acquired a substantially more developed health surveillance and treatment ability (13). COVID was first detected in Kyrgyzstan in March 2020, following a visit by a number of Kyrgyz muslims to the "Small Hajj" in Saudi Arabia; cases now stand at about 170,000 (August 2021), about 2.5% of the population.

Following WHO guidelines, laboratories tested nasal swab samples for SARS-CoV-2 using RT-PCR. Early in the outbreak, 13 laboratories for PCR diagnostics were opened in the country, including three mobile ones. Together with the local health, education and science ministries, international organizations, such as WHO, CDC, academic initiatives such as the Columbia University International Assistance Program (ICAP), and groups of foreign scientists, (from China and Russia), a series of training

sessions on PPE, laboratory testing, treatment and follow-up were organized. This international cooperative approach, following the international efforts mentioned earlier, allowed the Kyrgyz authorities to be better prepared to deal with the pandemic.

Currently, research is being conducted on serological assessment of population immunity in seven regions of the republic and in the cities of Bishkek and Osh using different age groups. ELISA is used to test for the presence of SARS-CoV-2-specific IgA, IgM and IgG. Based on these results, national immunity to this coronavirus infection in the Kyrgyz Republic will be known and appropriate action taken. No vaccine development is underway in the country, since there are no suitable facilities available. However, China has recently gifted doses of the "Sinopharm" COVID vaccine and, despite some local resistance, about 8% of the population has had at least one dose.

CRIMEAN-CONGO HEMORRHAGIC FEVER IN THE REPUBLIC OF TAJIKISTAN

WHO prioritizes Crimean-Congo haemorrhagic fever virus (CCHFV) as one of seven epidemic-prone diseases: a "public health emergency" with an "urgent need for accelerated research" and is the most widespread tick-borne viral haemorrhagic fever infection in the world (14).

CCHF is notoriously difficult to diagnose, because early symptoms, including fever, myalgia, diarrhea, nausea, and vomiting, are often indistinguishable from those of more common tropical diseases (15). CCHF is endemic in Central Asia while the incidence and prevalence in countries such as Tajikistan is not yet widely understood.

In 2012, Public Health England (PHE)'s Virology and Pathogenesis group was invited to support the development of molecular CCHFV diagnostics in Tajikistan by its Ministry of Health. Accordingly, a cooperative programme was developed to implement a standard RT-PCR assay (16) at the Institute of Preventative Medicine (IPM) in the capital, Dushanbe (**Figure 2**). This included a series of training workshops in the UK and Tajikistan supported by the UK IBSP. Over a 2-year period, PCR diagnostics for CCHFV became a standard capability in the IPM laboratories. Collaboration, including the exchange of samples between the UK and Tajikistan, which supported the continued development of new RT-PCR assays, built capacity in country (17) and supported the UK's capability to rapidly detect imported CCHF and reduce onward transmission in the UK National Health Service. Based on the successful implementation of this laboratory diagnostic assay, the cooperative programme went on to work up the development of a field-capable nucleic acid test for CCHFV using novel isothermal Replicase Polymerase Amplification (RPA) chemistry (18). This new tool is ideally placed for use in-low resource settings and can monitor CCHF outbreaks at the point-of-need, such as in remote rural regions in affected countries. Its implementation in Tajikistan has also contributed to major new CCHF disease control programmes in the country. The UK's International Biological Security Program (IBSP), which is a global partnership with aims to strengthen national health systems; support research on vaccines, drugs and diagnostics, was also active in several locations including Kazakhstan.

As evidenced here and other parts of the world, the lack of a rapid, simple and affordable diagnostic in these early stages of disease is a serious problem, which leads to the propensity of the virus to cause nosocomial outbreaks where mortality rates of up to 80% have been reported (19–21). In other austere environments and regions, obtaining reagents and consumables for diagnostics can be difficult to obtain. In rural settings, the situation is exacerbated by limited health care facilities and initial spill-over events from wildlife tick vectors that go unrecognized until community outbreaks sustained by human-to-human transmission develop (22). Such a situation exists in Tajikistan which, in addition to having one of the highest national burdens of CCHF, also has the dubious distinction of occupying territory where CCHF was first described in the 11th Century. Many severe cases have been recognized since the disease was first brought to modern medical attention over 60 years ago.

ROLE OF SCIENCE NETWORKS: FORMAL AND INFORMAL

Scientific collaboration networks can exist formally or informally and can focus around any given specific disease topic, a technology such as genomics and sequencing, or an emerging field such as ecoimmunology. Formal networks are most likely funded and organized whereas informal networks are a subset of researchers that may be connected in some manner such as through institutions, professional societies and past collaborations. To address infectious diseases and biosurveillance, DTRA BTRP created the more formal Biological Threat Reduction Networks (BTRN) (23). As the name implies, BTRNs aim to connect scientists around the world with the shared mission of reducing biological threats. With the several existing cooperative biological engagement programs mentioned, the primary objectives include strengthening capabilities in detection and diagnostics and to have these scientific and technical capabilities become sustainable. One of the best ways of creating sustainable capabilities within countries is to connect researchers and the technical professionals together enabling cooperation or sharing expertise and information, as well as combating misinformation.

Informal networks are the connections, professional relationships, and source of contacts that scientists often leverage throughout their careers. These contacts include fellow researchers, peer colleagues, and mentor/mentees that connect at scientific conferences and related collaborations. These scientists maintain informal networks independently. As science becomes more multidisciplinary across disparate fields, the breadth of the informal networks between researchers is becoming larger and more diverse. For example, infectious disease research requires the understanding the ecology of an emerging or endemic infectious disease system where epidemiologists may work with meteorologists, sociologists, wildlife biologists, and geographers. With unlimited access between researchers through the internet,

general connections are not endangered, however, trusted relationships and sustained connections are rarer. Cooperative engagement research is designed to build trusting and long-term relationships. The COVID-19 pandemic has led to an unprecedented amount of science and it was through the trusted collaborations that existed prior to the pandemic, that the critical initial data and information on the coronavirus was shared. Also, with the COVID-19 pandemic, most networks have had to move to become virtual networks. Having a low-cost virtual platform for connecting can help networks become sustainable into the future if and when funding ends.

During infectious disease outbreaks, both formal and informal networks are critical for a rapid and coordinated response. As it is often repeated, "if you exchange business cards on the first day of an outbreak, the pathogen has already won." The return of investment for cooperative engagement programs (5) became evident immediately in the 2020 COVID-19 pandemic. Country partner researchers and diagnosticians quickly moved to detect and diagnose SARS-2 as it moved into and across regions. Both through formal and informal networks, researchers reached out to each other for advice on the specifics of PCR diagnostics, sequencing of the SARS-2, and general information on the behavior of the disease in humans. The COVID-19 pandemic has shown that the time and effort over the past 15 years in cooperative engagement paid off in the faster exchange of information, data, samples, and has overall built trust between scientists and countries. Cooperative engagement research designed to understand One Health systems has shown a high return on investment and has reduced the threat of global infectious disease spread (24).

