

Advances in emerging coronavirus identification and tracing methods

Edited by

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Advances in emerging coronavirus identification and tracing methods

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Editorial: Advances in emerging coronavirus identification and tracing methods

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Editorial on the Research Topic

Advances in emerging coronavirus identification and tracing methods

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) gave rise to a viral pneumonia outbreak in China late 2019 and has continued spread rapidly across the globe becoming an unprecedented pandemic for over 3 years (Lu et al., 2020; Rambaut et al., 2020; Zhu et al., 2020). The World Health Organization (WHO) has reported more than 600 million confirmed coronavirus disease 2019 (COVID-19) cases and over six million deaths globally (<https://covid19.who.int>). Soon after the first COVID-19 case was reported and spread, the SARS-CoV-2 virus genome mutations gave rise to new variants, five of which have been classified as variants of concern (VOC) by the WHO (Alpha, Beta, Gamma, Delta, and Omicron), which also spread globally. SARS-CoV-2 VOCs can cause severe disease, increase infectivity, reduce neutralization by antibodies elicited from prior infections or vaccinations, and limit the efficacy of vaccine immunity (Karthikeyan et al., 2022; Oude Munnink and Koopmans, 2023). Therefore, rapid and convenient methods for detecting and tracing SARS-CoV-2 variants are required.

The Research Topic, “Advances in emerging coronavirus identification and tracing methods,” covered the latest developments and applications for differential diagnoses and origin tracing methods. The Research Topic contains 19 articles: five focused on monitoring the emergence of variants, 10 emphasized the development, evaluation, comparison, and application of efficient and easy-to-use methods (the targets of which are usually nucleic acids, antigens, and antibodies), and four expanded the application of mechanistic research.

The increasing prevalence of new SARS-CoV-2 variants can alter viral transmissibility, virulence, antigenicity, and recognition by the adaptive immune system triggered by prior vaccination; thus, characterizing and cataloging viral variants are crucial. Five articles were included related to this theme. First, Padilla-Blanco et al. performed a pilot study exploring viral variant circulation on Sicily Island, for which variation information is scarce. This study used reverse transcription polymerase chain reaction (RT-PCR) amplification and sequencing of selected viral genomic regions to characterize the variants, providing vital information on the predominant variants and their circulation during a specific pandemic wave in this insular region. Moreover, the SARS-CoV-2 virus evolves from mutations and the natural selection of variants. To address this issue, Tsuchiya et al. performed a comprehensive genomic analysis

of 112 SARS-CoV-2 strains detected in Japan and simultaneously processed the genomic data from all of Japan deposited in the Global Initiative on Sharing Avian Influenza Data to investigate the pattern of mutations and examine the relationship between amino acid changes and the transmissibility and severity of each strain or lineage. However, as stated in the article, the lack of experimental evidence employing recombinant SARS-CoV-2 with or without particular amino acid alterations to support the impact of mutations was a drawback of this study. Similarly, to understand the molecular determinants associated with mutation-driven evolution, [Pal et al.](#) adapted nanopore sequencing to investigate the molecular evolution of the SARS-CoV-2 genome during the first, second, and third COVID-19 waves in Uttar Pradesh, India, which helped identify the key mutational combinations that led to vaccination failures in India and shed light on how the virus's binding affinity changed. Moreover, to explore SARS-CoV-2 evolution in specific populations, [Hosaka et al.](#) targeted immunosuppressed patients in a nosocomial cluster in Japan, performing whole-genome sequencing analyses to examine the evolution of mutations in this cluster. As a result, they identified evidence of emerging mutation acquisition during transmission, emphasizing the necessity of improved infection control measures to prevent nosocomial infections among immunosuppressed patients. Finally, SARS-CoV-2 shedding via human feces has resulted in viral genome detection in human sewage. Therefore, [Barbé et al.](#) developed a wastewater-based sequencing process to track shifts in variant predominance using Oxford Nanopore Technology, which is time-efficient and cost-saving. Notably, constrained by the complexity of SARS-CoV-2 strains in the sewage matrix, this approach could only detect mutations in conjunction with a genome site instead of strains, which is indirect proof of the presence of a lineage. Together, these articles highlight the urgent need to develop detection and tracing methods for SARS-CoV-2 with high sensitivity, specificity, and throughput.

Three articles were collected that compared detection and tracing methods and statistically analyzed SARS-CoV-2 variants. The need for tools to identify SARS-CoV-2-positive individuals is urgent. Thus, [Cabrera et al.](#) conducted a study early in the pandemic that assessed diagnostics methods for SARS-CoV-2 positivity. [Wu et al.](#) also assessed the impact of the COVID-19 pandemic on the population, finding that SARS-CoV-2 antibody assays may have an adjunct role in the diagnosis and exclusion of COVID-19, especially high-throughput technologies, such as enzyme immunoassays or chemiluminescent immunoassays (CLIAs). Finally, [Windsor et al.](#) compared multiple SARS-CoV-2 serology reference materials to the WHO International Standard (WHO IS) to determine their utility as secondary standards using an international network of laboratories with high-throughput quantitative serology assays. Their findings indicated that the arbitrary WHO IS unit does not accurately compare SARS-CoV-2 serology results between different laboratories or methods. This study also showed that even after converting to international or binding antibody units, candidate secondary material results differed drastically between the laboratory methods. Currently, there are three main SARS-CoV-2 detection methods: traditional culture, immunological, and molecular methods. Culture is the gold standard for pathogen identification. However, this method

is technically demanding and time-consuming, thus, is not widely used for early SARS-CoV-2 screening. In contrast, molecular and immunological detection methods are common, simple, convenient, and rapid.

Additional seven research articles were included to analyze SARS-CoV-2 detection and tracing. First, [Lin et al.](#) developed an RAA/CRAA /CRISPR-Cas12a-mediated assay to specifically distinguish major SARS-CoV-2 variants. All reactions were conducted in sealed tubes without requiring complex equipments or facilities. Therefore, this simple and rapid assay could be implemented in resource-limited settings. Furthermore, these methods can be simplified for high-throughput multiplex screening in combination with sophisticated microfluidic devices. [Yu et al.](#) developed a visual nucleic acid detection method combining reverse transcription loop-mediated isothermal amplification and a vertical flow visualization strip (i.e., RT-LAMPVF), which does not require special equipment, has broad applicability, and is expected to achieve on-site real-time detection without needing to transport samples, making it especially useful for screening in airports and train stations. [Tanimoto et al.](#) quantified the SARS-CoV-2 RNA copy number using reverse transcription quantitative real-time PCR with primers and probes targeting the N gene, allowing for the detection of both wild-type and variant SARS-CoV-2 strains in sewage samples from two wastewater treatment plants in Kobe City, Japan, during the fourth and fifth COVID-19 pandemic waves (between February 2021 and October 2021). They found that quantitative RNA studies in sewage could be useful for administrative purposes related to public health, including issuing warnings and implementing preventive measures within sewage basins. Regarding immunological detection methods, [Wang et al.](#) obtained two monoclonal antibodies that recognized the recombinant porcine Delta coronavirus nucleocapsid protein, reporting high coincidence rates compared to reverse transcription-quantitative PCR results in only 15 min, allowing for rapid diagnosis and early control of the disease. Moreover, [Choi et al.](#) compared three CLIAs, three lateral flow immunoassays, and a surrogate virus neutralization test assay. With vaccine administration, routine antibody tests for COVID-19 were also initiated in general laboratories worldwide. Therefore, to select the most suitable serological assay for a particular laboratory environment, it is necessary to understand the characteristics of each assay. Additionally, [Cai et al.](#) evaluated non-specific reactivity in SARS-CoV-2 serological tests in 46,777 post-pandemic samples, reporting an unspecific reactivity incidence rate of 0.361% involving 14 disease spectrum categories. These results indicate that unspecific reactivity must be excluded when using serological antibody testing for COVID-19 epidemiological analysis or virus tracing. In addition, nanopore sequencing is increasingly used for whole-genome sequencing of SARS-CoV-2 since it is simple, fast, and provides real-time results. Finally, [Luo et al.](#) found that the Q20+ kit was more accurate than previous nanopore sequencing kits, especially for sequencing long amplicons, which could promote epidemic prevention and control and improve SARS-CoV-2 traceability analyses. An essential component, such as proteins or lipids, may influence viral replication and interaction activities. Therefore, several mechanistic studies

have explored these correlations, which could help expand treatment strategies.

To clarify whether non-structural proteins (nsps) are indispensable for viral replication and transcription, Jin et al. examined the replication activity of the viral replicon by deleting individual nsps. They discovered that the dependence of viral replication on individual nsps varied significantly, providing a new perspective on the role of nsps in viral replication and transcription. This information also suggests that nsps are a potential target for antiviral drug development. Strategies or approaches that could lead to therapeutic options for SARS-CoV-2 are also of interest. For example, Wang et al. utilized phage display to search for peptides that likely inhibited S protein binding to cellular angiotensin-converting enzyme 2 (ACE2). As a result, they identified two potential 12-aa peptides, which were further exploited to produce peptidomimetics, the intercepts of which were verified experimentally. It is worth emphasizing that these peptides or their derivatives may be developed into therapeutic regimens that interrupt virus-host attachment and hinder disease onset. Furthermore, Ishigaki et al. investigated the protective efficacy of the rDIs-S vaccine, a recombinant DI strain carrying the SARS-CoV-2 spike-encoding gene, against SARS-CoV-2 infection in a non-human primate model and heterologous human ACE2-expressing mouse model. The results indicated that vaccination with rDIs-S could prevent protein expression related to the severe pathogenic effects of SARS-CoV-2 infection and restore protein expression related to immune responses. Moreover, previous studies have reported dyslipidemia in patients with COVID-19, thus, Zhao et al. conducted an extensive study comparing serum lipid profiles among different cohorts and a bioinformatics analysis to explore the possible relationship between viral pathogenesis and metabolic reprogramming mechanisms, which may be a target for developing antivirals against SARS-CoV-2.

This review topic comprised studies on advances in coronavirus identification and tracking methods, emphasizing efficient and easy-to-use methods. These methods target nucleic acids, antigens, and antibodies and apply to coronavirus identification, differential diagnoses, and origin tracing.

Collectively, these studies considerably benefit the disease research field by presenting ways to cut off transmission routes and formulating epidemic prevention and control strategies for COVID-19.

Author contributions

JP was a guest associate editor of the Research Topic and wrote the paper text. Y-WT and ZS were guest associate editors of the Research Topic and edited the text, respectively. All authors contributed to the article and approved the submitted version.

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References

- Karthikeyan, S., Levy, J. I., De Hoff, P., Humphrey, G., Birmingham, A., Jepsen, K., et al. (2022). Wastewater sequencing reveals early cryptic SARS-CoV-2 variant transmission. *Nature* 609, 101–108. doi: 10.1038/s41586-022-05049-6
- Lu, H., Stratton, C. W., and Tang, Y. W. (2020). Outbreak of pneumonia of unknown etiology in Wuhan, China: the mystery and the miracle. *J. Med. Virol.* 92, 401–402. doi: 10.1002/jmv.25678
- Oude Munnink, B. B., and Koopmans, M. (2023). Tracking SARS-CoV-2 variants and resources. *Nat. Methods* 20, 489–490. doi: 10.1038/s41592-023-01833-y
- Rambaut, A., Holmes, E. C., O'Toole, A., Hill, V., McCrone, J. T., Ruis, C. et al. (2020). A dynamic nomenclature proposal for SARS-CoV-2 lineages to assist genomic epidemiology. *Nat. Microbiol.* 5, 1403–1407. doi: 10.1038/s41564-020-0770-5
- Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733. doi: 10.1056/NEJMoa2001017



Performance of Severe Acute Respiratory Syndrome Coronavirus 2 Serological Diagnostic Tests and Antibody Kinetics in Coronavirus Disease 2019 Patients

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Serological testing is recommended to support the detection of undiagnosed coronavirus disease 2019 (COVID-19) cases. However, the performance of serological assays has not been sufficiently evaluated. Hence, the performance of six severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) binding antibody assays [three chemiluminescence (CLIAs) and three lateral flow immunoassays (LFIAs)] and a surrogate virus neutralization test (sVNT) was analyzed in a total of 988 serum samples comprising 389 COVID-19-positives and 599 COVID-19-negatives. The overall diagnostic sensitivities of CLIAs and LFIAs ranged from 54.2 to 56.6% and from 56.3 to 64.3%, respectively. The overall diagnostic specificities of CLIAs and LFIAs ranged from 98.2 to 99.8% and from 97.3 to 99.0%, respectively. In the symptomatic group ($n = 321$), the positivity rate increased by over 80% in all assays > 14 days after symptom onset. In the asymptomatic group ($n = 68$), the positivity rate increased by over 80% in all assays > 21 days after initial RT-PCR detection. In LFIAs, negatively interpreted trace bands accounted for the changes in test performance. Most false-positive results were weak or trace reactions and showed negative results in additional sVNT. For six binding antibody assays, the overall agreement percentages ranged from 91.0 to 97.8%. The median inhibition activity of sVNT was significantly higher in the symptomatic group than in the asymptomatic group (50.0% vs. 29.2%; $p < 0.0001$). The median times to seropositivity in the symptomatic group were 9.7 days for CLIA-IgG, 9.2 and 9.8 days for two CLIAs-Total (IgM + IgG), 7.7 days for LFIA-IgM, 9.2 days for LFIA-IgG, and

8.8 days for sVNT-IgG, respectively. There was a strong positive correlation between the quantitative results of the four binding antibody assays and sVNT with Spearman ρ -values ranging from 0.746 to 0.854. In particular, when using LFIAs, we recommend using more objective interpretable assays or establishing a band interpretation system for each laboratory, accompanied by observer training. We also anticipate that sVNT will play an essential role in SARS-CoV-2 antibody testing and become the practical routine neutralizing antibody assay.

Keywords: SARS-CoV-2, antibody, performance, kinetics, binding antibody, neutralizing antibody

INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in December 2019 and has become a pandemic with continued transmission (Fong et al., 2020; Wu and McGoogan, 2020). In Korea, the first COVID-19 case was confirmed in January 2020 (Korea Centers for Disease Control and Prevention, 2021). The Korean government has wisely established a strategy against COVID-19 consisting of swift implementation of *in vitro* diagnostic (IVD) devices in disease prevention and control sites, early and extensive testing using accurate real-time reverse transcriptase-polymerase chain reaction (RT-PCR) testing, systematic contact tracing, and quarantine measures (Ministry of Food and Drug Safety, 2021). Therefore, it is conceivable that the proportion of undetected patients with COVID-19 is minimal (Song et al., 2020). Nonetheless, it is possible that the undiagnosed cases, including asymptomatic patients and symptomatic patients who visit the hospital later in disease and who test negative by molecular assays, may impede the effective control of disease spread (Bae et al., 2021; Zhang et al., 2021). Hence, serological testing is recommended to support the detection of such undiagnosed cases (Guo et al., 2020). Serological testing is also essential for surveys to know the epidemic curve and set the surveillance strategy, integral to pandemic control measures. Furthermore, serologic testing helps determine antibody kinetics to predict the infection severities and outcomes in SARS-CoV-2 infection. Combined with RT-PCR, detection of the production of immunoglobulin (Ig) class can be a valuable tool to enhance sensitivity and accuracy for the detection of COVID-19. Few studies have evaluated the seroconversion of IgG or M using several commercial serologic assays (Guo et al., 2020; Orner et al., 2021).