CONCLUSIONS

Continuing to operationalize cooperative research and infectious disease surveillance are essential complements to identify and mitigate the next outbreak. As illustrated in our three examples of cooperative biological research, prior efforts by agencies from many different countries have set up the scientific and medical communities in partner countries to deal rapidly and expertly with a biological threat outbreak. Global infectious disease outbreaks, such as the COVID-19 pandemic, require a global response. These agencies have primed personnel, with little prior skills, to recognize threats, deal with them and share techniques, data and ideas with colleagues across the world. Human and social factors such as trust building and political will influence the partnerships and networks. The activities described demonstrated the requisite trust needed to continue collaborations and avoid transactional one-off studies. Overall, the political will, which usually backs financial investment, has made COVID-19 easier to track, deal with clinically and attack through development of therapies and candidate vaccines. Sharing technology and ideas is only possible when partners are trained to understand their importance and carry out state-of-the-art techniques. Continuing these activities to enhance capabilities and capacities along with building trust will be required beyond the COVID-19 pandemic.

AUTHOR CONTRIBUTIONS

KY, FP, and KairT developed this concept along with JH, RH, JF, and SE. KaisT contributed content for the manuscript. All authors reviewed and agreed on the final submission.

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Building Scientific Capability and Reducing Biological Threats: The Effect of Three Cooperative Bio-Research Programs in Kazakhstan

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Cooperative research programs aimed at reducing biological threats have increased scientific capabilities and capacities in Kazakhstan. The German Federal Foreign Office's German Biosecurity Programme, the United Kingdom's International Biological Security Programme and the United States Defense Threat Reduction Agency's Biological Threat Reduction Program provide funding for partner countries, like Kazakhstan. The mutual goals of the programs are to reduce biological threats and enhance global health security. Our investigation examined these cooperative research programs, summarizing major impacts they have made, as well as common successes and challenges. By mapping various projects across the three programs, research networks are highlighted which demonstrate best communication practices to share results and reinforce conclusions. Our team performed a survey to collect results from Kazakhstani partner scientists on their experiences that help gain insights into enhancing day-to-day approaches to conducting cooperative scientific research. This analysis will serve as a basis for a capability maturity model as used in industry, and in addition builds synergy for future collaborations that will be essential for quality and sustainment.

Keywords: biosecurity, Kazakhstan, global health security, one health approach, vector-borne disease, zoonoses

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"*Life on Earth is at the ever-increasing risk of being wiped out by a disaster, such as sudden global nuclear war, a genetically engineered virus or other dangers we have not yet thought of.*"
Stephen Hawking

BACKGROUND

Countries from the Former Soviet Union (FSU) including Armenia, Azerbaijan, Georgia, Kazakhstan, Kyrgyzstan, Tajikistan, Ukraine, and Uzbekistan have partnered in various threat reduction, biosecurity, and related programs that engage its scientists in relevant biological research and infectious disease surveillance. The appeal of the Government of Kazakhstan as a partner stems from its work in the former Soviet Union's biological weapons program, the anti-plague surveillance network and a recent history of infectious disease management of hotspots such as those due to anthrax, brucellosis, plague, and tularemia. Earlier, in a compendium, we summarized some aspects of recent cooperative biological research in Kazakhstan, noting its infectious disease surveillance activity, history of scientific achievement, economy, and national research bibliometrics (1). In this paper, we examine the overall impact on scientific capability of three cooperative infectious disease research programs partnering with Kazakhstan (KZ): Germany's Federal Foreign Office's German Biosecurity Programme (GBP), the United Kingdom's (UK) International Biological Security Programme (IBSP), and the United States (US) Defense Threat Reduction Agency's Biological Threat Reduction Program (DTRA BTRP) (**Figure 1**). Our findings here reflect outputs of enhanced scientific capability and reduced biological threats by Kazakhstan from within and across partner programs.

While each of the programs has specific objectives (**Figure 1**), the overarching goals are to enhance biosafety and biosecurity, improve disease detection, surveillance and control and engage in cooperative research that will result in sustainable scientific advancements. A major focus of all three cooperative research programs is to build local scientific research capability while reducing biological threats. All three programs have contributed funding to improve infrastructure, cooperative research and related training. DTRA BTRP is a long-standing program with legacy FSU engagements in Armenia, Azerbaijan, Georgia, Kazakhstan, Ukraine, and Uzbekistan; the GBP funds projects in Georgia, Kazakhstan and Ukraine and the IBSP has supported work in Azerbaijan, Georgia, Kazakhstan, Tajikistan, as well as parts of the Middle East and Africa.

Since the early 2000s, the **DTRA BTRP** has funded biological threat reduction in Kazakhstan along three lines of work: biosafety and biosecurity, biosurveillance (i.e., capacities to detect, diagnose, and report disease) and cooperative biological research (CBR). DTRA BTRP has also funded investments for enhancement of facility and infrastructure for BSL-2 Zonal Diagnostic Laboratories (ZDL) in partner countries of the Former Soviet Union (FSU), including Kazakhstan. To reinforce these activities, BTRP recently spent $102 M on the construction of a Central Reference Laboratory (CRL) in Almaty, Kazakhstan. The CRL validation, which includes a biosafety level-3 (BSL-3)

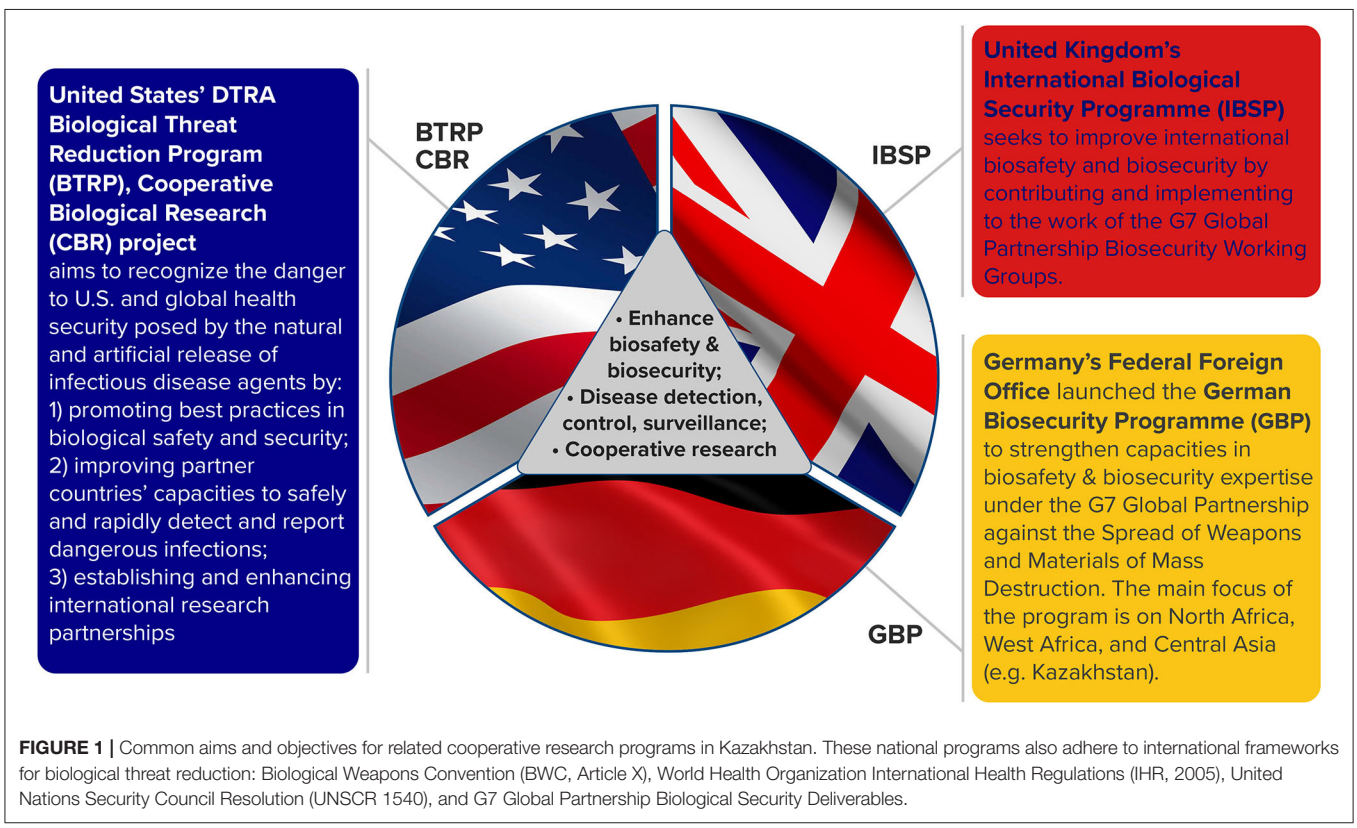

FIGURE 1 | Common aims and objectives for related cooperative research programs in Kazakhstan. These national programs also adhere to international frameworks for biological threat reduction: Biological Weapons Convention (BWC, Article X), World Health Organization International Health Regulations (IHR, 2005), United Nations Security Council Resolution (UNSCR 1540), and G7 Global Partnership Biological Security Deliverables.

laboratory, was completed in August 2017, and the facility was transferred to the Government of Kazakhstan on September 29, 2017. The CRL serves as the national diagnostic reference laboratory and an inter-ministerial agreement involving the Ministry of Health [National Scientific Center for Especially Dangerous Infections (NSCEDI)] with cooperation from the Ministry of Agriculture and the Ministry of Education and Science co-owns and operates the facility (2). The CRL operates BSL-3, animal biosafety level 3 (ABSL-3) and BSL-2 laboratories which have been designed to current international standards for biosafety and biosecurity.

DTRA BTRP's long standing engagement in the FSU region is also exemplified in Kazakhstan through cooperative biological research (CBR) under which 30 biosurveillance-related projects and studies have been implemented, utilizing over $25M in funding. In a previous paper, our team mapped these 30 projects, which spanned the periods 2005–2007, 2009–2014, and 2015–2018 (1). US contractors such as AECOM implemented these studies in concert with US and UK project collaborators and partner country scientists in Kazakhstan. More recently, DTRA BTRP has moved to a more traditional grant funding system where research collaborators submit proposals through a stand-alone competitive program. These opportunities are grouped into three categories by duration and approximate amount of funding (i.e., labor, material, and travel): 2–3 year, $1–3 M projects; 1–1.5 years, up to $1 M projects and 1 year, $100 K studies (only material and travel). In the absence of a current formal research office in Kazakhstan, through which an independent party typically manages the BTRP grant process, we recently and independently mapped research activities to illustrate the linkages, progression and evolution of the CBR program in Kazakhstan (1). From 2009 to 2014, three of the largest projects spawned eight follow-on projects, which emphasizes the interest from the partner country scientists in Kazakhstan in continuing previously funded cooperative research (1, 3). In addition, Kazakhstani scientists received training on topics such as biosafety, biosecurity, and laboratory diagnostics, which complemented their research activities. This integrated approach of parallel research and training serves as a model for all future cooperative activity.

The **DTRA BTRP** and the **UK IBSP** have collaborated through agreements between the US Department of Defense and the UK Ministry of Defense. Similar to the situation with the DTRA BTRP, the UK Government funds its scientists on a "per project" basis, including technical assistance to partner country scientists in Kazakhstan through training and research capacity that UK specialists delivered. This agreement spawned two projects: KZ-4, a multi-viral pathogen study that ran from 2005 to 2007, that evolved into KZ-29, a broader tick-borne pathogen surveillance project targeting Crimean-Congo hemorrhagic fever

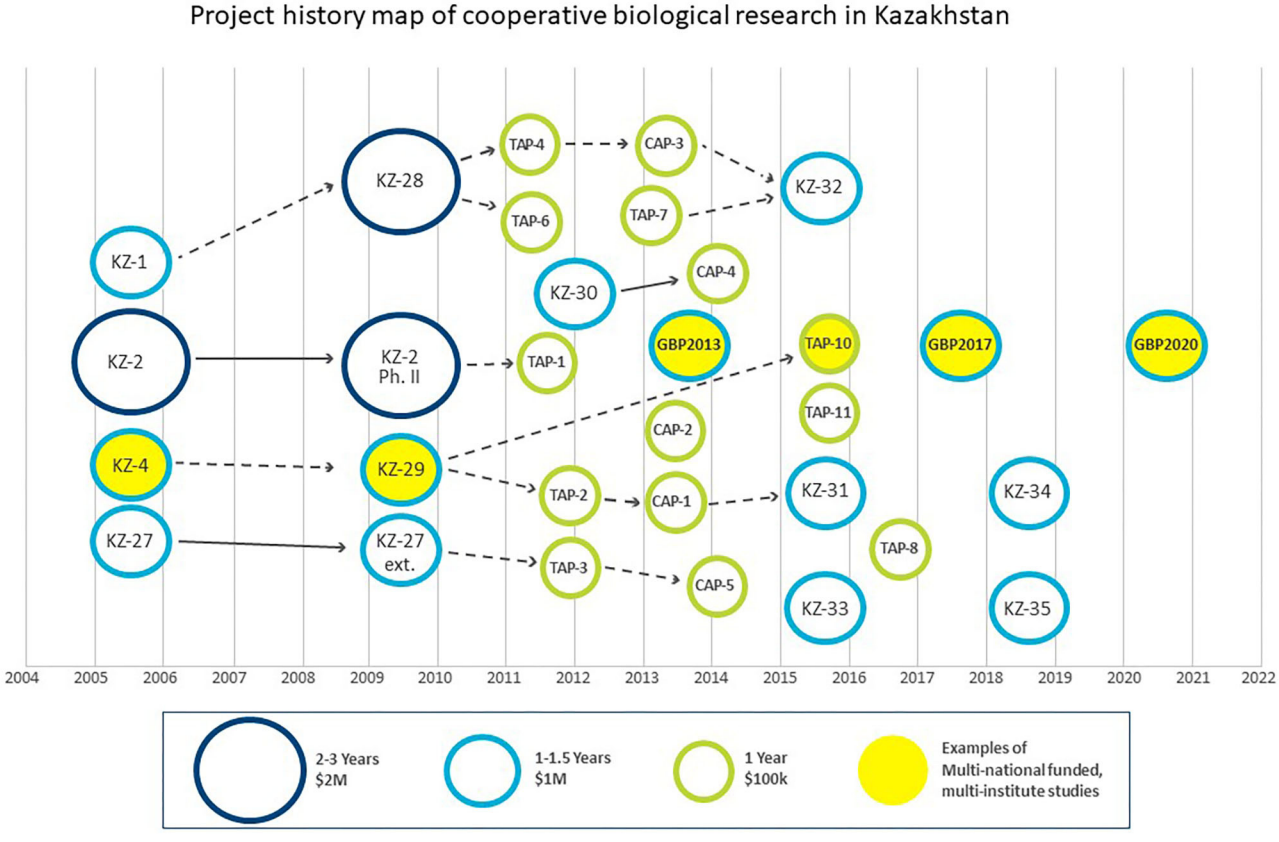

FIGURE 2 | DTRA BTRP (KZ projects, TAP studies) and GBP have funded over 30 studies since 2005. UK IBSP directly funded UK specialists for KZ-4 and KZ-29. A number of research grants awarded in 2020 or later are not shown.