Several types of assays have been developed for the detection of SARS-CoV-2 antibodies. As of September 9, 2021, the Korean Ministry of Food and Drug Safety approved 62 COVID-19 diagnostic reagents, including 28 PCR assays, 20 antigen assays, and 14 antibody assays (Innovative and Diagnostic Medical Device Policy Division, 2021). Among the 14 antibody assays, seven are lateral flow immunoassays (LFIAs), five are enzyme-linked immunosorbent assays (ELISAs), and two are chemiluminescent immunoassays (CLIAs). LFIAs, usually used at the point of care, detect antibodies using immunochromatographic chemistry. Manual or semiautomated

96-well ELISAs and fully automated CLIA/chemiluminescent microparticle immunoassays (CMIA) are available to measure specific antibody subclasses such as IgA, M, and G (Zhang et al., 2021). Most SARS-CoV-2 serologic assays have been developed to target antibodies for one of the two structural proteins: the most surface-exposed spike (S) protein that comprises S1 and S2 functional subunits or the most abundantly expressed nucleocapsid (N) protein. In addition, the receptor-binding domain (RBD), which is located in the S1 subunit and mediates viral entry, is a target for detecting SARS-CoV-2 antibodies (Satarker and Nampoothiri, 2020; Tai et al., 2020; Walls et al., 2020). Antibodies can be classified into two categories according to their responses to the virus: binding antibodies (bAbs) and neutralizing antibodies (nAbs). The bAbs act against the virus-infected cells via complement activation or opsonization; on the other hand, the nAbs bind to the viral structures that block viral attachment and entry for viral replication (Klasse, 2014; Zhang et al., 2021). The gold standard for detecting nAbs against SARS-CoV-2 is the conventional plaque reduction neutralization test, for which any live pathogen and biosafety level (BSL) 3 facility are essential, making routine evaluation difficult. Recently, an ELISA-based surrogate virus neutralization test (sVNT) designed to mimic the virus-host interaction using purified RBD and angiotensin-converting enzyme 2 (ACE2) was developed, which can be performed within 1–2 h in an ordinary BSL2 laboratory (Klasse, 2014; Tan et al., 2020). However, the performance and usefulness of serological assays for detecting SARS-CoV-2 bAbs or nAbs have not yet been thoroughly assessed. Here, we evaluated the diagnostic performance of seven SARS-CoV-2 serologic assays—six bAb assays and one nAb assay. Furthermore, we investigated the dynamic characteristics of the immune responses in patients with COVID-19.

MATERIALS AND METHODS

Sample Collection

A total of 988 serum samples were obtained from 786 patients, consisting of 199 COVID-19-positive patients confirmed using RT-PCR between March and November 2020, and 587 COVID-19-negative patients with no history of COVID-19 or any epidemiological relationship with COVID-19 between June 2019 and October 2020 at Chonnam National University Hospital (CNUH), Gwangju, South Korea. RT-PCR was performed using

TABLE 1 | Characteristics of SARS-CoV-2 antibody assays used in this study.

Assay	Manufacturer	Target Antibody	Antigen	Method	Analyzer	Cut-off	Sensitivity* [†] % (95%CI)	Specificity* % (95%CI)
SARS-CoV-2 IgG	Abbott	IgG	N	CMLA	ARCHITECT i2000SR	≥1.4 S/C	100 (95.9–100)	99.6 (99.1–99.9)
Elecsys Anti-SARS-CoV-2	Roche	Total (IgM + IgG)	N	ECLIA	Cobas e801	≥1.0 COI	99.5 (97.0–100)	99.8 (99.7–99.9)
ADVIA Centaur SARS-CoV-2 Total	Siemens	Total (IgM + IgG)	RBD in S1	CLIA	Centaur XP	≥1.0 S/CO	98.7 (93.2–99.8)	99.8 (99.5–99.9)
STANDARD F COVID-19 IgM/IgG Combo FIA	SD BIOSENSOR	IgM, IgG (separately)	N + S	LF-FIA	STANDARD F2400	≥1.0 COI	98.9 (93.8–99.9)	90.6 (85.0–94.7)
STANDARD Q COVID-19 IgM/IgG Combo	SD BIOSENSOR	IgM, IgG (separately)	N + S	LFIA	Manual	–	96.9 (91.3–99.4)	95.7 (92.3–97.9)
P4DETECT COVID-19 IgM/IgG	PRIME4DIA	IgM, IgG (separately)	N + S1	LFIA	Manual	–	96.7 (82.8–99.9)	100 (88.4–100)
SARS-CoV-2 Surrogate Virus Neutralization Test	GenScript	IgG (nAb)	RBD in S1	ELISA	ThunderBolt	≥30% [‡]	100 (87.1–100)	100 (95.8–100)

*Manufacturer specified sensitivity and specificity in each assay kit insert. [†]Sensitivity was based on samples collected ≥ 14 days after RT-PCR positive or symptom onset. [‡]Percentage inhibition (%) = $[1 - (\text{sampled optical density value/negative control optical density value})] \times 100$. CI, confidence interval; CLIA, chemiluminescence immunoassay; CMLA, chemiluminescent microparticle immunoassay; COI, cut-off index; COVID-19, coronavirus disease 2019; ECLIA, electrochemiluminescence immunoassay; IgG, immunoglobulin G; IgM, immunoglobulin M; LFIA, lateral flow immunoassay; LF-FIA, lateral flow fluorescence immunoassay; N, nucleocapsid protein; nAb, neutralizing antibody; RBD, receptor-binding domain; S1, subunit 1 of spike protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; S/C, sample/calibrator.

the PowerChek 2019-nCoV RT-PCR Kit (KogeneBiotech, Seoul, Korea). Serum remnants from blood samples retrieved for routine laboratory tests were aliquoted and stored at -80°C before the assays. This study was approved by the Institutional Review Board (IRB) of CNUH (IRB No. CNUH-2020-223). The IRB waived the requirement for informed consent because of the retrospective nature of this study.

Severe Acute Respiratory Syndrome Coronavirus 2 Antibody Assays

Seven serological assays were assessed in this study: three CLIAs [SARS-CoV-2 IgG (Abbott, Chicago, IL, United States); Elecsys Anti-SARS-CoV-2 (Roche, Basel, Switzerland); ADVIA Centaur SARS-CoV-2 Total (Siemens, Munich, Germany)], three LFIAs [STANDARD F COVID-19 IgM/IgG Combo FIA (SD Biosensor Inc., Gyeonggi-do, Korea), briefly SDF; STANDARD Q COVID-19 IgM/IgG Combo (SD Biosensor Inc.), briefly SDQ; P4DETECT COVID-19 IgG/IgM (PRIME4DIA Co., Gyeonggi-do, Korea), briefly P4D], and one SARS-CoV-2 sVNT kit (GenScript Biotech Co., NJ, United States) (Table 1). All samples were analyzed using six SARS-CoV-2 bAb assays. Because of insufficient sample volumes, only 418 serum samples, consisting of 385 samples from COVID-19-positive patients and 33 samples from COVID-19-negative patients with false-positive results from at least one of the binding antibody assays, were subjected to the SARS-CoV-2 sVNT testing. All assays were performed at the Diagnostic Immunology Laboratory of CNUH according to the manufacturer's instructions.

Statistical Analysis

The sensitivity [true positive/(true positive + false negative)], specificity [true negative/(false positive + true negative)], positive predictive value [PPV: sensitivity $\times$ prevalence/(sensitivity $\times$ prevalence + (1 - specificity) $\times$ (1 - prevalence))], negative predictive value [NPV: specificity $\times$ (1 - prevalence)/((1 - sensitivity) $\times$ prevalence + specificity $\times$ (1 - prevalence))], and accuracy [sensitivity $\times$ prevalence + specificity $\times$ (1 - prevalence)] for the three CLIAs and three LFIAs were calculated based on RT-PCR results and the history of COVID-19 or epidemiological relationship with COVID-19. For LFIAs, the separated and combined results of IgM and IgG were included in the performance analysis. The detection rates of SARS-CoV-2 antibody assays in known COVID-19-positive samples were assessed based on the number of days post symptom onset in the symptomatic group and the number of days post the initial positive RT-PCR detection in the asymptomatic group. The degree of agreement between assays was quantified using the agreement percentage and Cohen's kappa (κ) value and further evaluated by McNemar's test of asymmetry (McHugh, 2012; Perkmann et al., 2020). The Fisher's exact test was performed to calculate *p*-values for differences in proportions between assays. Normality tests were performed using the D'Agostino-Pearson test. The Mann-Whitney *U*-test was used to compare assay level results based on the number of days post symptom onset. The Spearman correlation coefficient (ρ) was used to measure the strength and direction of the correlation between four bAb assays vs. sVNT (Laerd Statistics, 2020). Statistical significance was set

at $p < 0.05$. All statistical analyses were performed using the MedCalc Diagnostic Test Evaluation Calculator¹ and GraphPad Prism software (version 5.03).

RESULTS

Study Population and Sample Characteristics

The clinical and demographic characteristics of the patients and samples used in this study are summarized in **Table 2**. Of a total of 988 serum samples, 389 (39.4%) were obtained from 199 COVID-19-positive patients [59.8% female; median age (IQR), 56 (38–67) years], whereas 599 (60.6%) were obtained from 587 COVID-19-negative patients [62.4% female; median age (IQR), 54 (38–68) years]. Of the 389 samples from COVID-19-positive patients, single samples were from 102 (51.3%) patients, and multiple samples were from 97 (48.7%) patients: two samples were from 48 patients; three from 27 patients; four from 12 patients; five from six patients; six from one patient; eight from one patient; and nine from two patients. The multiple samples from one patient were serially collected at different time points, showing that about one or two samples per week were collected as follows: the first samples (patient number = 97) were collected at 2 days post the diagnosis of COVID-19; the second ($n = 97$) at 9 days; the third ($n = 49$) at 12 days; the fourth ($n = 22$) at 17 days; the fifth ($n = 10$) at 18.5 days; the sixth ($n = 4$) at 16 days; the seventh ($n = 3$) at 19 days; the eighth ($n = 3$) at 23 days; and the ninth ($n = 2$) at 28.5 days (**Supplementary Table 1**). The median number of multiple samples given by the same patient was three. Of the 389 positive samples, 321 (82.5%) and 68 (17.5%) were obtained from 158 symptomatic and 41 asymptomatic COVID-19 patients, respectively. Of the 599 samples from COVID-19-negative patients, most (96.5%) were one sample per patient; 144 (24%) were collected during the pre-pandemic period from June 2019 to November 2019, which were all antinuclear antibody (ANA)-positive; and 455 (76%) were collected during the pandemic period from December 2019 to October 2020, consisting of 88 ANA-positive, 340 viral-infected or positive for antibodies other than SARS-CoV-2, and 27 bacterial or parasite antibody-positive.

Overall Diagnostic Performance of Severe Acute Respiratory Syndrome Coronavirus 2 Antibody Assays

The diagnostic performance of SARS-CoV-2 bAb assays is described in **Table 3**. The diagnostic sensitivity of the CLIAs ranged from 54.2 to 56.6%, with no significant difference between the assays ($p > 0.05$). The sensitivity of the LFIAs ranged from 56.3 to 64.3%, showing a significant difference between SDF and P4D ($p = 0.0279$). SDF showed the highest sensitivity among the six assays, which was significantly different from all the other assays except SDQ. The diagnostic specificity of the CLIAs ranged from 98.2 to 99.8%, with a significant difference between Roche

TABLE 2 | Characteristics of samples tested for SARS-CoV-2 antibody assays in this study.

Characteristics	Patients	Samples
Total number, n	786	988
Female/male, n	485/301	
Age, median (IQR), year	55 (38–68)	
COVID-19 positive patients, n (%)	199 (25.3)	389 (39.4)
Female/male, n	119/80	
Age, median (IQR), year	56 (38–67)	
Patient with given multiple samples, n (%)		
1 sample	102 (51.3)	102 (26.2)
2 samples	48 (24.1)	96 (24.7)
3 samples	27 (13.6)	81 (20.8)
4 samples	12 (6.0)	48 (12.3)
5 samples	6 (3.0)	30 (7.7)
6 samples	1 (0.5)	6 (1.5)
8 samples	1 (0.5)	8 (2.1)
9 samples	2 (1.0)	18 (4.6)
Symptomatic (Days after the onset of symptoms), n (%)	158 (79.4)	321 (82.5)
1–7 days		98 (30.5)
8–14 days		111 (34.6)
15–21 days		59 (18.4)
22–28 days		27 (8.4)
≥ 29 days		26 (8.1)
Asymptomatic (Days after initial RT-PCR detection), n (%)	41 (20.6)	68 (17.5)
1–7 days		47 (69.1)
8–14 days		13 (19.1)
15–21 days		5 (7.4)
22–28 days		1 (1.5)
≥ 29 days		2 (2.9)
COVID-19 negative patients, n (%)	587 (74.6)	599 (60.6)
Female/male, n	366/221	
Age, median (IQR), year	54 (38–68)	
Patient with given multiple samples, n (%)		
1 sample	578 (98.5)	578 (96.5)
2 samples	7 (1.2)	14 (2.3)
3 samples	1 (0.2)	3 (0.5)
4 samples	1 (0.2)	4 (0.7)
Pre-pandemic (Before December 2019)		144 (24.0)
ANA-positive		144 (100)
Pandemic (Since December 2019)		455 (76.0)
ANA-positive		88 (19.3)
Viral antibody-positive		340 (74.7)
Bacterial or parasite antibody-positive		27 (5.9)

ANA, antinuclear antibody; COVID-19, coronavirus disease 2019; IQR, interquartile range; n, number; RT-PCR, reverse transcription-polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

and Siemens ($p = 0.0061$). The specificity of the LFIAs ranged from 97.3% to 99.0%, without any significant difference between the assays. There was no significant difference in the pooled sensitivity and specificity between CLIAs and LFIAs. The Roche had the highest PPV (84.9%), whereas the SDQ had the lowest (28.5%). The NPV of each assay was comparable, ranging from

¹ www.medcalc.org

99.2% to 99.4%. The accuracy of the CLIAs ranged from 97.5% to 99.1%, and that of the LFIs ranged from 96.7% to 98.3%.

Interpretation of Trace Bands in Lateral Flow Immunoassay

The relatively strong and weak bands observed in SDQ and P4D were interpreted as distinctively positive and trace bands, respectively, by visual observation, whereas those in SDF were presented as index values using the fluorescence-based automated analyzer. Three observers reached a consensus through discussion of the trace bands observed in the SDQ and P4D. We compared the SDF index values between distinct positive and trace bands in SDQ and P4D according to the antibody type (Table 4). The proportion of IgM trace bands was significantly lower in SDQ than that in P4D (19.3% vs. 27.8%, $p = 0.0471$), whereas the proportion of IgG trace bands was comparable between SDQ and P4D (6.5% vs. 11.3%, $p = 0.1109$). In both assays, the trace bands were more frequently detected for IgM compared with IgG (19.3% vs. 6.5%, $p < 0.0001$ for SDQ; 27.8% vs. 11.3%, $p < 0.0001$ for P4D, respectively). The mean SDF indices were significantly lower in samples with trace bands compared to those with distinctively positive bands in SDQ and P4D (mean $\pm$ SD, 2.29 ± 1.23 vs. 7.12 ± 3.37 , $p < 0.0001$ for SDQ-IgM; 3.80 ± 5.13 vs. 15.01 ± 4.83 , $p < 0.0001$ for SDQ-IgG; 3.41 ± 1.82 vs. 7.99 ± 3.11 , $p < 0.0001$ for P4D-IgM; and 9.50 ± 6.44 vs. 16.31 ± 3.11 , $p < 0.0001$ for P4D-IgG, respectively). If the trace bands were considered negative, the sensitivity of SDQ-IgM and P4D-IgM would have decreased significantly (56.0% vs. 45.2%, $p = 0.0033$; 49.9% vs. 36.0%, $p = 0.0001$, respectively), and the specificity of SDQ-IgM would have increased significantly (97.7% vs. 99.7%, $p = 0.0039$).

Sensitivity of Severe Acute Respiratory Syndrome Coronavirus 2 Antibody Assays at Different Time Stages With and Without Symptoms

The diagnostic sensitivities of the six bAb assays in 389 samples from COVID-19-positive patients according to the presence or absence of symptoms and different time stages are shown in Table 3 and Figures 1A–E. The 321 samples from symptomatic COVID-19 patients were subdivided according to the number of days post symptom onset as follows: 1–7 days, 98 (30.5%) sera; 8–14 days, 111 (34.6%) sera; 15–21 days, 59 (18.4%) sera; 22–28 days, 27 (8.4%) sera; and ≥ 29 days, 26 (8.1%) sera. The 68 samples from asymptomatic COVID-19 patients were subdivided based on days after initial RT-PCR detection as follows: 1–7 days, 47 (69.1%) sera; 8–14 days, 13 (19.1%) sera; 15–21 days, 5 (7.4%) sera; 22–28 days, 1 (1.5%) sera; and ≥ 29 days, 2 (2.9%) sera. In the symptomatic group, the sensitivities of all six serological assays increased over 80% > 14 days after symptom onset. In the asymptomatic group, the sensitivities of both SDF and SDQ reached over 80% 8–14 days after initial RT-PCR detection, while those of the other assays reached over 80% > 21 days. In LFIs, in the first 2 weeks of illness, the sensitivity of IgM was higher

than that of IgG. The sensitivity of IgG began to exceed that of IgM after 15 days and was completely reversed after over 29 days for all LFIs.

A total of 385 samples from 196 COVID-19-positive patients were evaluated using the SARS-CoV-2 sVNT (Table 3 and Figure 1F). The sVNT quantifies the inhibitory activity of the RBD-targeting nAbs, and the result is expressed as percentage inhibition (%I) = $[1 - (\text{sampled optical density value/negative control optical density value})] \times 100$. The median value of the nAb inhibition activity of the total COVID-19 samples was 44.3%. The median inhibition activity was significantly higher in the symptomatic group than in the asymptomatic group (50.0% vs. 29.2%; $p < 0.0001$). At 1–7 days after symptom onset, 32.3% were positive, with a median inhibition activity of 20.3% (cut-off: 30%). At 8–14 days, 64.6% were positive, with a median inhibition activity of 46.8%. At 15–21 days, 94.9% were positive, with a median inhibition activity of 85.2%. At 22–28 days, 96.3% were positive, with a median inhibition activity of 89.5%. After ≥ 29 days, the positive rate was 96.0%, and the inhibition activity was 81.0%.

Specificity and False-Positive Results in Coronavirus Disease 2019-Negative Samples

The specificities of the three CLIAs and three LFIs in COVID-19-negative samples are described in Table 3. The overall false-positive rate of the CLIAs ranged from 0.2 to 1.8%, and that of the LFIs ranged from 0.2 to 2.7%. Among a total of 599 COVID-19-negative samples, a total of 34 samples were found to be false-positive for at least one of six serologic assays (Supplementary Table 2). Of those 455 COVID-19-negative samples collected during the pandemic period, 27 showed false-positive (5.9%). Of those 144 COVID-19-negative samples collected in the pre-pandemic period, seven showed false-positive (4.9%). Fisher's exact test showed no significant difference in proportions between the two groups ($p = 0.8363$). Among the 34 discordant (false-positive) samples, 20 (58.8%) showed false-positive results in one assay, 11 (32.4%) in two assays, and 3 (8.8%) in three assays. For CLIAs, the three false-positive results using the Abbott were weakly positive, so was the single false-positive result using the Roche. The 11 false-positive results obtained using the Siemens exhibited a wide range of positivity, from weak to strong. For LFIs, IgM demonstrated more false-positive results than IgG. Using SDF, 42.9% (6/14) of the false-positive results were weakly positive. Using SDQ, 87.5% (14/16) were weakly positive (trace band), and using P4D, 83.3% (5/6) were weakly positive (trace band). To validate the 34 false-positive samples, we performed additional sVNT, except for one sample (No. 608) due to insufficient sample volume. Among the 33 validated samples, only one (sample No. 501) was weakly positive for sVNT (cut-off: 30% inhibition). The sample No. 501, positive only for SDF-IgG, had an ANA-positive feature and was obtained during the pre-pandemic period.

TABLE 3 | Diagnostic performance of SARS-CoV-2 antibody assays according to days after symptom onset.

	Abbott	Roche	Siemens	CLIA	SDF			SDQ			P4D			LFIA	sVNT [†]	
	IgG	Total	Total	pooled	IgM	IgG	IgM/IgG	IgM	IgG	IgM/IgG	IgM	IgG	IgM/IgG	pooled	IgG	%I [‡]
Overall diagnostic performance*																
Sensitivity, %	55.3	54.2	56.6	55.4	58.9	54.8	64.3	56.0	55.0	61.7	49.9	47.8	56.3	59.3	62.3	44.3
Specificity, %	99.5	99.8	98.2	99.2	98.5	99.2	97.7	97.7	99.7	97.3	99.2	99.8	99.0	98.5	—	—
PPV, %	65.6	84.9	34.8	61.8	40.4	53.2	32.2	29.3	74.0	28.5	50.8	83.2	49.3	41.9	—	—
NPV, %	99.2	99.2	99.2	99.2	99.3	99.2	99.4	99.2	99.2	99.3	99.1	99.1	99.2	99.3	—	—
Accuracy, %	98.8	99.1	97.5	98.5	97.8	98.4	97.1	97	98.9	96.7	98.3	99	98.3	97.8	—	—
Sensitivity in positive COVID-19 samples																
Symptomatic, days post symptom onset																
Total (n = 321), %	60.4	59.2	59.8	59.8	62.3	60.1	68.2	59.2	59.8	65.7	53.6	54.2	61.1	56.3	65.6	50.0
1–7 days (n = 98), %	23.5	22.5	22.5	22.8	33.7	26.5	36.7	28.6	22.5	30.6	22.5	21.4	26.5	27.7	32.3	20.3
8–14 days (n = 111), %	59.5	57.7	58.6	58.6	64.9	60.4	68.5	61.3	60.4	65.8	56.8	53.2	61.3	61.4	64.6	46.8
15–21 days (n = 59), %	91.5	89.8	91.5	90.9	91.5	88.1	96.6	89.8	91.5	96.6	86.4	88.1	94.9	91.5	94.9	85.2
22–28 days (n = 27), %	100	96.3	96.3	97.5	85.2	92.6	96.3	85.2	88.9	96.3	77.8	85.2	92.6	88.9	96.3	89.5
≥ 29 days (n = 26), %	92.3	96.2	96.2	94.9	69.2	88.5	92.3	69.2	96.2	96.2	57.7	73.1	80.8	80.4	96.0	81.0
Asymptomatic, days after initial RT-PCR detection																
Total (n = 68), %	30.9	30.9	41.2	34.3	42.7	29.4	45.6	41.2	32.4	42.7	32.4	17.7	33.8	35.3	47.1	29.2
1–7 days (n = 47), %	12.8	12.8	27.7	17.8	21.3	12.8	23.4	23.4	12.8	23.4	19.2	10.6	21.3	18.7	25.5	15.1
8–14 days (n = 13), %	69.2	69.2	69.2	69.2	92.3	61.5	92.3	84.6	76.9	84.6	61.5	30.8	61.5	71.8	92.3	45.5
15–21 days (n = 5), %	60.0	60.0	60.0	60.0	100.0	60.0	100.0	80.0	60.0	80.0	60.0	20.0	60.0	68.9	100	54.4
22–28 days (n = 1), %	100	100	100	100	0.0	100	100	100	100	100	0.0	0.0	0.0	55.6	100	30.9
≥ 29 days (n = 2), %	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	56.1
Specificity in negative COVID-19 samples																
Pre-pandemic (n = 144), % (n)	100	100	99.3	—	97.9	98.6	96.5	97.2	100	97.2	98.6	100	98.6	—	—	—
False positive, n	0	0	1	—	3	2	5	4	0	4	2	0	2	—	—	—
Pandemic (n = 455), % (n)	99.3	99.8	97.8	—	98.7	99.3	98.0	97.8	99.6	97.4	99.3	99.8	99.1	—	—	—
False positive, n	3	1	10	—	6	3	9	10	2	12	3	1	4	—	—	—

*Since the PPV, NPV, and accuracy are dependent on disease prevalence, the rate of the accumulated confirmed cases of COVID-19 in South Korea, 1.7% (on July 2021), was counted as disease prevalence for the calculation. [†]Only 385 samples from COVID-19 positive patients were evaluated with the SARS-CoV-2 sVNT. Among the 321 samples from symptomatic patients, the sVNT was available only in 317 samples because of the limited sample volume: 1–7 days (n = 96), 8–14 days (n = 110), 15–21 days (n = 59), 22–28 days (n = 27), ≥ 29 days (n = 25). [‡]Median percentage inhibition. Abbott, SARS-CoV-2 IgG (Abbott); CLIA, chemiluminescent immunoassay; COVID-19, coronavirus disease 2019; LFIA, lateral flow immunoassay; n, number; NPV, negative predictive value; %I, percentage inhibition = $[1 - (\text{sampled optical density value/negative control optical density value})] \times 100$; PPV, positive predictive value; P4D, P4DETECT COVID-19 IgM/IgG (PRIME4DIA); Roche, Elecsys Anti-SARS-CoV-2 (Roche); RT-PCR, reverse transcription-polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SDF, STANDARD F COVID-19 IgM/IgG Combo FIA (SD BIOSENSOR); SDQ, STANDARD Q COVID-19 IgM/IgG Combo (SD BIOSENSOR); Siemens, ADVIA Centaur SARS-CoV-2 Total (Siemens).

Agreement Between the Severe Acute Respiratory Syndrome Coronavirus 2 Antibody Assays

The overall/positive/negative percent agreement, Cohen's κ -values, and McNemar's test of asymmetry between the six SARS-CoV-2 antibody assays are presented in **Table 5** and **Supplementary Table 3**. The agreement percentages ranged from 91.0 to 97.8%, with the κ -values ranging from 0.734 to 0.935. For CLIAs, Abbott and Roche showed the highest agreement rate (97.4%, $\kappa = 0.923$), whereas, for LFIAs, SDF-IgG and SDQ-IgG showed the highest agreement rate (97.8%, $\kappa = 0.935$). Comparing the agreement rates of LFIAs with those of CLIAs, the IgG of all LFIAs showed the highest agreement rate with Abbott-IgG (SDF: 96.4%, $\kappa = 0.894$; SDQ: 97.0%, $\kappa = 0.911$; and P4D: 94.8%, $\kappa = 0.842$). Despite a good or very good overall inter-assay agreement, significant differences were shown using

McNemar's test between CLIA and LFIA (in particular, SDF and SDQ) (**Table 5**).

Kinetics of the Binding and Neutralizing Antibodies in Patients With Coronavirus Disease 2019

Kinetic analysis of symptomatic COVID-19 patients who demonstrated seroconversion based on the quantitative results of CLIA, SDF-IgM/IgG, and sVNT was performed (**Figure 2**). The seroconversion was detected in 135 serial samples from 44 patients by Abbott, 121 serial samples from 41 patients by Roche, 132 serial samples from 39 patients by Siemens, 125 serial samples from 37 patients by SDF-IgM, 139 serial samples from 42 patients by SDF-IgG, and 133 serial samples from 41 patients by sVNT. The distribution of time to seropositivity (TTP) was calculated by interpolating the positive cut-off line to the curve using the four-parameter logistic (4PL) equation (AAT Bioquest Inc, 2022).

TABLE 4 | The comparison of the distinct positive bands and the trace bands in LFIA by visual reading in compliance to the index of LFIA by fluorescence-based automated reading.

	SDQ		P4D	
	IgM	IgG	IgM	IgG
Confirmed as positive, N	218	214	194	186
Distinct positive band				
N	176	200	140	165
%	80.7	93.5	72.2	88.7
SDF index (COI), mean $\pm$ SD	7.12 $\pm$ 3.37	15.01 $\pm$ 4.83	7.99 $\pm$ 3.11	16.31 $\pm$ 3.11
Trace band				
N	42	14	54	21
%	19.3	6.5	27.8	11.3
SDF index (COI), mean $\pm$ SD	2.29 $\pm$ 1.23	3.80 $\pm$ 5.13	3.41 $\pm$ 1.82	9.50 $\pm$ 6.44
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
By reclassifying “trace” as “negative”				
Sensitivity, %	45.2	51.4	36	42.4
Specificity, %	99.7	100	100	99.8

COI, cut-off index; LFIA, lateral flow immunoassay; N, n, number; P4D, P4DETECT COVID-19 IgM/IgG (PRIME4DIA); SD, standard deviation; SDF, STANDARD F COVID-19 IgM/IgG Combo FIA (SD BIOSENSOR); SDQ, STANDARD Q COVID-19 IgM/IgG Combo (SD BIOSENSOR).

The median TTPs were as follows in the ascending order: 7.7 days for SDF-IgM, 8.8 days for sVNT, 9.2 days for Siemens and SDF-IgG, 9.7 days for Abbott, and 9.8 days for Roche. In addition, TTP for each assay was also analyzed in the asymptomatic group. The median TTPs were as follows in the ascending order: 7.2 days for sVNT (patient $n = 3$), 7.5 days for SDF-IgG (patient $n = 3$), 8.8 days for SDF-IgM (patient $n = 2$), 9.6 days for Roche (patient $n = 4$), 10.2 days for Siemens (patient $n = 2$), and 10.6 days for Abbott (patient $n = 3$).

Correlation of Surrogate Virus Neutralization Test With Binding Antibody Assays

The quantitative results of the three CLIAs, SDF-IgM/IgG, and sVNT acquired from COVID-19-positive samples were used to analyze the correlation between assays (Figure 3). The results showed a strong correlation between sVNT and other assays, with the Spearman ρ -values ranging from 0.746 (sVNT vs. SDF-IgM) to 0.854 (sVNT vs. Siemens).

DISCUSSION

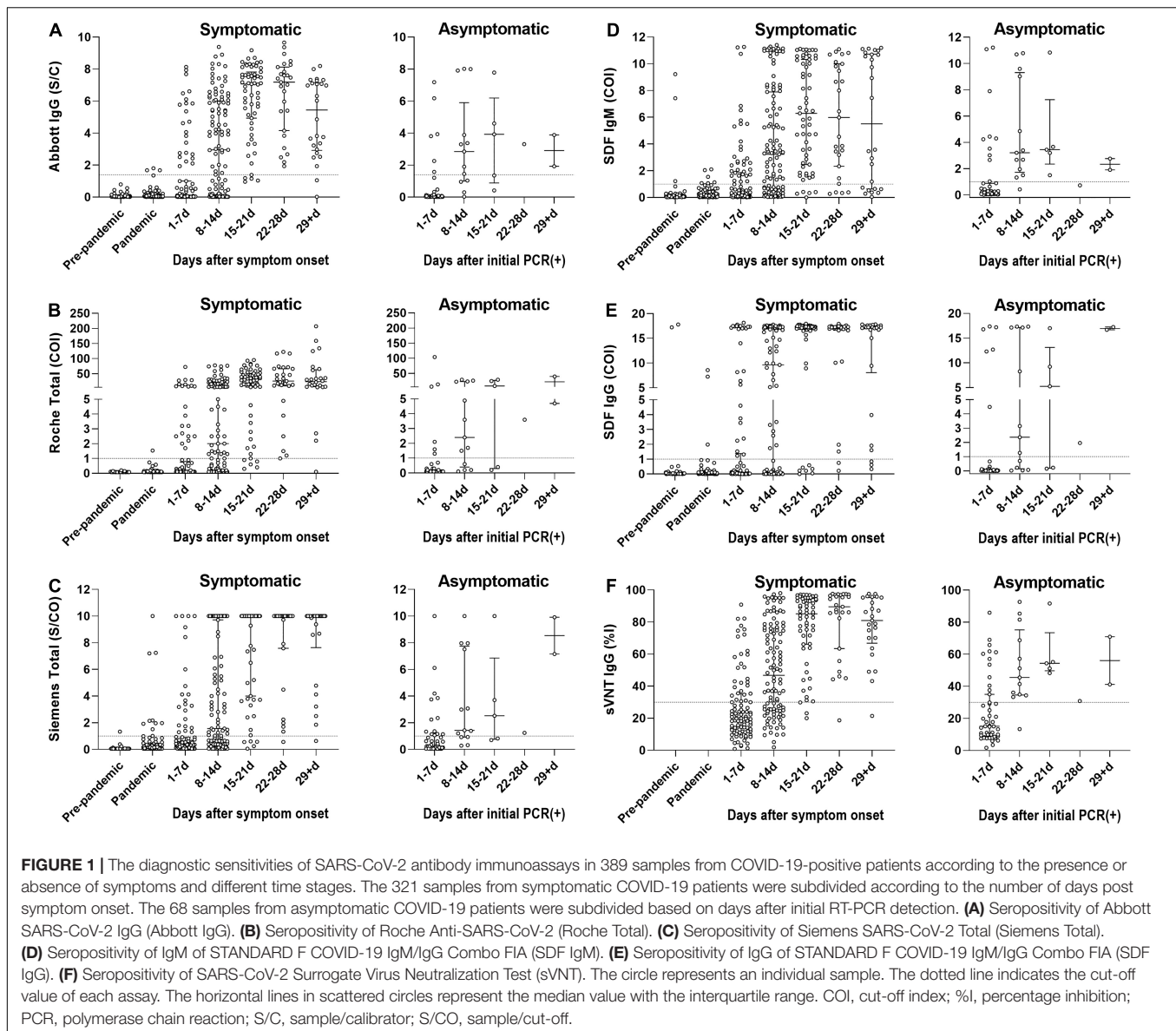
To the best of our knowledge, this is the most extensive single-center evaluation of SARS-CoV-2 antibody tests in Korea, assessing the diagnostic performance of six different SARS-CoV-2 bAb assays and the activity and kinetics of neutralizing antibodies in a large set of COVID-19 samples.