(CCHF) virus, hantaviruses, tick-borne encephalitis (TBE) viruses and tick-borne rickettsiae that ran from 2009 to 2014 (1–3) (**Figure 2**). In 2020, DTRA awarded a new cooperative research grant for $1.5 M over three years to US, UK, and Kazakhstani scientists, who will continue study of the CCHF and TBE viruses endemic in Kazakhstan through novel sequencing and bioinformatics approaches. This activity illustrates the important continuity built in to the cooperative DTRA program, targeting issues of interest to all parties and involving many of the same personnel on all sides of the program.

Although **DTRA BTRP** and **GBP** have not formally collaborated, their respective efforts have developed very much in parallel (**Figure 2**). In 2013, the GBP was launched to foster responsible behavior in life sciences, strengthen national health security and focus on capacity development. From 2013 to 2021, the GBP supported three projects in Kazakhstan with a total expenditure of €2.5 M, utilizing the Bundeswehr Institute of Microbiology and the Deutsche Gesellschaft für Internationale Zusammenarbeit to implement this work through research and training. The initial effort was the establishment of a *German Kazakh Network for the diagnosis of infectious diseases caused by potential B-Agents* (2013–2016), followed by two projects called *German Kazakh Network for Biosafety and Biosecurity* (2017–2019 and 2020–2022) (4). Five national Kazakh institutions with expertise in arthropod and rodent vectors, especially dangerous pathogens or molecular biology were involved in the projects: the Kazakh National Medical University, the Scientific Practical Center for Sanitary and Epidemiological Expertise (SPCSEEM), NSCEDI including Taldykorgan and Uralsk Anti-plague Stations (UAPS), the Research Institute for Biological Safety Problems and the National Center for Biotechnology Almaty branch.

The **DTRA** and **GBP** projects have independently cooperated with three of the same institutes in Kazakhstan: SPCSEEM, NSCEDI, and UAPS. Examples of this cooperation include the aforementioned KZ-29 (as well as TAP-10, which was a 1-year study on tick-borne encephalitis virus, *Coxiella burnetii*, and *Brucella* species presence in livestock milk), in addition to the three GBP projects mentioned earlier (**Figure 2**). The KZ-29 project resulted in two publications (3, 5) and 21 conference presentations (1). Within the three German projects, two serological patient studies (Fevers of Unknown Origin), a tick-borne disease and a rodent-borne disease study were conducted. Scientific results and the progress of the projects were presented in 41 oral talks and 21 poster presentations at national and international scientific conferences. Work published from GBP involvement included articles describing the results of studies on CCHF and TBE viruses, rickettsiae and orthohantaviruses (6–9). As a result of awareness and post-project coordination of the DTRA and GBP programs on tick-borne diseases research (DTRA TAP-10, GBP), a joint publication on tick-borne encephalitis virus in North Kazakhstan is in final preparation.

Unquestionably, these three cooperative bio-research programs all resulted in better collaboration, increased communication, and significant investment in building scientific research capabilities in Kazakhstan (1, 3, 4). At the government-to-government level, there is agreement and interest to further this work which is demonstrated by the increase in the absolute number of projects. However, there have been no specific studies on what long-term impacts these programs have on the day-to-day approach to scientific research in Kazakhstan; thus it is hard to assess capability maturity and related performance and quality. Nevertheless, what our informal observations tell us is that increased awareness and visibility of these programs to the scientific community through conference presentations serve as good models for future similar international collaborations.

In view of the above uncertainties and to gain more formal insight into the impact of these international programs, our team developed a questionnaire that consisted of 21 questions, structured as yes or no, multiple choice and short answers.

The questions focused on an institute's research capability and were grouped in categories for demographics, standard processes for pursuing research funding and after-action and "lessons learned" processes. In addition, we asked about simple metrics, including the number of scientific conferences attended, the number of presentations given, and the number of publications per author per year, as well as the number of grants applied for in the last 5 years. The contact list was developed and the questionnaire was sent to 37 participants who were colleagues involved in bio-research and biosecurity programs as participants in at least one of the projects mentioned earlier. These individuals represented five institutes and two universities. For ease of completion, one of our team members who is a native Kazakh and Russian speaker translated the survey into Russian and this was provided as a Microsoft Word document attachment. Surveys were sent as requests to support this current manuscript construction and, in addition to scientists, recipients also included institute scientific secretaries who could best answer questions related to metrics. The University at Buffalo's Institutional Review Board reviewed the questionnaire which was submitted under IRB ID STUDY00004695, and deemed it to be "Exempt"from further IRB scrutiny.

The results represent a qualitative analysis from a limited sample ($n = 11$) of completed questionnaires (seven males and four females). The respondents represented five institutes and two universities that employed between 11 and 99 scientists in their home departments. The responses came from those at different positions of seniority, including institution director, laboratory head, and scientific secretary. Of the 11 respondents, 5 were in the 40–49 age category, 3 were in the 30–39 age category, 2 were in the 50–59 age category, and 1 was in the 60–69 age category.

When asked who decides which research funding opportunities are applied for, half responded that a laboratory/division/department head was the decision maker, followed by ministerial leadership and institute director. Respondents split on the question about whether their institute had an overall strategy for pursuing funding. The majority of respondents answered that their institute does not have a standard approach to identify and prioritize opportunities. The funding sources that the institutes pursued was consistent: mainly existing and historical sources (both domestic and foreign), followed by new opportunities, private, and foreign funding. Although the institutes represented employed up to 99 scientists, half responded that 11–49 of the scientists were

involved in pursuing research funding and half responded that <10 scientists were involved.

Respondents stated that the three most important factors that the institutes considered when applying for research funding were the source, amount and scope. In addition, institutes also reported the importance of continuing existing research capabilities, ability to expand and applicability of the research to the institute's mission.

When applying for research funding, the majority of respondents stated that their institutes provided guidance, standard documents, tools, and training; however, two respondents stated the opposite. On the question of whether institutes held debriefs after a funding submission, all respondents stated this occurred internally but only half stated this occurred externally. Nearly all respondents stated that their institutes tracked applications and reviewed "lessons learned" to provide continuous improvement.

The majority of respondents stated they attended 1–3 international and 1–3 national conferences annually, as well as some local conferences. The number of publications varied from 2 to 18 distributed across different indices: Web of Science, Scopus, and the Russian Science Citation index. The number of proposals applied for in the past 5 years varied from 3 to 36 according to the respondent's job function.

DISCUSSION

As evidenced from funding records, US DTRA BTRP, GBP, and UK IBSP have each invested and engaged in cooperative research projects in Georgia and Kazakhstan. Kazakhstan's important role in biological threat reduction and biosecurity is reflected in the over $25 M in cooperative research projects awarded over the last 20 years. US DTRA BTRP milestones for infrastructure have been achieved through the construction and commissioning of two ZDLs from 2009 to 2010 and the Central Reference Laboratory in 2019; similar work was achieved in Tbilisi, Georgia (10). In discussions with our colleagues in Kazakhstan, the impact of these related programs is and will be substantial, particularly for Kazakhstan's current and next generation of scientists, confirmed through their more frequent participation at international conferences and a greater number of peer-reviewed publications in international journals. Our earlier compendium discussed a roadmap and framework to grow a research program and incorporate the value of simple metrics, such as number of publications, conference presentations, proposals submitted, and proposals secured (1) and this seems to be taking place. Successful partnerships with German, UK, and US researchers continue to raise awareness and visibility of this work in the global scientific community. Looking ahead, investments in these partnerships appear to be seen as relevant for the future of global biological threat reduction and public health. As mentioned, the DTRA BTRP program has started making awards through the mid-2020s for research proposals submitted through their annual funding opportunity calls.