Several SARS-CoV-2 antibody assays have been developed and evaluated. However, owing to the variable factors affecting diagnostic accuracy, the clinical implications remain uncertain. The reported performance of antibody assays varies widely by factors such as the size of patients or samples, the type of analytical method, the type of antigen, the population used as the control samples, or the timing of sample collection

(Lisboa Bastos et al., 2020; Chvatal-Medina et al., 2021; Jarrom et al., 2022). Recently, a meta-analysis reported that the sensitivity of antibody tests using CLIAs ranged from 48.1 to 100%, and that of LFIAs ranged from 14.4 to 100% (Lisboa Bastos et al., 2020). In our study, the pooled sensitivity of CLIAs was 55.4%, and that of the LFIAs was 56.1%, with no significant difference between the two groups. Among the CLIAs, the Abbott was used to detect IgG, and there was no significant difference in the IgG detection rate between CLIAs and LFIAs. Among the LFIAs, the sensitivity of the combination of IgM and IgG was higher than that of each Ig class, consistent with findings from previous studies (Chen et al., 2020; Yun et al., 2021). Other studies have also recommended measuring both IgM and IgG in the first days of illness to reduce the risk of false-negative results, which may be due to dynamic antibody titer changes (Krajewski et al., 2020; Sun et al., 2020).

We analyzed the PPV, NPV, and accuracy with the rate of the accumulated confirmed cases (1.7%) in Korea. The PPV of Roche and Abbott was similar to the results of Park et al. (2022), who calculated the PPV using several exemplary COVID-19-prevalent populations. The PPV of Siemens was relatively lower than that of other CLIAs, with lower specificity compared to previous findings (Florin et al., 2021; Yun et al., 2021; Park et al., 2022). As the CLIAs for the SARS-CoV-2 antibody are not routinely used in our laboratory work, the reagent evaluation for this study was performed only for a short period. Therefore, insufficient optimization of the analytical system might be one reason for the poor performance (Kumleben et al., 2020; Ye et al., 2021).

Usually, the LFIA result appears in color-changing bands interpreted by visual inspection, which can easily be influenced by the observer's experience and subjectivity. Those ambiguous bands would be a critical issue in using LFIAs for SARS-CoV-2 antibody detection because reclassifying trace bands as “negative” can change the test performance, as shown in our analysis (Table 4). A previous study underlined the importance of seropositive threshold determination, observer training, and



LFIA analytical tools such as digital image analysis to improve objectivity (Whitman et al., 2020).

As reported in previous studies, our results showed that the antibody detection rate of the symptomatic group increased over 80% > 14 days after symptom onset in all assays (Sun et al., 2020; Nicholson et al., 2021; Yun et al., 2021). However, the rate in the asymptomatic group reached over 80% > 21 days after initial RT-PCR detection. This result might be due to the proportion difference in the early (1–7 days) stages of illness between the two groups. According to the Korean government's rapid COVID-19 response system, the asymptomatic COVID-19 patients were generally confirmed through contact tracing; hence, the early stage proportion would be higher [Table 3: 30.5% (98/321) vs. 69.1% (47/68)]. Another explanation could be that a lower viral load in asymptomatic individuals leads to a lower seropositivity rate, as reported by Wellinghausen et al. (2020).

Additionally, we identified the primary RT-PCR cycle threshold (Ct) values tested at CNUH for 148 of the 158 symptomatic patients and 34 of the 41 asymptomatic patients. The Ct values of both the envelope (*E*) and RNA-dependent RNA polymerase (*RdRp*) genes revealed approximately one cycle bias between the symptomatic and asymptomatic groups with no significant difference [*E* gene: mean $\pm$ SD, 24.44 $\pm$ 6.38 vs. 23.43 $\pm$ 5.64, $p = 0.4064$; *RdRp* gene: 25.33 $\pm$ 6.22 vs. 24.35 $\pm$ 5.38; $p = 0.4495$ (data not shown)]. However, since one cycle difference in PCR suggests twice the viral load, we could assume that the viral load of asymptomatic patients might be only about half of that of symptomatic patients in this study.

A previous study on false-positive results of SARS-CoV-2 antibody tests in samples stored before the pandemic reported that the false-positive rate of the LFIAs was higher than that of the ELISAs (1.8% vs. 0.6%) (Latiano et al., 2021). This was consistent

TABLE 5 | Agreement rate analysis between the SARS-CoV-2 antibody assays using agreement percentage (%), Cohen's kappa (κ), and McNemar's test.

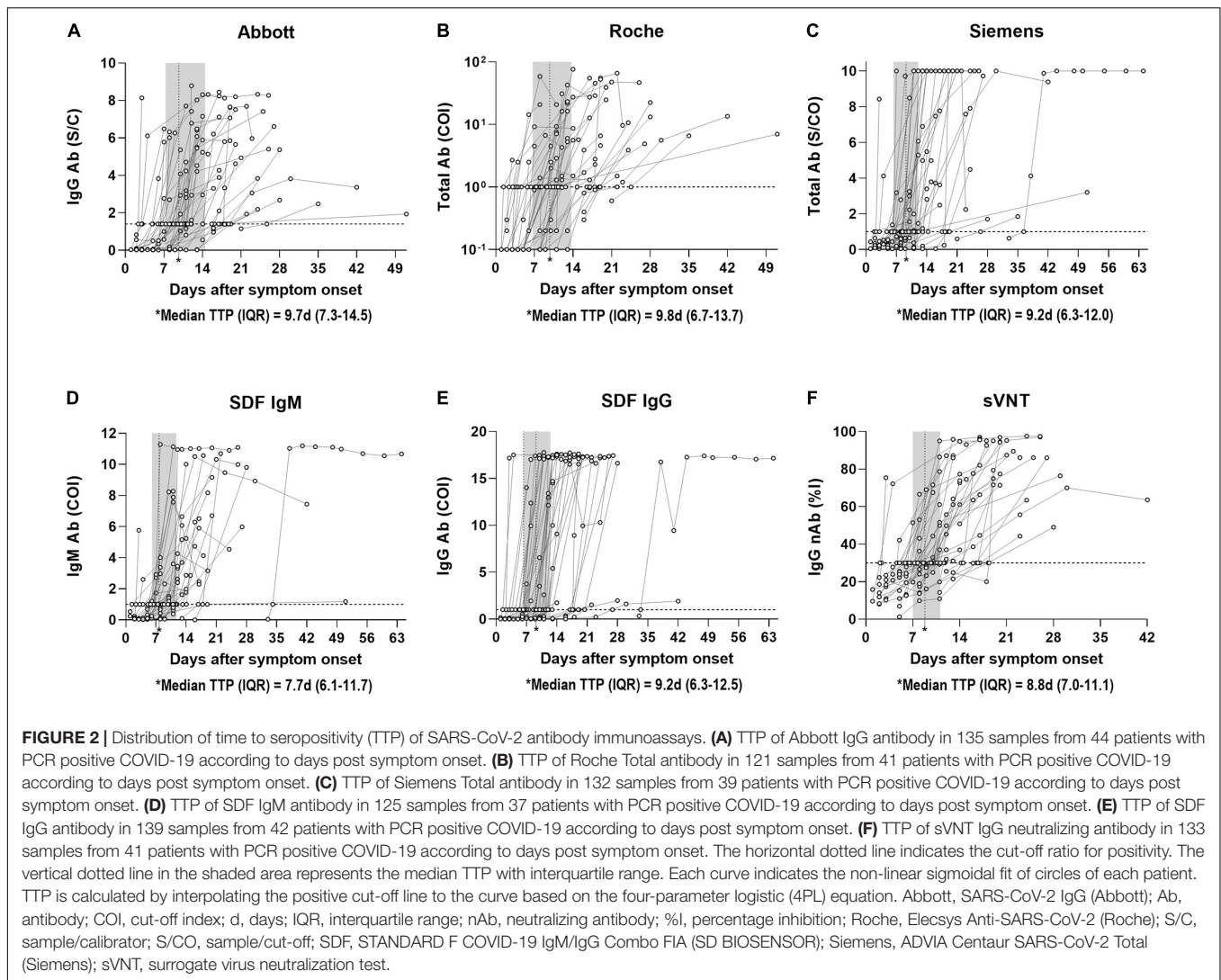
% (κ) p-value*	Abbott- IgG	Roche- Total	Siemens- Total	SDF- IgM	SDF- IgG	SDF- IgM/ IgG	SDQ- IgM	SDQ- IgG	SDQ- IgM/ IgG	P4D- IgM	P4D- IgG	P4D- IgM/ IgG
Abbott-IgG												
Roche-total	97.4 (0.923) 0.3268											
Siemens-total	94.0 (0.830) 0.1182	94.2 (0.834) 0.0171										
SDF-IgM	93.3 (0.812) 0.0193	92.5 (0.787) 0.0037	92.0 (0.779) 0.4996									
SDF-IgG	96.4 (0.894) 0.8676	96.0 (0.787) 0.4292	94.0 (0.830) 0.1182	92.7 (0.795) 0.0251								
SDF-IgM/IgG	93.9 (0.836) < 0.0001	93.3 (0.818) < 0.0001	92.8 (0.809) < 0.0001	97.4 (0.931) < 0.0001	95.3 (0.874) < 0.0001							
SDQ-IgM	93.7 (0.822) 0.0987	93.1 (0.803) 0.0212	92.4 (0.788) 1.0000	96.8 (0.911) 0.3768	93.3 (0.810) 0.1096	96.8 (0.911) < 0.0001						
SDQ-IgG	97.0 (0.911) 0.8551	96.4 (0.893) 0.6171	94.2 (0.835) 0.0637	92.1 (0.777) 0.0174	97.8 (0.935) 0.8312	93.9 (0.835) < 0.0001	92.1 (0.777) 0.0608					
SDQ-IgM/IgG	94.7 (0.856) < 0.0001	94.1 (0.838) < 0.0001	93.2 (0.818) 0.0034	94.5 (0.854) 0.0207	94.7 (0.856) < 0.0001	96.4 (0.906) 0.2433	97.6 (0.935) < 0.0001	94.5 (0.854) < 0.0001				
P4D-IgM	92.8 (0.784) 0.0327	92.6 (0.776) 0.1602	91.9 (0.763) 0.0005	94.8 (0.850) < 0.0001	92.8 (0.784) 0.0327	92.8 (0.801) < 0.0001	96.7 (0.902) < 0.0001	92.6 (0.777) 0.0611	94.8 (0.850) < 0.0001			
P4D-IgG	94.8 (0.842) < 0.0001	94.6 (0.834) 0.0010	92.9 (0.788) < 0.0001	91.0 (0.734) < 0.0001	96.3 (0.885) < 0.0001	91.6 (0.764) < 0.0001	92.4 (0.774) < 0.0001	96.3 (0.885) < 0.0001	92.6 (0.789) < 0.0001	91.0 (0.734) 0.1691		
P4D-IgM/IgG	94.4 (0.840) 0.4185	94.2 (0.833) 0.1120	93.1 (0.806) 0.5443	94.4 (0.845) 0.1056	95.0 (0.857) 0.3914	95.0 (0.867) < 0.0001	96.3 (0.895) 0.3239	94.8 (0.851) 0.2626	96.5 (0.904) < 0.0001	97.4 (0.922) < 0.0001	94.4 (0.845) < 0.0001	

*Calculated using McNemar's test. Abbott, SARS-CoV-2 IgG (Abbott); P4D, P4DETECT COVID-19 IgM/IgG (PRIME4DIA); Roche, Elecsys Anti-SARS-CoV-2 (Roche); SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SDF, STANDARD F COVID-19 IgM/IgG Combo FIA (SD BIOSENSOR); SDQ, STANDARD Q COVID-19 IgM/IgG Combo (SD BIOSENSOR); Siemens, ADVIA Centaur SARS-CoV-2 Total (Siemens).

with our results, in which the overall false-positive rates of the LFIAs were of a more expansive range than those of the CLIAs (Table 3). A large portion of the false-positive results was weak or trace, and the additionally performed sVNT was negative, except for in one sample collected in the prepandemic period. Among a total of 455 samples collected in the pandemic period, 9, 12, and 4 were found to be false positive for SDF-IgM/IgG, SDQ-IgM/IgG, and P4D-IgM/IgG, leading to assay specificities of 98.0, 97.4, and 99.1%, respectively. A comparative analysis for specificity, sensitivity, PPV, and NPV of all three LFIAs for varying seroprevalences (1, 5, and 10%) of SARS-CoV-2 antibody was shown in **Supplementary Table 4**. At seroprevalence of 10%, all LFIAs had unsatisfactory or acceptable PPVs of 78.3, 72.2, and 87.7% for SDF-IgM/IgG, SDQ-IgM/IgG, and P4D-IgM/IgG, but at seroprevalence of 1%, these values dropped to unacceptably low levels of 24.7, 19.1, and 39.3 for SDF, SDQ, and P4D. However, at varying seroprevalence of 1, 5, and 10%, NPVs ranging from 95.3 to 99.6% were acceptably high

levels. Collectively, these findings suggest that LFIA tests may be useful in a high seroprevalence setting, in which COVID-19 is widely spread.

In SARS-CoV-2 antibody testing, false-positive results (non-specific or cross-reactive) can arise from endogenous factors such as rheumatoid factors, heterophil antibodies, lysozymes, complements, other cross-antigens (e.g., similar epitopes between SARS-CoV-2 and other human coronaviruses), or exogenous interferences such as inadequate specimen quality and unsatisfactory test kit optimization (Ye et al., 2021). A previous study in sub-Saharan Africa showed that pre-pandemic plasma samples, which either had the S proteins of HCoV-OC43, HCoV-HKU-1, HCoV-NL63, and HCoV-229E or the N proteins of HCoV-NL63, and HCoV-229E, were serological cross-reactive against the S and N proteins of SARS-CoV-2 (Tso et al., 2021). Previous studies have also reported cross-reactivity with autoantibodies such as ANA and other viral infections such as cytomegalovirus (Jääskeläinen et al., 2020;

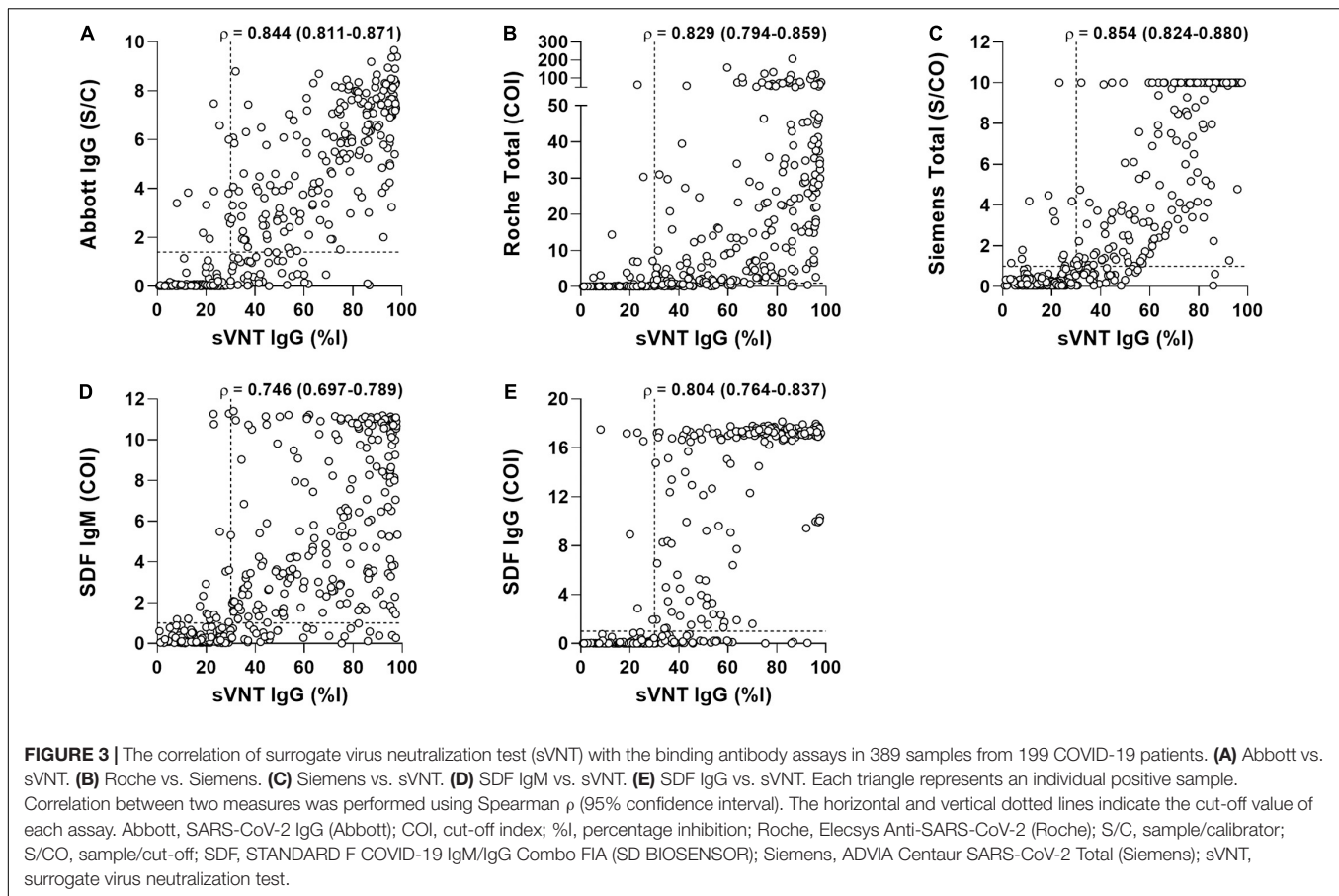


Nicholson et al., 2021). In practice, false-positive cases are difficult to rule out; therefore, test subjects should be selected wisely, recognizing the limitations of serological tests when applying them to asymptomatic, healthy subjects with no history of SARS-CoV-2 exposure (Latiano et al., 2021).

In the agreement rate analysis, all assays showed good agreement. Among the CLIAs, the Abbott vs. Roche comparison had a higher agreement rate than the Siemens vs. Abbott or Roche comparison, consistent with other studies (Yun et al., 2021; Park et al., 2022). This result might be due to the difference in the target protein—Abbott and Roche target an epitope of the N protein, and Siemens targets the S protein. Among the LFIAs, SDF-IgG and SDQ-IgG showed the highest agreement rates, likely because both assays are from the same manufacturer and target the same IgG.

The sVNT test indirectly detects the function of neutralizing SARS-CoV-2 antibodies that block the interaction between the viral spike RBD and the host ACE2 receptor. The positive rate of sVNT in sera collected > 14 days after symptom onset

was similar to previous findings (Tan et al., 2020; Nicholson et al., 2021; Yun et al., 2021). Interestingly, the symptomatic group showed higher inhibition activity than the asymptomatic group, although the positive rate was reversed (Table 3 and Figure 1F). These data might align with the statement that the asymptomatic group consisted of a higher proportion of early stage illnesses and might have a lower viral load (Wellinghausen et al., 2020). However, this could be due to the sample size imbalance (8 vs. 112) between asymptomatic and symptomatic groups at that time frame (≥ 15 days category), requiring a further study using more sample size of asymptomatic cases. In our study, the bAb IgM assay showed the earliest seroconversion at 7.7 days, followed by the nAb IgG assay at 8.8 days and the bAb IgG assay at 9.2–9.8 days, which supports previous data for the utility and clinical importance of using IgM antibodies for SARS-CoV-2 diagnosis (Ng et al., 2020; Orner et al., 2021). The index values of the three CLIAs and SDF-IgM/IgG vs. sVNT percentage inhibition were strongly correlated (Figure 3), and the use of the same target protein could



explain the strongest correlation between Siemens and sVNT (Yun et al., 2021).

Our study had some limitations. First, the negative samples were from patients in the pre-pandemic and pandemic periods who had no history of COVID-19 or epidemiological relationship with COVID-19. They did not undergo additional PCR confirmation. Nevertheless, reflecting the government's strict response policy to COVID-19 and the low disease prevalence of COVID-19 at that time in Korea, the samples from patients with no history of COVID-19 or any epidemiological relationship with COVID-19 could be considered COVID-19-negative. Second, false-negative (or even undetected) results cannot be ruled out due to the possibility of insufficient optimization of the assay systems, as we evaluated the assays only for a short period. Finally, we only proposed the fragmentary kinetics of the antibodies detected in this study. Because the samples used in this study were serum remnants from blood samples retrieved for routine laboratory tests, the multiple samples from one patient were serially collected at different time points (one or two samples per week) but having irregular time intervals, providing only estimated TTPs for individual patients calculated by interpolating the positive cut-off line to the curve using the 4PL equation. Moreover, the SARS-CoV-2 antibody response is correlated with various factors, including primary infection or reinfection of COVID-19, symptom onset, disease severity, fever, age, and sex (Schlickeiser et al., 2021). As the national contact tracing system

was widely and strictly applied to all patients from the beginning of the pandemic period in Korea, the contact history with COVID-19 patients in this study were thoroughly investigated through the contact tracing system. As a result of the analysis, all of these patients had a current but no previous contact history with COVID-19 patients in the pandemic period, indicating that all of our patients had a primary infection, not re-infection. In addition, we analyzed the difference of antibody kinetics between symptomatic and asymptomatic groups. However, the sample size of the asymptomatic group was too small to calculate the p -values. More serial follow-up data and large-sized samples from well-evaluated individuals may be needed for intense antibody kinetic analyses.

In conclusion, our study offers a detailed comparison of three CLIAs, three LFIAs, and an sVNT assay. With the initiation of vaccines administration, routine antibody test for COVID-19 has been started in general laboratories all over the world. Therefore, to choose the most suitable serological assays for a particular laboratory environment and situation, it is necessary to understand the characteristics of each assay. The interpretation of antibody assay results should also be performed with caution. The patient's contact history, symptoms, the time of illness, measured assays, target antibodies, and the antigens used should also be considered. In particular, for LFIAs, it is recommended that more objectively interpreted assays are used, and a band interpretation system should be established for each laboratory

with sufficient observer training. We also expect that routinely available sVNT will play an essential role in the laboratory where nAb testing is desired.

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 Institutional Review Board (IRB) of CNUH (IRB No. CNUH-2020-223). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

H-WC, SYL, and S-JKe designed the study, analyzed, and discussed the data. H-WC, C-HJ, S-JKa, EJW, and SYL generated the data. H-WC and SYL ran the statistical analysis. H-WC and S-JKe wrote the primary manuscript. All authors were involved in writing and had final approval of the submitted and published versions of the manuscript.

REFERENCES

- AAT Bioquest Inc (2022). *Quest Graph™ Four Parameter Logistic (4PL) Curve Calculator*. Available online at: <https://www.aatbio.com/tools/four-parameter-logistic-4pl-curve-regression-online-calculator> (accessed February 8, 2022).
- Bae, S., Lim, J. S., Kim, J. Y., Jung, J., and Kim, S. H. (2021). Transmission characteristics of SARS-CoV-2 that hinder effective control. *Immune Netw.* 21:e9. doi: 10.4110/in.2021.21.e9
- Chen, S. Y., Lee, Y. L., Lin, Y. C., Lee, N. Y., Liao, C. H., Hung, Y. P., et al. (2020). Multicenter evaluation of two chemiluminescence and three lateral flow immunoassays for the diagnosis of COVID-19 and assessment of antibody dynamic responses to SARS-CoV-2 in Taiwan. *Emerg. Microbes Infect.* 9, 2157–2168. doi: 10.1080/22221751.2020.1825016
- Chvatal-Medina, M., Mendez-Cortina, Y., Patiño, P. J., Velilla, P. A., and Rugeles, M. T. (2021). Antibody Responses in COVID-19: a Review. *Front. Immunol.* 12:633184. doi: 10.3389/fimmu.2021.633184
- Florin, L., Maelegher, K., Vandewal, W., Bernard, D., and Robbrecht, J. (2021). Performance evaluation of the siemens SARS-CoV-2 total antibody and IgG antibody test. *Lab. Med.* 52, e147–e153. doi: 10.1093/labmed/lmab027
- Fong, C. H., Cai, J. P., Dissanayake, T. K., Chen, L. L., Choi, C. Y., Wong, L. H., et al. (2020). Improved detection of antibodies against SARS-CoV-2 by microsphere-based antibody assay. *Int. J. Mol. Sci.* 21:6595. doi: 10.3390/ijms21186595
- Guo, L., Ren, L., Yang, S., Xiao, M., Chang, D., Yang, F., et al. (2020). Profiling early humoral response to diagnose novel coronavirus disease (COVID-19). *Clin. Infect. Dis.* 71, 778–785. doi: 10.1093/cid/ciaa310
- Innovative and Diagnostic Medical Device Policy Division (2021). *List of COVID-19 in Vitro Diagnostic Devices Officially Approved by MFDS*. Available online at: https://www.mfds.go.kr/eng/brd/m_65/list.do (accessed September 16, 2021).
- Jääskeläinen, A. J., Kuivanen, S., Kekäläinen, E., Ahava, M. J., Loginov, R., Kallio-Kokko, H., et al. (2020). Performance of six SARS-CoV-2 immunoassays in comparison with microneutralisation. *J. Clin. Virol.* 129:104512. doi: 10.1016/j.jcv.2020.104512

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The reagents were provided in-kind: SARS-CoV-2 IgG by Abbott, Elecsys Anti-SARS-CoV-2 by Roche, ADVIA Centaur SARS-CoV-2 Total by Siemens, STANDARD F COVID-19 IgM/IgG Combo FIA and STANDARD Q COVID-19 IgM/IgG Combo by SD Biosensor, and P4DETECT COVID-19 IgG/IgM by PRIME4DIA.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.881038/full#supplementary-material>

- Jarrom, D., Elston, L., Washington, J., Prettyjohns, M., Cann, K., Myles, S., et al. (2022). Effectiveness of tests to detect the presence of SARS-CoV-2 virus, and antibodies to SARS-CoV-2, to inform COVID-19 diagnosis: a rapid systematic review. *BMJ Evid. Based Med.* 27, 33–45. doi: 10.1136/bmjebm-2020-111511
- Klasse, P. J. (2014). Neutralization of virus infectivity by antibodies: old problems in new perspectives. *Adv. Biol.* 2014:157895. doi: 10.1155/2014/157895
- Korea Centers for Disease Control and Prevention (2021). *Coronavirus Disease-19 Main Website*. Available at online: <http://ncov.mohw.go.kr/en> (accessed September 16, 2021).
- Krajewski, R., Gołębiewska, J., Makuch, S., Mazur, G., and Agrawal, S. (2020). Update on serologic testing in COVID-19. *Clin. Chim. Acta* 510, 746–750. doi: 10.1016/j.cca.2020.09.015
- Kumleben, N., Bhopal, R., Czypionka, T., Gruer, L., Kock, R., Stebbing, J., et al. (2020). Test, test, test for COVID-19 antibodies: the importance of sensitivity, specificity and predictive powers. *Public Health* 185, 88–90. doi: 10.1016/j.puhe.2020.06.006
- Laerd Statistics (2020). *Spearman's Rank-Order Correlation. Statistical Tutorials and Software Guides*. Available online at: <https://statistics.laerd.com/statistical-guides/spearman-rank-order-correlation-statistical-guide.php> (accessed January 5, 2022).
- Latiano, A., Tavano, F., Panza, A., Palmieri, O., Niro, G. A., Andriulli, N., et al. (2021). False-positive results of SARS-CoV-2 IgM/IgG antibody tests in sera stored before the 2020 pandemic in Italy. *Int. J. Infect. Dis.* 104, 159–163. doi: 10.1016/j.ijid.2020.12.067
- Lisboa Bastos, M., Tavaziva, G., Abidi, S. K., Campbell, J. R., Haraoui, L. P., Johnston, J. C., et al. (2020). Diagnostic accuracy of serological tests for covid-19: systematic review and meta-analysis. *BMJ* 370:m2516.
- McHugh, M. L. (2012). Interrater reliability: the kappa statistic. *Biochem. Med.* 22, 276–282. doi: 10.11613/bm.2012.031
- Ministry of Food and Drug Safety (2021). *Ministry of Food and Drug Safety White Paper*. Available online at: https://www.mfds.go.kr/eng/brd/m_61/view.do?seq=122 (accessed September 16, 2021).