There are many cultural, technical, and programmatic challenges associated with these cooperative bio-research programs that include the language barrier, lack of diagnostic capabilities and varying expectations among funders, recipients and stakeholders (1, 3, 4). Among the large funding programs, there is a challenge in managing the bureaucratic inertia of many implementing partners which often changes with different contract awards and can result in a lack of program continuity. Staff turnover on both sides leads to gaps in science and program knowledge. Regarding capability and capacity building, in earlier CBR projects short timelines and limited in-person interactions did not favor successful training and mentoring for developing *in vitro* diagnostic assays. Instead, commercial kits were purchased akin to an instant food option. While this was effective in the short term it is not sustainable in the long term. This is a "lesson learned" that has led to changes in newer contracts. Limitations in exchanging sample material with foreign partners, which is largely not permitted in Kazakhstan even for research (a measure of scientific transparency) were recently offset by permissions to exchange genomic sequencing data in an electronic form (4, 11). Most importantly, building capacity requires strong mentorship and trust among collaborators and partners who are working together on the same goals, objectives, and strategies.

Obtaining responses to our survey was challenging, especially since this was an unfunded effort that did not allow us to pay respondents. Our team addressed needs for access and ease-of-use by emailing a Word document with the survey in English and Russian, as noted earlier, and we assume that this was helpful. Although some respondents had participated in earlier surveys, we recognized that they did not generally have much experience with such requests for information. This may reflect the fact that, in many countries, grass roots opinions are rarely used in planning future research activities. Thus, the difficulty observed with participation in this survey may be the result of lack of experience especially from junior level scientists and continuing local practice. In reviewing our survey responses, we also observed that the nature of many of the answers was typical of institutions and organizations without standard or repeatable processes for capturing research funding.

Regarding peer reviewed publications, we learned that, in Kazakhstan, quality and prestige of a publication often depends on indices for Scopus, Web of Science, and the Russian Science Citation Index. One other encouraging aspect noted, however, is that more Kazakhstani partner country scientists are now first or senior authors in recent publications from cooperative studies. This is a welcome change, we believe brought about by the influence of cooperative international research programs, since in earlier years, it was US and UK project collaborators who initiated and authored joint publications. In recent years, Kazakh partner country scientists, especially those who have become proficient in English and actively collaborate in international programs, seem also to have become more successful in their scientific careers in general. Overall, this requires strong mentorship among collaborators to develop a goal and strategy. For example, if early work was fronted by scientists from the funder country, partner country scientists should be, and are being, consulted and invited to contribute as co-authors. A successful evolution of work, from the funders' perspective, would later show those partner country scientists as

lead and senior authors with the earlier scientists from funder as middle authors. This transition is already evident, where strong teams can achieve these metrics through collaboration and, with limited resources, publish work not funded by foreign collaborators.

One final point worth mentioning was the lack of consensus among respondents on whether standard grant-getting procedures existed in their workplace, and who was responsible for developing and submitting proposals. Possessing such a structure and framework for grants is grounded within organizational values and objectives, such as striving to be the top research institution for a given region, country or sector. These high-level goals and pathways to maturing capabilities seem to be missing in many places in partner countries. If that can be changed, perhaps as a spin-off from cooperative research involvement, the route to developing better self-sustained research programs may in the future be accepted as having an agile playbook to capture external funding across multiple sources, as well as developing a diverse portfolio of capabilities and expert staff.

Recognizing and encouraging successful networks, such as the ones we have described, are important for growing and sustaining collaborations which in turn mature into sustainable new capabilities (11–14). In this work, singular projects among DTRA BTRP/IBSP and DTRA BTRP/GBP funded work created networks among common Kazakhstani partner scientists at common institutes. Ideally, diverse teams that represent not just various disciplines and backgrounds but also cross national boundaries help reinforce creativity and avoid one-dimensional group thought. The networks we have described also demonstrate strong communication among peers and a refreshing transparency, both of which promote ideas and trust, and work toward resolving difficult issues such as access to benefits and sharing challenges, to increase further scientific capability. In that context, while there is only limited collaborative activity among the foreign funding partners at the executive level, each partner's scientific staff who implement the research awards are in direct working contact with staff from other partners. Most importantly, however, is that foreign scientists have established long-term trusting relationships with Kazakh colleagues that continue outside of funding periods and have, in some cases, lasted for close to 20 years. Such relationships form the real core of cooperative research efforts, in that they have an enduring benefit, rather than a transient one, in which lines of communication remain open and where technical issues and future plans can be freely and honestly discussed.

FUTURE PROSPECTS

In light of the COVID-19 pandemic, it is obvious that scientific and medical cooperation to enhance preparedness and response capability to global health events cannot be allowed to wait until a disaster is upon us. In the sense research starts with basic efforts that lead to applied and translational activity, and infectious disease surveillance provides the data that drive the process. These efforts all require investment in training and research, such as we have described for the Cooperative Biological Threat Reduction initiatives in this article.

There are numerous other cooperative research programs such as NIH Fogarty and ICAP that should be continued to reinforce existing networks and keep generate awareness for such activities. In Kazakhstan, there are additional instances of international collaboration other than the three programs we have described. For example, a memorandum of understanding was signed in 2019 between Ohio State University and the Kazakh National Agrarian University to collaborate on COVID-19 vaccine work, while the International Science and Technology Committee (ISTC) has been active there for many years, approving research work funded from its multinational contributors. Kazakhstani scientists also mentioned positive experiences through two international exchanges programs. The US Borlaug Fellowship Program offers a collaborative research program by pairing early-career scientists with a US mentor to study agricultural and veterinary topics. The Bolashak International Scholarship funded by the Government of Kazakhstan provides all-expenses-paid studies at the world's top universities and recipients return to Kazakhstan to provide 5 years of work service. The Ministry of Education and Science of Kazakhstan also has a Strategic Plan for 2020–2024 specific to further developing science under three priorities with various measures similar to what we have described (15). The benefits of these experiences help develop the next generation of leaders who can further mature their respective research programs in Kazakhstan, based on the formation of cooperative scientific arrangements.

AUTHOR CONTRIBUTIONS

KY, KT, FP, and JH developed the concept. EM, YS, ZS, AS, SF, SE, RH, and AR contributed to the manuscript. All authors reviewed and confirmed this work.

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Ideal Test Time for Coronavirus Disease 2019 Contact Tracing

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Background: Epidemiological contact tracing is a powerful tool to rapidly detect SARS-CoV-2 infection in persons with a close contact history with COVID-19-affected patients. However, it remains unclear whom and when should be PCR tested among the close contact subjects.

Methods: We retrospectively analyzed 817 close contact subjects, including 144 potentially SARS-CoV-2-infected persons. The patient characteristics and contact type, duration between the date of the close contact and specimen sampling, and PCR test results in PCR positive and negative persons were compared.

Results: We found that male gender {adjusted odds ratio 1.747 [95% confidence interval (CI) 1.180–2.608]}, age $\geq$ 60 [1.749 (95% CI 1.07–2.812)], and household contact [2.14 (95% CI 1.388–3.371)] are independent risk factors for close contact SARS-CoV-2 infection. Symptomatic subjects were predicted 6.179 (95% CI 3.985–9.61) times more likely to be infected compared to asymptomatic ones. We could observe PCR test positivity between days 1 and 17 after close contact. However, no subject could be found with a Ct-value $<$ 30, considered less infective, after day 14 of close contact.