- Ng, D. L., Goldgof, G. M., Shy, B. R., Levine, A. G., Balcerak, J., Bapat, S. P., et al. (2020). SARS-CoV-2 seroprevalence and neutralizing activity in donor and patient blood. *Nat. Commun.* 11:4698. doi: 10.1038/s41467-020-18468-8
- Nicholson, S., Karapanagiotidis, T., Khvorov, A., Douros, C., Mordant, F., Bond, K., et al. (2021). Evaluation of 6 commercial SARS-CoV-2 serology assays detecting different antibodies for clinical testing and serosurveillance. *Open Forum Infect. Dis.* 8:ofab239. doi: 10.1093/ofid/ofab239
- Orner, E. P., Rodgers, M. A., Hock, K., Tang, M. S., Taylor, R., Gardiner, M., et al. (2021). Comparison of SARS-CoV-2 IgM and IgG seroconversion profiles among hospitalized patients in two US cities. *Diagn. Microbiol. Infect. Dis.* 99:115300. doi: 10.1016/j.diagmicrobio.2020.115300
- Park, Y., Hong, K. H., Lee, S. K., Hyun, J., Oh, E. J., Lee, J., et al. (2022). Performance comparison of five SARS-CoV-2 antibody assays for seroprevalence studies. *Ann. Lab. Med.* 42, 71–78. doi: 10.3343/alm.2022.42.1.71
- Perkmann, T., Perkmann-Nagele, N., Breyer, M. K., Breyer-Kohansal, R., Burghuber, O. C., Hartl, S., et al. (2020). Side-by-Side comparison of three fully automated SARS-CoV-2 antibody assays with a focus on specificity. *Clin. Chem.* 66, 1405–1413. doi: 10.1093/clinchem/hvaa198
- Satarker, S., and Nampoothiri, M. (2020). Structural proteins in severe acute respiratory syndrome coronavirus-2. *Arch. Med. Res.* 51, 482–491. doi: 10.1016/j.arcmed.2020.05.012
- Schlickeiser, S., Schwarz, T., Steiner, S., Wittke, K., Al Beshar, N., Meyer, O., et al. (2021). Disease severity, fever, age, and sex correlate with SARS-CoV-2 neutralizing antibody responses. *Front. Immunol.* 11:628971. doi: 10.3389/fimmu.2020.628971
- Song, S. K., Lee, D. H., Nam, J. H., Kim, K. T., Do, J. S., Kang, D. W., et al. (2020). IgG Seroprevalence of COVID-19 among individuals without a history of the coronavirus disease infection in Daegu, Korea. *J. Korean Med. Sci.* 35:e269. doi: 10.3346/jkms.2020.35.e269
- Sun, B., Feng, Y., Mo, X., Zheng, P., Wang, Q., Li, P., et al. (2020). Kinetics of SARS-CoV-2 specific IgM and IgG responses in COVID-19 patients. *Emerg. Microbes Infect.* 9, 940–948. doi: 10.1080/22221751.2020.1762515
- Tai, W., He, L., Zhang, X., Pu, J., Voronin, D., Jiang, S., et al. (2020). Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: implication for development of RBD protein as a viral attachment inhibitor and vaccine. *Cell Mol. Immunol.* 17, 613–620. doi: 10.1038/s41423-020-0400-4
- Tan, C. W., Chia, W. N., Qin, X., Liu, P., Chen, M. I., Tiu, C., et al. (2020). A SARS-CoV-2 surrogate virus neutralization test based on antibody-mediated blockage of ACE2-spike protein-protein interaction. *Nat. Biotechnol.* 38, 1073–1078. doi: 10.1038/s41587-020-0631-z
- Tso, F. Y., Lidenge, S. J., Peña, P. B., Clegg, A. A., Ngowi, J. R., Mwaiselage, J., et al. (2021). High prevalence of pre-existing serological cross-reactivity against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in sub-Saharan Africa. *Int. J. Infect. Dis.* 102, 577–583. doi: 10.1016/j.ijid.2020.10.104
- Walls, A. C., Park, Y. J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veasler, D. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 181, 281–292. doi: 10.1016/j.cell.2020.02.058
- Wellington, N., Plonné, D., Voss, M., Ivanova, R., Frodl, R., and Deininger, S. S. A. R. S. (2020). CoV-2-IgG response is different in COVID-19 outpatients and asymptomatic contact persons. *J. Clin. Virol.* 130:104542. doi: 10.1016/j.jcv.2020.104542
- Whitman, J. D., Hiatt, J., Mowery, C. T., Shy, B. R., Yu, R., Yamamoto, T. N., et al. (2020). Evaluation of SARS-CoV-2 serology assays reveals a range of test performance. *Nat. Biotechnol.* 38, 1174–1183. doi: 10.1038/s41587-020-0659-0
- Wu, Z., and McGoogan, J. M. (2020). Characteristics of and important lessons from the coronavirus Disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the Chinese Center for Disease Control and Prevention. *JAMA* 323, 1239–1242. doi: 10.1001/jama.2020.2648
- Ye, Q., Zhang, T., and Lu, D. (2021). Potential false-positive reasons for SARS-CoV-2 antibody testing and its solution. *J. Med. Virol.* 93, 4242–4246. doi: 10.1002/jmv.26937
- Yun, S., Ryu, J. H., Jang, J. H., Bae, H., Yoo, S. H., Choi, A. R., et al. (2021). Comparison of SARS-CoV-2 antibody responses and seroconversion in COVID-19 patients using twelve commercial immunoassays. *Ann. Lab. Med.* 41, 577–587. doi: 10.3343/alm.2021.41.6.577
- Zhang, Y. V., Wiencek, J., Meng, Q. H., Theel, E. S., Babic, N., Sepiashvili, L., et al. (2021). AACC practical recommendations for implementing and interpreting SARS-CoV-2 emergency use authorization and laboratory-developed test serologic testing in clinical laboratories. *Clin. Chem.* 67, 1188–1200. doi: 10.1093/clinchem/hvab051

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Pilot Investigation of SARS-CoV-2 Variants in the Island of Sicily Prior to and in the Second Wave of the COVID-19 Pandemic

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After 2 years of the COVID-19 pandemic, we continue to face vital challenges stemming from SARS-CoV-2 variation, causing changes in disease transmission and severity, viral adaptation to animal hosts, and antibody/vaccine evasion. Since the monitoring, characterization, and cataloging of viral variants are important and the existing information on this was scant for Sicily, this pilot study explored viral variants circulation on this island before and in the growth phase of the second wave of COVID-19 (September and October 2020), and in the downslope of that wave (early December 2020) through sequence analysis of 54 SARS-CoV-2-positive samples. The samples were nasopharyngeal swabs collected from Sicilian residents by a state-run one-health surveillance laboratory in Palermo. Variant characterization was based on RT-PCR amplification and sequencing of four regions of the viral genome. The B.1.177 variant was the most prevalent one, strongly predominating before the second wave and also as the wave downsized, although its relative prevalence decreased as other viral variants, particularly B.1.160, contributed to virus circulation. The occurrence of the B.1.160 variant may have been driven by the spread of that variant in continental Europe and by the relaxation of travel restrictions in the summer of 2020. No novel variants were identified. As sequencing of the entire viral genome in Sicily for the period covered here was restricted to seven deposited viral genome sequences, our results shed some light on SARS-CoV-2 variant circulation during that wave in this insular region of Italy which combines its partial insular isolation with being a major entry point for the African immigration.

Keywords: SARS-CoV-2, Sicily (Italy), B.1.160 variant, B.1.177 variant, COVID-19 in autumn 2020

INTRODUCTION

In the late December 2019, the novel human-infecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was reported in Wuhan, Hubei Province, China (ProMED-mail, 2020; Zhu et al., 2020). Since then, this virus spread around the globe, causing a global pandemic, known as coronavirus disease 2019 (COVID-19). Up to 3 February 2022, the WHO (Coronavirus Resource Center, 2021; World Health Organization [WHO], 2022a) recorded > 380 million infections worldwide and almost 5.7 million COVID-19 deaths. The history of this pandemic is punctuated by the emergence of novel viral variants differing from the one described originally, some of which are variants of concern due to their increased infectivity and potential escape from immunity derived from prior infection with earlier variants, or, since January 2021, from vaccination (Worobey et al., 2020; Funk et al., 2021). Variants result from spontaneous mutations in the viral genome that introduce changes in the encoded proteins, particularly on the *spike* (S) gene which encodes the spike glycoprotein (S protein), the key mediator of the interaction of the virus with the host cells, leading to infection (Chakraborty et al., 2022). Thus, the monitoring of the emergence of novel viral variants is crucial, as variants can result in changes in viral transmissibility, virulence, antigenicity, and recognition by the adaptive immune system triggered by prior infection or vaccination (Funk et al., 2021). For example, in early 2020, SARS-CoV-2 sequences which included the D614G mutation in the S protein exhibited higher human transmissibility than the original variant of the Wuhan outbreak, resulting in the occurrence of this mutation in all the variants that have circulated afterward (Korber et al., 2020; Volz et al., 2021).

Because of the strict control measures on mobility and social distancing imposed in Europe during the spring of 2020, COVID-19 cases dwindled, and the appearance of new variants was minimized (Hodcroft et al., 2021). Subsequent relaxation of restrictions, including the resumption of travel in the summer of 2020, led to the re-emergence of the disease throughout Europe, with the spread of new variants (Hodcroft et al., 2021). In June 2020, the B.1.177 variant emerged in Spain and spread throughout Europe, becoming in some countries (such as Iceland, Ireland, and Spain), the major circulating variant (Hodcroft et al., 2021). Relative to the early Wuhan isolates, the B.1.177 variant presented variant-defining mutations in the S, *nucleocapsid* (N), and *ORF10* genes, causing the amino acid changes S:A222V (S protein), N:A220V (nucleocapsid phosphoprotein, abbreviated N protein) and ORF10:V30L (ORF10 protein). The corresponding nucleotide changes were C2222T, C28932T, and G29645T (numbered according to the SARS-CoV-2 reference genome, GenBank Accession Number: NC_045512.2) (Hodcroft et al., 2021). Later variants appearing in Europe during 2020 exhibited other mutations in the S protein, such as the S:S477N mutation of the B.1.160 variant, or, in separate clusters, of other S protein mutations, such as D80Y, S98F, and N439K (Hodcroft et al., 2021). None of these mutations seemed to importantly increase viral transmission or virulence relative to the B.1.177

variant, or to cause evasion from antibodies (or later on from vaccines) (Hodcroft et al., 2021; Xie et al., 2021). Therefore, the incorporation of these changes and the temporal success of some of them appear to be a consequence of genetic bottlenecks created by the low circulation of the SARS-CoV-2 virus after the period of generalized movement restriction throughout Europe (Hodcroft et al., 2021).

In any case, as already indicated, the S protein is of particular interest concerning the incorporation of changes and development of variants, as it is largely responsible for viral attachment to the host cell *via* interaction with the cellular receptor for this virus, the angiotensin-converting enzyme 2 (ACE2) (Jackson et al., 2022). Mutations leading to alterations in the amino acid sequence of the S protein can strongly modify viral fitness (Lan et al., 2020). This is exemplified by the B.1.1.7, B.1.351, and P.1 variants, which presented eight, six, and ten mutations in their S protein, respectively (Gómez et al., 2021), with concomitant 71% increased transmissibility in the case of B.1.1.7 (Bian et al., 2021), while the B.1.351 and P.1 variants decreased the effectivity of therapeutic antibodies and vaccines (Hoffmann et al., 2021).

Up to 3 February 2022, the WHO reported > 11 million confirmed COVID-19 cases and > 147,000 deaths due to this disease in Italy (World Health Organization [WHO], 2022b). Regional reference laboratories (ISS, 2021a), such as the one of Palermo that is centrally involved in this study have played in Italy a paramount role in the detection of infected people. The Palermo institute surveys the Italian island of Sicily, which hosts a population of about 5 million inhabitants. By the end of sample gathering for this study (mid-December 2020), the number of COVID-19 cases diagnosed in Sicily was 84,835. However, for the period studied here only in seven cases the viral genome was sequenced and deposited in the GISAID public databank (EPI_ISL_2308744, EPI_ISL_2308745, EPI_ISL_2308746, EPI_ISL_2308747, EPI_ISL_2308749, EPI_ISL_3274295, and EPI_ISL_910332) (ISS, 2021b). Therefore, knowledge was scant regarding the nature of the viral lineages circulating in Sicily during the second wave of the disease.

This pilot study contributes to remediating this lack of knowledge by characterizing the variants circulating in Sicily at the end of the prevaccinal period. For achieving this goal, we have used 54 SARS-CoV-2-positive samples collected by our institute of Palermo from inhabitants of Sicily, largely in September and early October, before the beginning and in the growth phase of the second pandemic wave, and in early December 2020, when the second wave started to decline (Table 1). We searched for variants *via* sequencing of selected viral genomic regions encompassing the defining mutation sites of the B.1.177 lineage, thus being able to assess the relative prevalence of this variant. In the samples found not to belong to the B.1.177 lineage, we also analyzed a partial sequence of the S gene that encodes a part of the receptor-binding domain (RBD) of the S protein. This highly variable sequenced region hosts a number of key sequence changes found in SARS-CoV-2 variants, including B.1.160 and the variants of concern B.1.1.7, B.1.351, and P.1 (Gómez et al., 2021), making it appropriate for pilot searching of these variants, which in other parts of Europe began to increase

their prevalence in December 2020¹. Because of its variability, this region also appears favorable, in principle, for the detection of the novel variants.

MATERIALS AND METHODS

Procurement of Samples

Between September and December 2020, a total of 20,258 nasopharyngeal swabs from individuals suspected of having COVID-19 were brought to the Virology Department at Istituto Zooprofilattico Sperimentale della Sicilia (Sicily, Italy) and were analyzed by RT-PCR for SARS-CoV-2. Positive SARS-CoV-2 amplification was obtained in 7,206 (35.57%) samples, while 11,933 (58.90%) tested negative and 1,119 (5.52%) were SARS-CoV-2 inconclusive because only one target gene for SARS-CoV-2 was positive or the Ct value for one or more targets were ≥ 37 (Ct cutoff positive value for assay targets). Some of the swab samples belonged to the migrants from many African countries and were the subject of a previous investigation (Tramuto et al., 2021). For this study, we analyzed SARS-CoV-2 positive samples collected from the Sicilian residents between September and December 2020. **Table 1** summarizes the samples used, while **Figure 1** shows the time of sample collection throughout the period of the second wave of COVID-19 in Sicily. For practical reasons, we were only able to examine about 50 samples (final, 54 samples). The initial part of the Results and Discussion describes the criteria used for sample selection.

Ethical Approval

This study was conducted with the approval of the ethics committee of Cardenal Herrera CEU University, Valencia, Spain (n. CEI20/083 released on 10/09/2020), and it is in agreement with the Helsinki Declaration.

Molecular SARS-CoV-2 Detection

This step was performed in the Virology Department at Istituto Zooprofilattico Sperimentale della Sicilia “A.Mirri” (Palermo, Italy). First, the total RNA was extracted by MagMAXTM Viral/Pathogen Nucleic Acid Isolation Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, United States) employing the KingFisher Flex 96 automatic nucleic acid extractor (Thermo Fisher Scientific, Waltham, MA, United States) of QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) following the instructions of the manufacturer.

Second, SARS-CoV-2 RNA detection was preliminarily performed by real-time reverse transcription polymerase chain reaction (rRT-PCR), according to the protocol of TaqPathTM COVID-19 CE-IVD RT-PCR Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, United States). This multiplex assay contains three primer/probe sets specific to different SARS-CoV-2 genomic regions: *ORF1ab*, *S*, and *N* genes (TaqPath COVID-19 CE-IVD RT-PCR Kit, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, United States). The

amplification was carried out by using a QuantStudio 6 Flex Real-Time PCR Systems (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, United States). Finally, once SARS-CoV-2 positivity was confirmed, RNA extracted previously from 54 positive samples was sent to the Health Sciences Faculty of UCH-CEU University (Valencia, Spain) preserved in dry ice to prevent RNA degradation.

Molecular Variants Characterization

RNA was thawed immediately before the reverse transcription protocol. cDNA was generated by NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal) and was stored at -20°C . Briefly, 8 μl of each thawed RNA sample was reverse transcribed in a 20 μl reaction mixture containing 10 μl of NZYRT 2 $\times$ Master Mix and 2 μl of NZYRT Enzyme Mix. The reaction mixture was incubated at 25°C for 10 min, at 50°C for 30 min followed by heat inactivation for 5 min at 85°C . Finally, 1 μl of NZY RNase H from *Escherichia coli* was added to remove RNA bound to cDNA and the final reaction mixture was incubated at 37°C for 20 min. The reverse transcribed products were stored at -20°C .

To detect the three specific mutations characterizing the B.1.177 variant (see section “Introduction,” and **Table 2**, reactions 1–3), three primer pairs were designed to amplify the targeted genomic regions by qPCR. The samples identified as “non-B.1.177 variant” (because they did not carry the full set of three specific mutations of the B.1.177 variant, see section “Introduction”) were subjected to an additional qPCR protocol (**Table 2**, reaction 4) for amplification, within the *S* gene, of a highly variable region which encodes a part of the RBD (Gómez et al., 2021).

Primer pairs were designed using Primer 3² to obtain a melting temperature around 60°C and a GC content of 40–60%, and to avoid dimerization, hybridization to unwanted sites, and the presence of secondary structures that could interfere with the amplification process. qPCR reactions were carried out by following the protocol given in the NZYSpeedy qPCR Green Master Mix (2 $\times$) (NZYTech, Portugal), which relies on SYBR green intercalation to generate the fluorescent signal. We used 2 μl of cDNA as the template in a 20- μl reaction mixture containing 10 μl of NZYSpeedy qPCR Green Master Mix (2 $\times$), 0.4 μl of 20 μM each primer, and 7.2 μl of nuclease-free water. The temperature program for all qPCR reactions was the same: (i) hot start: 2 min at 95°C ; (ii) amplification: 40 cycles, with one cycle consisting of 5 s at 95°C and 30 s at 60°C ; and (iii) melting: 30 s at 95°C , 30 s at 65°C , and 30 s at 95°C . Amplified DNA products were visually confirmed by agarose gel electrophoresis with fluorescent identification of bands of the expected size in the gel (**Table 2**). During each qPCR run, negative control using water as a template, and positive control (human sample) belonging to the B.1.177 variant were included.

Sequencing

After qPCR amplification, the desired amplified fragments were Sanger sequenced using as sequencing primer the forward primer utilized in the amplification. Given the small size of

¹<https://covariants.org>

²<https://primer3.ut.ee/>

TABLE 1 | Sample information.

ID	Date	Ct for the targeted regions			Variant identified	Additional mutations
		ORF1ab	S	N		
PA57583	01/09/2020	13.72	14.91	15.42	B.1.177	
PA57891	02/09/2020	10.94	10.94	12.78	B.1.177	
PA58234	03/09/2020	11.02	11.42	13.43	B.1.177	
PA58236	03/09/2020	15.39	14.62	16.26	B.1.177	
PA58243	03/09/2020	17.15	17.34	17.96	B.1.177	
PA58968	04/09/2020	12.57	11.89	13.4	B.1.177	
PA58981	04/09/2020	13.94	13.51	15.8	B.1.177	
PA5991	04/09/2020	11.17	10.97	11.68	B.1.177	
PA59042	04/09/2020	11.95	12.02	13.33	B.1.177	
PA59067	04/09/2020	13.97	11.88	14.7	B.1.177	
PA59059	04/09/2020	22.62	22.73	25.41	Pre-existing Wuhan	
PA62148	14/09/2020	21.15	17.97	22.36	B.1.177	
PA62720	15/09/2020	11.59	11.7	12.69	B.1.177	
PA62743	15/09/2020	13.39	13.32	13.64	B.1.177	
PA65252	17/09/2020	16.66	16.57	19.02	B.1.177	
PA65276	17/09/2020	16.27	15.35	18.97	B.1.177	
PA65285	17/09/2020	13.67	13.51	15.34	B.1.177	
PA67704	23/09/2020	19.72	15.6	19.89	B.1.177	
PA67793	23/09/2020	22.97	19.39	23.46	Pre-existing Wuhan	
PA77591	15/10/2020	11.94	11.44	13.97	B.1.177	
PA80503	21/10/2020	11.67	11.81	14.93	B.1.177	
PA117525	04/12/2020	10.2	10.25	12.04	B.1.177	
PA117545	04/12/2020	11.31	11.8	13.99	B.1.177	
PA117667	04/12/2020	10.76	11.13	12.78	B.1.177	
PA117741	04/12/2020	16.8	14.71	16.79	Undetermined	G28875T (N_S201I)
PA117895	04/12/2020	13.46	11.29	15.63	B.1.177	
PA117912	04/12/2020	11.77	11.94	12.73	B.1.177	
PA118201	04/12/2020	12.21	12.38	15.79	Undetermined	G28875T (N_S201I)
PA118273	04/12/2020	10.87	11.56	12.49	B.1.177	
PA118338	04/12/2020	16.64	16.34	16.95	Undetermined	G28903T (N_M210I) C28905T (N_A211V)
PA118376	04/12/2020	11.67	11.31	12.36	Undetermined	G28875T (N_S201I)
PA118507	04/12/2020	16.02	15.09	17.31	B.1.177	
PA118573	04/12/2020	11.59	12.13	13.59	B.1.160	
PA118732	04/12/2020	11.69	11.23	11.96	B.1.177	
PA118586	04/12/2020	11.51	11.55	14.08	B.1.160	
PA118615	04/12/2020	12.07	12.54	15.29	B.1.177	
PA118625	04/12/2020	13.96	12.89	14.46	B.1.160	
PA118642	04/12/2020	12.64	12.06	14.65	B.1.177	
PA118659	04/12/2020	14.46	14.68	14.42	B.1.160	
PA118723	04/12/2020	12.22	12.22	12.57	Pre-existing Wuhan	
PA117797	04/12/2020	10.52	10.25	11.86	B.1.177	
PA118148	04/12/2020	11.58	12.47	13.14	B.1.177	
PA120227	09/12/2020	11.93	12.47	12.79	B.1.160	
PA120229	09/12/2020	11.42	11.35	11.69	B.1.160	
PA120230	09/12/2020	11.73	12.89	13.98	B.1.177	T29685C (ORF10)
PA120241	09/12/2020	12.34	12.11	13.89	B.1.177	T29685C (ORF10)
PA1120370	09/12/2020	10.74	10.83	11.48	Pre-existing Wuhan	
PA120623	09/12/2020	11.64	11.7	13.2	B.1.160	
PA120628	09/12/2020	8.25	8.86	9.76	B.1.177	
PA120636	09/12/2020	13.35	12.98	14.32	Pre-existing Wuhan	
PA120695	09/12/2020	10.74	9.54	12.84	B.1.177	
PA120704	09/12/2020	12.06	11.29	12.51	B.1.177	
PA120758	09/12/2020	11.15	12.69	14.57	B.1.177	
PA120812	09/12/2020	11.92	11.82	13.6	B.1.177	

The columns show sample number, collection date, Ct from ORF1ab, S, and N RNA regions, variant identified, and presence of additional mutations not found in the B.1.177, B.1.160 or pre-existing Wuhan variants. Pre-existing Wuhan indicates that in all the sequenced regions (including the S region amplified in the 4th PCR reaction of **Table 2**) the sample conformed to the reference sequence. In line with current trends, the observation of the synonymous change in ORF10 (labeled ORF10 in the Additional mutations column) did not prevent our consideration of these samples as belonging to the B.1.177 variant revealed by the sequenced regions. In contrast, the non-synonymous mutation in the N gene (labeled in the last column as N_ preceding the amino acid change between parentheses) led us to consider the sample as having an undetermined variant.

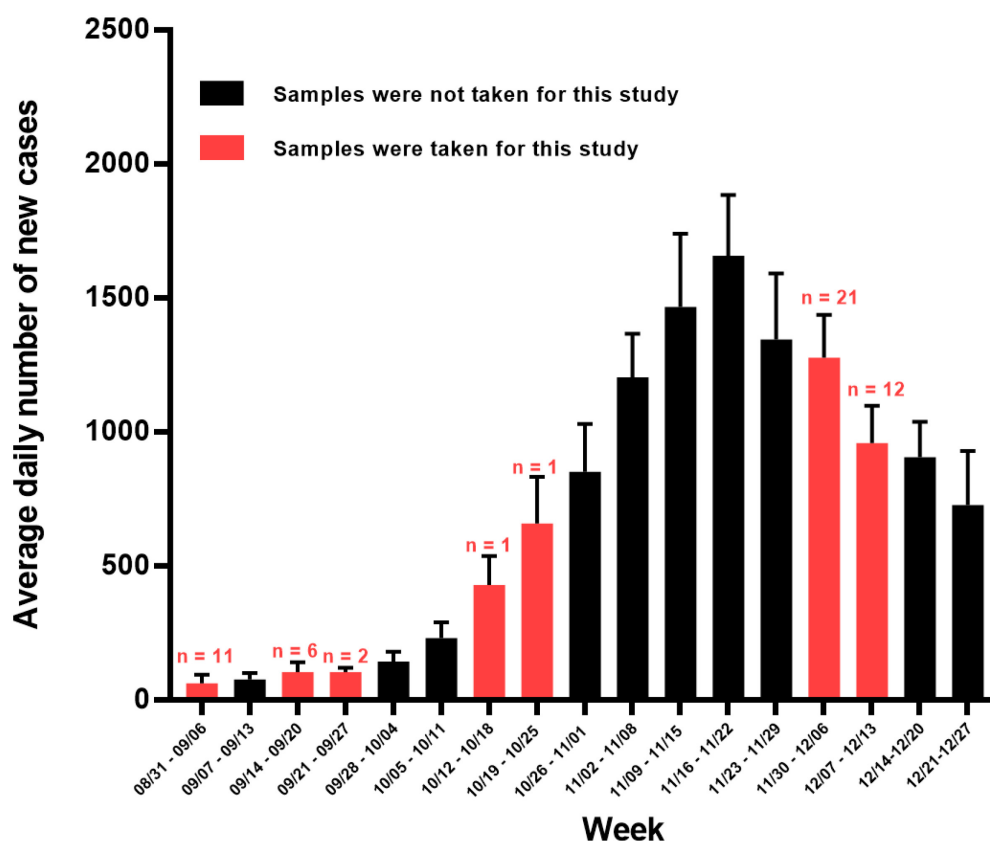


FIGURE 1 | Average daily number of new SARS-CoV-2 positive cases per week in Sicily between 31 August 2020 and 27 December 2021. Red bars denote weeks in which samples were taken for this study (number of samples taken shown above red bars) whereas non-sampled weeks are colored black. Error lines show SDs. Data for Sicily were downloaded from the GitHub repository (<https://github.com/pcm-dpc/COVID-19/tree/master/dati-regioni>).

TABLE 2 | Primer pairs used in PCR amplifications to detect SARS-CoV-2 variants after partial sequencing, as detailed in the corresponding columns.

PCR reaction	Gene	Sequences of primers	Amplified region			Targeted genetic mutation	Possible SARS-CoV-2 tested variant
			Start position	End position	Size (bp)		
1	S	F: 5'-GGACCTTGAAGGAAAACAGG-3' R: 5'-GAACCATTTGGTAGATTTGCCA-3'	22,160	22,239	80	C22227T	B.1.177
2	N	F: 5'-GCAGTCAAGCCTCTTCTCGT-3' R: 5'-TTGAACCAGCTTGAGAGCAA-3'	28,871	28,964	94	C28932T	
3	ORF10	F: 5'-ATTGCAACAATCCATGAGCA-3' R: 5'-TAGGGAGGACTTGAAAGAGCC-3'	29,556	29,704	149	G29645T	
4	S (RBD)	F: 5'-CCGCATCATTTTCCACTTTT-3 R: 5'-AAACAGTTGCTGGTGCATGT-3'	22,728	23,124	397	A23063T	B.1.1.7
						A23063T G23012A G22813T	B.1.351
						A23063T G23012A A22812G	P.1
						G22992A	B.1.160
						C22879A	Cluster S_N439K

the amplicons (Table 2), only the forward DNA sequence was determined for each sequencing reaction. DNA purification and sequencing were carried out by a core sequencing

service (Genomic Department, Principe Felipe Research Centre, Valencia, Spain) in an ABI Prism 3730 automated sequencer (Applied Biosystems, Foster City, CA, United States). All

sequences were subjected to BLASTN³ to identify related SARS-CoV-2 sequences deposited in the GenBank database. BioEdit ver. 7.2.5 software (Hall, 1999) was used for nucleotide and corresponding amino acid sequences alignment, and for analysis and calculation of the degree of identity of the retrieved sequences.

Statistical Analysis

For the statistical analysis, R software was used (R Core Team, 2021). We divided the samples into two groups: those collected pre-December 2020 (September and October 2020) and those sampled during December 2020. A non-parametric Fisher's exact test was used to compare the proportion of samples which belonged to the different SARS-CoV-2 variants analyzed in this study. *P*-value was calculated from 2-sided test using 0.05 as the significance level.

RESULTS AND DISCUSSION

The characteristics of the 54 samples analyzed in this study, including the dates of collection and molecular information derived from the present studies, are summarized in **Table 1**. We focused on the early part of the downslope of Sicily's second wave (**Figure 1**) when the B.1.160 viral lineage was spreading through continental Europe (Hodcroft et al., 2021). For this period, we randomly chose 2 days (4 and 9 of December). From the samples collected on these 2 days, we randomly selected 33 of them obtained from unrelated individuals among those samples that had high viral loads, reflected in Ct values < 18 for the three genes examined in the diagnostic qRT-PCR (*ORF1ab*, *S*, and *N* genes, see section "Materials and Methods"). This last criterion sought to maximize success in molecular studies. To obtain insight into the variants circulating before the second wave and in the early stages of it, we included 21 additional samples in the study. Eleven of these samples were obtained from 1 to 4 September 2020, in advance of the wave; a further 8 samples were collected on 12–23 September 2020, closer to the beginning of the wave; and single samples, each from 15 and 21 October 2020, were from the early phase of the wave. Due to the paucity of cases and samples during the month of September, four samples for this month (one taken 4 September and the other three in the second half of the month) had at least one Ct value > 18 (although all Ct values were < 26) (**Table 1**).

For each sample, we amplified and sequenced three SARS-CoV-2 genomic regions (**Table 2**, PCR reactions 1–3) encompassing the three specific nucleotide mutations of the B.1.177 lineage, C2222T, C28932T, and G29645T, mapping to the *S*, *N*, and *ORF10* genes, respectively. Of the 54 samples, 38 (70.4%) corresponded to the B.1.177 variant (**Figure 2A**), two of which hosted a synonymous nucleotide change in the sequenced region of the *ORF10* gene (**Table 1**). We compared the relative prevalence of this variant before and at the start of the second wave with that during the downslope of the wave (**Figure 1**). With this goal in mind, we distributed the samples into two

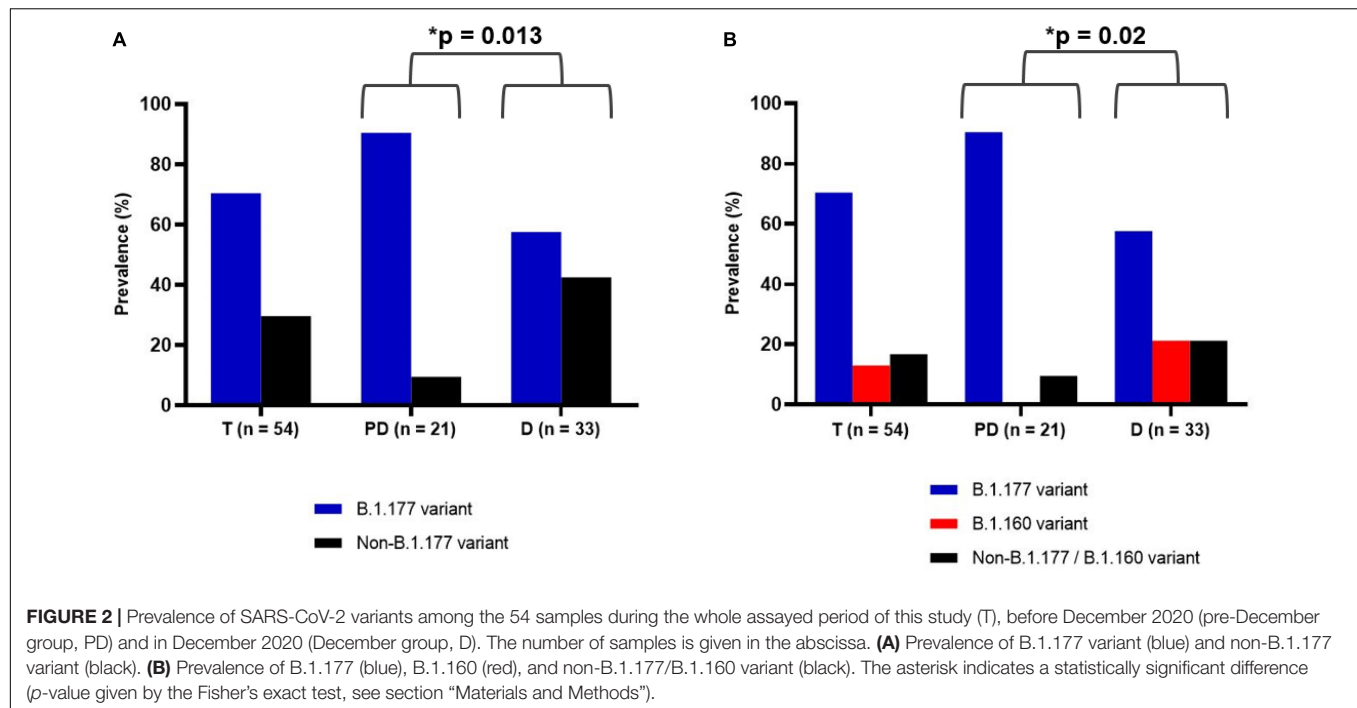
groups, the "pre-December 2020" group, which included the 21 samples collected in September and October of 2020; and the "December 2020" group, which included the 33 samples gathered in December 2020 (**Figure 2**). While 90.47% (19/21) of the samples in the pre-December 2020 group corresponded to the B.1.177 variant, this variant was only found in 57.6% (19/33) of the samples in the December 20 group (**Figure 2A**). A Fisher's exact test was carried out to statistically compare the relative prevalence of the B.1.177 variant among the samples in these two groups. A *p* = 0.013 confirmed that the difference in the relative prevalence of this variant before December 2020 and in December 2020 was statistically significant.