Conclusions: Based on our results, we suggest that contact tracing should be performed on the high-risk subjects between days 3 and 13 after close contacts.

Keywords: coronavirus disease 2019, contact duration, contact tracing, cycle threshold, risk factor, household contact

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INTRODUCTION

The ongoing coronavirus disease (COVID-19) pandemic is one of the biggest global challenges for the healthcare and economic systems (1). COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has affected more than 150 million individuals and caused over 2.3 million deaths as of February 21, 2021 (2). The case fatality rates of COVID-19 differ from country to country, depending on the healthcare systems and health policies (3). Exceeding the healthcare capacity of intensive care units could lead to the collapse of the medical services and trigger a mortality rate increase (4, 5). During the pandemic, several aspects of the characteristics of SARS-CoV-2 have been unveiled (6), indicating that asymptomatic and pre-symptomatic carriers are potentially important infection sources. To control the spread of infection, it is important to intervene in the transmission chain. Adequate preventive measures, such as social distancing, hand hygiene, and wearing masks, are recommended for the public (7). At the national level, it is

necessary to apply quarantine policies for infected and suspected cases, including asymptomatic ones (8, 9).

COVID-19 affected 0.4 million individuals and caused 6.4 thousand deaths in Japan as of February 9, 2021 (10). Close contacts identified using the Japanese guideline bore a high infection probability (11). Yokohama is the second largest city in Japan, and the number of COVID-19 cases was ∼18,800 (0.5% of the population) as of February 9, 2021 (12). Three different COVID-19 waves occurred in Japan: April 2020, July to August 2020, and January 2021. The number of infected persons was relatively low compared to that in other countries. A potential reason for the low infection numbers could be thanked to active epidemiological surveys and contact tracing, which turned prove to be effective virus control policies in Japan (13). The public health centers of local governments conducted active epidemiological surveys for contact tracing driven by the intention to minimize cluster outbreaks and limit spread of the infection. These surveys enable the early detection and isolation of asymptomatic COVID-19 patients. Since February 2020, Yokohama City University Hospital has provided an outpatient clinic for contact tracing, in collaboration with the local government. As the COVID-19 transmission dynamics in the close contact of infected individuals are not understood well-enough, the outpatient clinic for contact tracing has been targeting this particular population. However, the risk factors for SARS-CoV-2 infection in the close contact cohort are still unknown. Moreover, it also remains elusive when to screen the close contact persons. In this study, we investigated the risk factors for SARS-CoV-2 infection between close contact persons and COVID-19 patients and established the ideal timeframe of sample collection for screening.

MATERIALS AND METHODS

This single-center retrospective cohort study aimed at optimizing epidemiological COVID-19 contact tracing surveys at Yokohama City University Hospital from February 1, 2020, to January 31, 2021. All patients who visited our outpatient clinic for COVID-19 contact tracing during the study period were included in the investigation. The close contacts of patients with COVID-19 were identified by the public health center in Japan according to the official criteria: contact with <1 m distance, contact >15 min, and contact without wearing adequate masks with or without symptoms. This policy was maintained throughout this study period. When the public center noticed the close contact cases regarding to the criteria, the public center performed a contact tracing investigation and PCR test for all subjects who met the official criteria as soon as possible. All close contacts were randomly allocated to a specialized medical institution for investigation by the public health center. The exclusion criteria included overseas travelers and those with repeated visits for negative PCR confirmation. There is no repetitive test per person included in the analysis. After excluding 105 subjects, finally 817 subjects were retrospectively analyzed. The following patient characteristics were collected: age, sex, contact type (household, verbal interactions such as meetings at workplaces, eating a meal together, and other types of close contact), duration between the date of the close contact and specimen sampling, and PCR test results. When the contact was continuous, the duration of contact was calculated starting from the onset date of the patient in contact. We also analyzed whether the patient was symptomatic or asymptomatic. The symptoms included fever, respiratory symptoms, digestive symptoms, and loss of smell or taste. Patient data were retrospectively examined using medical records. The Institutional Review Board of Yokohama City University Hospital approved this study (approval number B200200047). For all patients, consent for participation for this retrospective study was obtained by disclosing the clinical study, including the description of opt-out (https://www.yokohama-cu.ac.jp/amedrc/ethics/ethical/fuzoku_optout.html).

Outpatient Clinic for Contact Tracing

Patients referring to our hospital were placed in a separate outpatient clinic in the emergency room. After the clinical interview, nasopharyngeal swab or saliva samples were collected for PCR testing using a nasopharyngeal swab and transport media (COPAN, Brescia, Italia) or 2 mL of saliva. Saliva sampling specimens were preferred after their approval for PCR testing in June 2020. A nasopharyngeal swab was used for testing subjects before June 2020 and those who ate or drink within 30 min of sampling specimens. The applied collection method was chosen individually after consultation with the patient. The collected PCR samples were packed securely and sent to the Yokohama City Institute of Public Health for PCR testing. The PCR testing was performed according to the Manual for Detection of Pathogen 2019-nCoV provided by the National Institute of Infectious Disease in Japan (14).

Statistical Analysis

The results are presented as the mean for the quantitative data and frequency (percentage) for the categorical data. Continuous data are presented as means and 95% confidence intervals (CIs) or medians and interquartile ranges (IQRs). Data were analyzed by performing two-tailed Mann–Whitney *U*-test for comparisons of continuous variables between two groups and Fisher's exact test for comparisons of categorical data. Multivariate logistic regression analyses were performed to investigate the predictors of SARS-CoV-2 positivity, and adjustments were made for potential confounders: male sex, age (including age $\geq$ 60 years), presence of symptoms, sampling specimens, and type of contact. The results of bivariate analysis indicated that the listed factors contributed significantly to SARS-CoV-2 positivity. Simple linear regression analysis was used for analyzing the association between the date after the contact and the Ct values of PCR specimens. $P < 0.05$ were considered statistically significant. All statistical analyses were performed using the JMP Pro 15 (SAS Institute Inc., Cary, NC, USA) and Prism 7.9 J softwares for Windows and the Prism 9.0 software for Macintosh (GraphPad Software, Inc., San Diego, CA, USA).

TABLE 1 | Screened characteristics of the subjects in epidemiological close contact tracing.

		N (%)	PCR positive	PCR negative	*P*-value
Gender	male	409 (50.1)	83 (57.6)	326 (48.4)	0.0536
	female	408 (49.9)	61 (42.4)	347 (51.6)	
Age	Age ≥ 60	137 (16.8)	33 (22.9)	104 (15.5)	0.0363*
	Age < 60	680 (83.2)	111 (77.1)	569 (84.5)	
Subject symptoms	Symptomatic	116 (14.2)	54 (37.5)	62 (9.2)	<0.0001*
	Asymptomatic	701 (85.8)	90 (62.5)	611 (90.8)	
Specimen sampling	Nasopharyngeal swab	505 (61.8)	96 (66.7)	409 (60.8)	0.1874
	Saliva	312 (38.2)	48 (33.3)	265 (39.4)	
Types of contacts	Household contact	523 (64.0)	109 (75.7)	414 (61.5)	0.0011*†
	Eat together	142 (17.4)	16 (11.1)	126 (18.7)	
	Talk together	109 (13.3)	14 (9.7)	95 (14.1)	
	Other	43 (5.3)	5 (3.5)	38 (5.6)	

*Statistically significant by Fisher's exact test.
† Compared with eat together, talk together, and others.

RESULTS

Outpatient Characteristics for Contact Tracing

Between February 1, 2020, and January 31, 2021, a total of 922 outpatients allocated by the public health center underwent a medical examination at our hospital. Of these outpatients, 105 were excluded due to our exclusion criteria. Hence, a total of 817 consecutive patients were enrolled in this study. The mean participant age was 36.2 years (range: 0–91 years), and 409 patients (50.1%) were men. The mean duration between the contact and sample collection was 7.0 days (range: 0–19 days). All subjects were asymptomatic or had very mild symptoms at the time of visit. A total of 701 (85.8%) patients were asymptomatic and 505 (61.8%) patients were assessed using nasopharyngeal swab samples. The most common contact type was household contact (523, 64.0%), followed by eating together (142, 17.4%), talking (109, 13.3%), and others (43, 5.3%) (**Table 1**).

PCR Positivity

Overall, 144 (17.6%) patients tested positive for SARS-CoV-2 by PCR. In total, 19.0% (96/505) and 15.4% (48/312) of the patients tested positive for SARS-CoV-2 by PCR using a nasopharyngeal swab and saliva samples, respectively. Our univariate analysis showed that the number of household contact subjects (75.7%) was significantly higher in the PCR positive group compared with the PCR negative group. Moreover, the numbers of subjects with symptoms (37.5 vs. 9.2%) and subjects aged ≥60 years (22.9 vs. 15.5%) were higher in the PCR positive group compared with PCR negative group (**Table 1**). Our multivariate analysis, performed to exclude confounding biases, showed that male gender (adjusted odds ratio, 1.747), age ≥60 (1.749), being symptomatic at hospital visit (6.179), and household contact (2.14) were risk factors for SARS-CoV-2 infection for the close contact subjects with COVID-19 (**Table 2**).

TABLE 2 | Multivariate analysis of risk factors for SARS-CoV-2 infection on the close contact persons of patients with COVID-19.

	Adjusted odds ratio [95% confidence interval]
Male gender	1.747 [1.18, 2.608]*
Age ≥ 60 year old	1.749 [1.07, 2.812]*
Symptomatic	6.179 [3.985, 9.61]*
Nasopharyngeal swab†	1.226 [0.8184, 1.855]
Household contact‡	2.14 [1.388, 3.371]*

*Statistically significant.
† Compared with saliva specimens.
‡ Compared with eat together, talk together, and others.

PCR Positivity and Duration Between Close Contact and Specimen Collection

We analyzed the duration after close contact to specimen collection and PCR positivity of the subjects having close contact with COVID-19. Of the 817 subjects, the PCR positivity rates were the highest on day 11 (32.6%) and were higher than 10% between days 3 and 15 (**Figure 1A**). Of 701 asymptomatic subjects at the time of specimen collection, the PCR positivity rate was over 10% between days 4 and 13, and the highest rate of PCR positivity could be observed on day 11 (25.0%) (**Figure 1B**). There were no subjects with Ct values <30 observed on days 14–17.

Association Between Duration of Specimen Collection and Ct Values of PCR Positive Subjects

Since positive PCR results alone do not mean that infectious virus is shed, based on previous reports (6), we analyzed the Ct values of the 144 PCR positive samples. The results of the analysis and the duration from specimen collection after close contact are shown in **Figure 2**. The Ct values were larger (reflecting

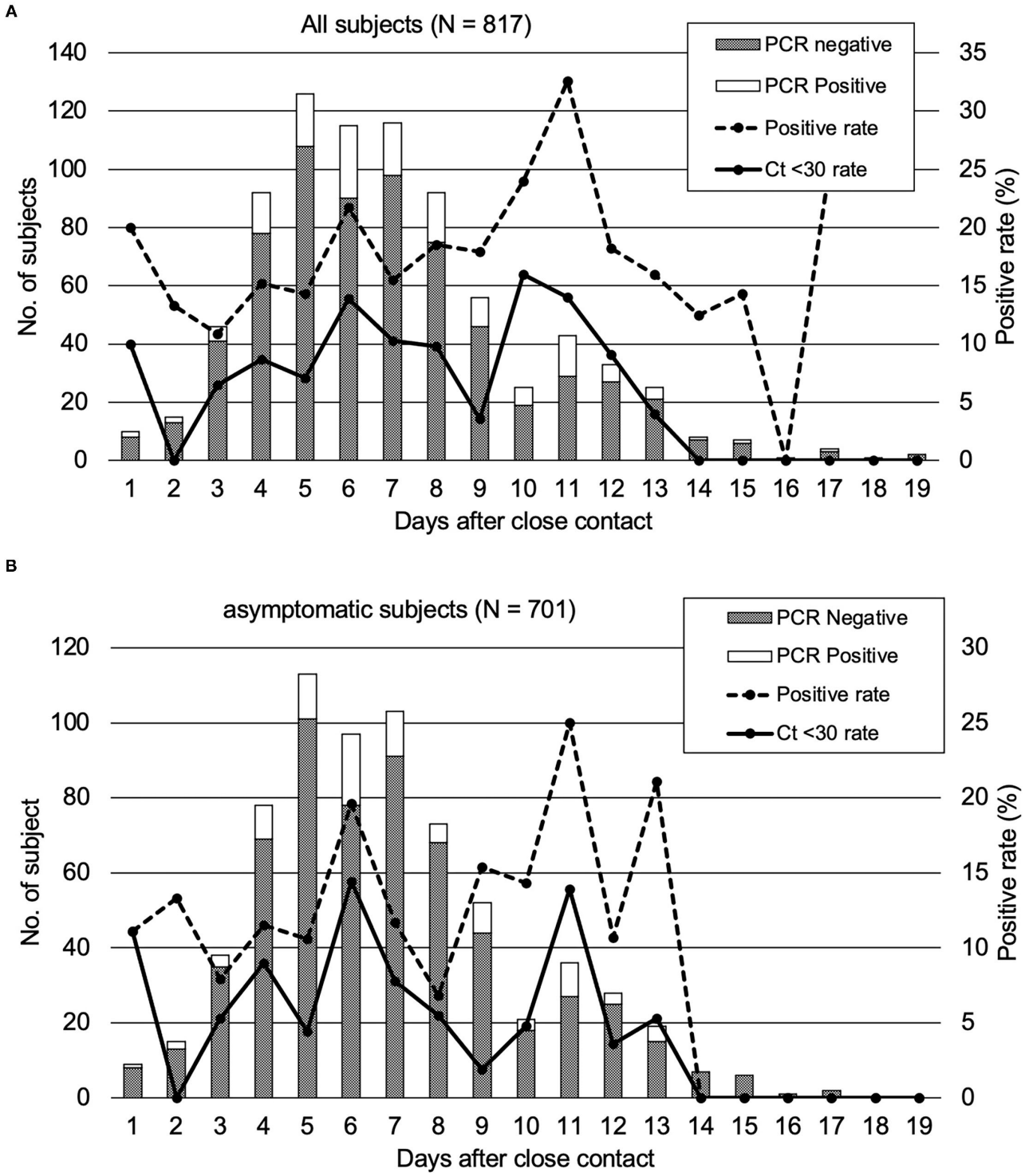

FIGURE 1 | PCR positivity of the subjects who underwent close contact PCR tests. The rates of the subjects with a high viral load (Ct < 30) were also plotted. **(A)** All close contact subjects in our study. **(B)** asymptomatic subjects.

reduced viral amounts) as time passed after the close contact. We found positive correlation between the Ct value and the days after contact (Ct: y = 0.3979 x + 25.87 [slope: 95% CI, −0.0203–0.8161]) (p = 0.062). As the time since specimen collection increased, the Ct-values tended to increase, indicating a decrease in the viral load of the specimens. According to this

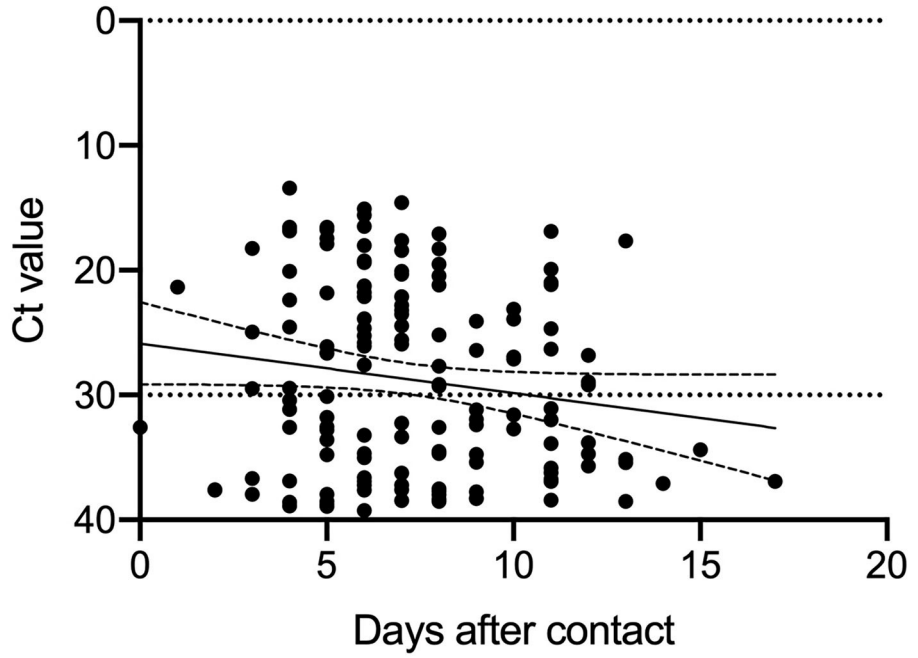

FIGURE 2 | Ct values of the specimens taken from the close contact tracing subjects.

equation, 11.6–17.5 days were required to achieve a Ct-value ≥30. **Figure 1A** shows the subjects with a high viral load (Ct ≤ 30) and the days after close contact. PCR positivity could be detected between days 3 and 13 with the highest rate of subjects with a high viral load on day 10 (16.0%).

DISCUSSION

In this study, we established two major results. First, on the epidemiological close contact tracing, male gender, age ≥ 60, and household contacts were independent infection risk factors for the close contacts of infected persons. Furthermore, symptomatic subjects were considered to be highly suspicious of being infected with SARS-CoV-2. Second, the close contact persons of patients with COVID-19 presented PCR positivity for up to 17 days following the close contact, although subjects with a high viral load (Ct ≤ 30) could be found up to day 13.

PCR positivity was detected in close contacts up to 17 days after the contact. The subjects tested between days 14 and 17 after the close contact were suggested still possessed viral RNA in the saliva or nasopharyngeal specimens but not infective to other persons (**Figures 1A,B**). No subject exhibited high viral load (Ct-value < 30) on days 14–17. Therefore, they were considered to exhibit a reduced viral load and less infectious to other people. In order to intervene in the second infection from the close contact subjects of patients with COVID-19, a contact tracing test had to be performed between days 3–13 after the close contact to prevent further expansion. This approach was applicable for all the subjects with a close contact history regardless their symptomatic or asymptomatic status at the time of being PCR tested. Saliva specimens were reported to be less sensitive for PCR testing than nasopharyngeal specimens (15). In contrast, another research has reported that saliva specimens were more sensitive for PCR testing in asymptomatic or mild COVID-19 patients (16).

The Ct value was reportedly dependent on the period from infection and useful for determining infectivity (17). That is, as the Ct value reflects the viral load, the subjects with higher Ct-values (Ct ≥ 30) are thought to be potentially less infectious. In particular, the samples with Ct-values over 30 were no longer cultured and did not show infectivity (6, 18). Hence, during the contact tracing, the infectious potential lasts for ∼2 weeks after the contact even in the asymptomatic subjects. The period at risk of infectious potential that we established is consistent with the previous estimation of the COVID-19 incubation and elimination periods. Considering the infectious potential, the PCR positive subjects, even the asymptomatic ones, should be properly quarantined to break the transmission chain.

Male gender, age ≥ 60, household contact, and symptomatic subjects were the four independent infection risk factors for the close contacts in this study. It is reasonable to think that symptomatic patients were mostly PCR positive. The gender-based immunity difference could also be considered as an underlying mechanism. About gender differences in COVID-19 epidemiology, a previous study reported that more male than female patients might tend to be severe by the disease (19). Therefore, there are reasons to speculate that men are more susceptible to COVID-19 than women. Among the contact types, household contact was the most common and an independent risk factor for COVID-19. Therefore, the strategy of isolating the infected persons within the household could be reasonable.

According to our study, the potential reason for the low infection numbers in Japan may be effective contact tracing, which successively detected to isolate the symptomatic and asymptomatic COVID-19 patients. At the same time, the manual survey for contact tracing driven by public health centers of local governments has a limit when the infection spreads explosively. The survey at the time of infection spread prone to staff shortages and delays in investigations. An emergency volunteer-run contact tracing survey was reported to fall short of adequate time and information (20). Furthermore, only 3 days delay of isolation of infectious person was reported leading to infection control failure (21). For that reason, digital contact tracing is expected, but ethical issues have been pointed out. Therefore, it is important to inform people of the high-risk situation (male gender, age $\geq$ 60, household contact, and symptomatic subjects) we have reported and to enable voluntary quarantine for 2 weeks. In that sense, the results of our study contribute to the infection control of SARS-Cov-2.

Nevertheless, our study has certain limitations. First, it consisted of a retrospective review at a single center with a limited number of participants. The characteristics of our patients referred by the public health center might depend on the study region and hospital characteristics. It was difficult for us to continuously pursue the subjects and obtain PCR specimens. The situations such as knowledge and approach to COVID-19 and the number of newly diagnosed COVID-19 patients had changed during this study, this study was possibly heterogenous in the beginning and the end of this study. The number of positive cases may have been underestimated if some subjects became positive at any timepoint after our examination. We were not able to directly compare nasopharyngeal swab specimens and saliva on the same subject. It was a limitation that we were not able to employ nasopharyngeal swab or saliva for PCR test throughout this study. The contact with patients with COVID-19 was self-reported, and the closeness of the contact and infection probability might depend on the situation reported. Therefore, further large-scale epidemiological studies would be required to obtain more concrete evidence on the SARS-CoV-2 transmission dynamics in the tracing of close contacts.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Yokohama City University Hospital (B200200047). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SM and HK contributed to the conception and design of the study, acquisition of data, and analysis and interpretation of data. SM, HK, NT, KS, HO, CK, and SU collected the clinical data. SM wrote the draft. HK conducted the statistical analysis. HN and TY supervised all aspects of this study. All authors revised critically, approved the final version of the manuscript, and attest to meeting the four ICMJE authorship criteria.

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