To identify which SARS-CoV-2 variants appeared alongside the lowering in the relative prevalence of B.1.177 in the downslope of the second wave, we amplified and sequenced the central part of the genomic region that encoded the RBD part of the *S* protein (see section "Materials and Methods" and **Table 2**) in the 16 non-B.1.177 viral samples. The amplified region was chosen because it is highly variable and its sequence would allow the identification of the B.1.1.7, B.1.351, and P.1 lineages (Bayarri-Olmos et al., 2021) and the B.1.160 lineage and a smaller cluster defined by the N439K *S* protein mutation (Hodcroft et al., 2021). Most of these variants considerably increased in prevalence in Europe around December 2020 (see text footnote 1). Through alignment with the SARS-CoV-2 reference genome (GenBank Accession Number: NC_045512.2), we detected that the only two non-B.1.177 sequences in the pre-December 2020 group (9.52%, 2/21) were identical in this region to the reference genome. On the other hand, 7 of the 14 non-B.1.177 samples gathered in the December 2020 group (21.2%, 7/33), contained the G22992A non-synonymous mutation. This mutation corresponds to the specific S477N substitution in the *S* protein of the B.1.160 variant (**Figure 2B**). Therefore, we were able to distribute the 54 samples in three sets: set (1) B.1.177 variant (*n* = 38), set (2) B.1.160 variant (*n* = 7), and set (3) non-B.1.177/B.1.160 variants (*n* = 9), of which 5 and 4 represented, respectively, the pre-existing Wuhan sequence and undetermined variants. The differences in the proportions of samples in these three sets in the pre-December 2020 and December 2020 groups were statistically significant (*p* = 0.020) (**Figure 2B**).

Interestingly, six samples presented additional mutations in the *ORF10* or *N* gene (**Table 1**). We already mentioned two of these samples (PA120230 and PA120241, **Table 1**), which contained a synonymous mutation in *ORF10* in addition to the three specific mutations that characterize the B.1.177 variant. The other four samples belonged to set (3), non-B.1.177/B.1.160, and represented previously undetermined variants. The RBD mutations found in these samples were proven by BLASTN analysis (see text footnote 3) to have been previously detected multiple times, although they had not been used to define or to participate in defining any lineage.

Our finding of the predominance of the B.1.177 variant and the timing of the appearance of the B.1.160 variant and of undetermined variants replicates the findings in other parts of Europe in approximately the same period (Hodcroft et al., 2021; see text footnote 1). This indicates that the insular character does

³<http://blast.ncbi.nlm.nih.gov>



not result in a particular pattern of variants reflecting isolation. Yet, further exploration of the undetermined variants should be implemented through a whole-genome sequencing approach to test the possibility that these variants could be “private” to Sicily and novel, arising locally or *via* immigration from other continents (largely Africa). The discussion on whether the slightly increased affinity for the ACE2 receptor of the S protein found in the B.1.160 viral variant (Chen et al., 2020), or the relaxation of restrictions in mobility throughout Europe caused the increase in the B.1.160 viral variant remains unanswered for Europe, and for Sicily. On this island, heavy business and touristic travel in and out of it may be a reason for the similarity of Sicily to the remainder of the continent in terms of variants prevalence. However, our failure to identify in any sample the B.1.1.7, B.1.351, or P.1 variant suggests some delay in the colonization of Sicily by these lineages, which present clear differential traits concerning viral biology and ability to spread across the population, as best exemplified in the increased transmissibility of B.1.1.7 (Davies et al., 2021). Further studies focusing on later periods in the pandemic are needed to analyze the spread of these novel variants in Sicily. These studies would clarify if, as in many other regions of Europe (Funk et al., 2021), these variants became predominant in late December 2020 and January 2021, a period of resurgence of the number of cases that could be equated to a second wave within the second wave. In any case, our pilot study suggests that in the period studied no SARS-CoV-2 variant of significantly higher transmissible potential than the consensus one emerged in Sicily.

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 below: <https://www.ncbi.nlm.nih.gov/genbank/>, OM510944; <https://www.ncbi.nlm.nih.gov/genbank/>, OM510945; <https://www.ncbi.nlm.nih.gov/genbank/>, OM510946; <https://www.ncbi.nlm.nih.gov/genbank/>, OM510947; <https://www.ncbi.nlm.nih.gov/genbank/>, OM510948; <https://www.ncbi.nlm.nih.gov/genbank/>, OM510949; <https://www.ncbi.nlm.nih.gov/genbank/>, OM510950; <https://www.ncbi.nlm.nih.gov/genbank/>, OM510951; and <https://www.ncbi.nlm.nih.gov/genbank/>, OM510952.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Bioethical Committee of Universidad Cardenal Herrera CEU. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AG, CR-G, VR, GP, and EM conceived the study. FG, GP, AP, and MT performed molecular SARS-CoV-2 detection in all the samples. MP-B did the molecular variants characterization. CR-G, EM, and MP-B analyzed the results. CS, VV, MP-O, and MP-B did the epidemiological analysis. CS, MP-B, EM, GP, FG, AG, VR, and CR-G analyzed the data and were responsible for writing the manuscript. All authors contributed to this last task, making substantial intellectual contributions, and having read, corrected, and approved the manuscript.

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REFERENCES

- Bayarri-Olmos, R., Jarlhelt, I., Johnsen, L. B., Hansen, C. B., Helgstrand, C., Bjelke, J. R., et al. (2021). Functional effects of receptor-binding domain mutations of SARS-CoV-2 B.1.351 and P.1 variants. *Front. Immunol.* 12:757197. doi: 10.3389/fimmu.2021.757197
- Bian, L., Gao, F., Zhang, J., He, Q., Mao, Q., Xu, M., et al. (2021). Effects of SARS-CoV-2 variants on vaccine efficacy and response strategies. *Expert Rev. Vaccines* 20, 365–373. doi: 10.1080/14760584.2021.1903879
- Chakraborty, C., Bhattacharya, M., and Sharma, A. R. (2022) Present variants of concern and variants of interest of severe acute respiratory syndrome coronavirus 2: their significant mutations in S-glycoprotein, infectivity, re-infectivity, immune escape and vaccines activity. *Rev. Med. Virol.* 32:e2270. doi: 10.1002/rmv.2270
- Chen, J., Wang, R., Wang, M., and Wei, G. W. (2020). Mutations strengthened SARS-CoV-2 infectivity. *J. Mol. Biol.* 432, 5212–5226. doi: 10.1016/j.jmb.2020.07.009
- Coronavirus Resource Center (2021). *Coronavirus COVID-19 Global Cases by the Center for Systems Science and Engineering (CSSE). Johns Hopkins Coronavirus Resource Center*. Available online at: <https://coronavirus.jhu.edu/map.html> (Accessed December 10, 2021).
- Davies, N. G., Abbott, S., Barnard, R. C., Jarvis, C. I., Kucharski, A. J., Munday, J. D., et al. (2021). Estimated transmissibility and impact of SARS-CoV-2 lineage B.1.1.7 in England. *Science* 372:eabg3055. doi: 10.1126/science.abg3055
- Funk, T., Pharris, A., Spiteri, G., Bundle, N., Melidou, A., Carr, M., et al. (2021). Characteristics of SARS-CoV-2 variants of concern B.1.1.7, B.1.351 or P.1: data from seven EU/EEA countries, weeks 38/2020 to 10/2021. *Euro. Surveill.* 26:2100348. doi: 10.2807/1560-7917.ES.2021.26.16.2100348
- Gómez, C. E., Perdiguero, B., and Esteban, M. (2021). Emerging SARS-CoV-2 variants and impact in global vaccination programs against SARS-CoV-2/COVID-19. *Vaccines (Basel)* 9:243. doi: 10.3390/vaccines9030243
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41, 95–98.
- Hodcroft, E. B., Zuber, M., Nadeau, S., Vaughan, T. G., Crawford, K. H. D., Althaus, C. L., et al. (2021). Spread of a SARS-CoV-2 variant through Europe in the summer of 2020. *Nature* 595, 707–712. doi: 10.1038/s41586-021-03677-y
- Hoffmann, M., Arora, P., Groß, R., Seidel, A., Hörnich, B. F., Hahn, A. S., et al. (2021). SARS-CoV-2 variants B.1.351 and P.1 escape from neutralizing antibodies. *Cell* 184, 2384–2393. doi: 10.1016/j.cell.2021.03.036
- ISS (2021a). *Epidemia COVID-19. Aggiornamento Nazionale 18 Agosto 2021*. Available online at: <https://www.epicentro.iss.it/coronavirus/aggiornamenti> (Accessed August 23, 2021)
- ISS (2021b). *Epidemia COVID-19. Aggiornamento Nazionale 22 Dicembre 2020*. Available online at: https://www.epicentro.iss.it/coronavirus/bollettino/Bollettino-sorveglianza-integrata-COVID-19_22-dicembre-2020 (Accessed August 23, 2021)
- Jackson, C. B., Farzan, M., Chen, B., and Choe, H. (2022). Mechanisms of SARS-CoV-2 entry into cells. *Nat. Rev. Mol. Cell. Biol.* 23, 3–20. doi: 10.1038/s41580-021-00418-x
- Korber, B., Fischer, W. M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., et al. (2020). Tracking changes in SARS-CoV-2 spike: evidence that D614G increases infectivity of the COVID-19 virus. *Cell* 184, 812–827. doi: 10.1016/j.cell.2020.06.043
- Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., et al. (2020). Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2-receptor. *Nature* 581, 215–220. doi: 10.1038/s41586-020-2180-5
- ProMED-mail (2020). Available online at: <https://promedmail.org/promed-post/?id=6864153>. (Accessed January 30, 2022).
- R Core Team (2021). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.
- Tramuto, F., Reale, S., Presti, A. L., Vitale, F., Pulvirenti, C., Rezza, G., et al. (2021). Genomic analysis and lineage identification of SARS-CoV-2 strains in migrants accessing Europe through the Libyan route. *Front. Public Health.* 9:632645. doi: 10.3389/fpubh.2021.632645
- Volz, E., Hill, V., McCrone, J. T., Price, A., Jorgensen, D., O’Toole, Á, et al. (2021). Evaluating the effects of SARS-CoV-2 spike mutation D614G on transmissibility and pathogenicity. *Cell* 184, 64–75. doi: 10.1016/j.cell.2020.11.020
- World Health Organization [WHO] (2022a). *COVID-19 Weekly Epidemiological Update*. Geneva: World Health Organization.
- World Health Organization [WHO] (2022b). *COVID-19*. Geneva: World Health Organization.
- Worobey, M., Pekar, J., Larsen, B. B., Nelson, M. I., Hill, V., and Joy, J. B. (2020). The emergence of SARS-CoV-2 in Europe and North America. *Science* 370, 564–570. doi: 10.1126/science.abc8169
- Xie, X., Liu, Y., Liu, J., Zhang, X., Zou, J., Fontes-Garfias, C. R., et al. (2021). Neutralization of SARS-CoV-2 spike 69/70 deletion, E484K and N501Y variants by BNT162b2 vaccine-elicited sera. *Nat. Med.* 27, 620–621. doi: 10.1038/s41591-021-01270-4
- Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733. doi: 10.1056/NEJMoa2001017

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Altered Lipid Profile in COVID-19 Patients and Metabolic Reprogramming

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Background: Coronavirus disease 2019 (COVID-19) is a global pandemic. Previous studies have reported dyslipidemia in patients with COVID-19. Herein, we conducted a retrospective study and a bioinformatics analysis to evaluate the essential data of the lipid profile as well as the possible mechanism in patients with COVID-19.

Methods: First of all, the retrospective study included three cohorts: patients with COVID-19, a healthy population, and patients with chronic obstructive pulmonary disease (COPD). For each subject, serum lipid profiles in the biochemical data were compared, including triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C). Furthermore, bioinformatics analyses were performed for exploring the biological or immunological mechanisms.

Results: In line with the biochemical data of the three cohorts, the statistical result displayed that patients with COVID-19 were more likely to have lower levels of TC and HDL-C as compared with healthy individuals. The differential proteins associated with COVID-19 are involved in the lipid pathway and can target and regulate cytokines and immune cells. Additionally, a heatmap revealed that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections were possibly involved in lipid metabolic reprogramming. The viral proteins, such as spike (S) and non-structural protein 2 (Nsp2) of SARS-CoV-2, may be involved in metabolic reprogramming.

Conclusion: The metabolic reprogramming after SARS-CoV-2 infections is probably associated with the immune and clinical phenotype of patients. Hence, metabolic reprogramming may be targeted for developing antivirals against COVID-19.

Keywords: COVID-19, lipid, SARS-CoV-2, dyslipidemias, metabolic reprogramming

INTRODUCTION

The infections of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) led to the coronavirus disease 2019 (COVID-19) pandemic and have been a threat to public health across the world. There are 464,809,377 confirmed cases of COVID-19, including 6,062,536 deaths as of March 20, 2022 (World Health Organization [WHO], 2022). Public health and social and economic growth have been enormously influenced by the COVID-19 pandemic. SARS-CoV-2 contains four structural proteins, [envelope (E), membrane (M), nucleocapsid (N), and spike (S)], 16 non-structural proteins (Nsp1 to Nsp16), and eight accessory proteins (orf3a, orf6, orf7a, orf7b, orf8, orf9b, orf9c, and orf10). These proteins are involved in the viral life cycle and viral interaction with the host. Although the interaction between SARS-CoV-2 and the host is a moot point, the scientific community steadily gained an understanding of pathogenesis in the past.

Although the interactions between the immune system and lipid metabolism during SARS-CoV-2 infections remain unclear, the new development of tumor and metabolism study can provide new ideas and methods for the influence of SARS-CoV-2 infections. Interestingly, lipids are involved in viral pathogenesis and the pathophysiology of viral disease (Nie et al., 2020). Lipids not only constitute virus envelope but also involve viral replication and invasion. The composition of viruses and cells always includes lipids involved in membrane fusion and replication during the entry and the release from the host cell membrane. Previous studies (Wei et al., 2020; Jin et al., 2021; Mahat et al., 2021) have shown that lipid profiles, such as the total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), in patients with COVID-19 are significantly altered. TC increases in the cell membrane, which benefits the virus entry to the host cells and the membrane fusion (Theken et al., 2021). The alterations of lipid profile in patients with COVID-19 seem to be proportional to the clinical phenotype and might be a target for risk evaluation. In addition, TC can regulate T-cell-mediated immune response and constitute T-cell receptors (TCRs) as a critical regulator, directly or indirectly (Bietz et al., 2017; Puleston et al., 2020). A previous study about lymphocytic choriomeningitis virus (LCMV) also showed that the clearance of the LCMV was significantly delayed in hypercholesterolemic mice, and LCMV-specific CD8⁺ T cells were suppressed (Ludewig et al., 2001). Cholesterol accumulation reduced the activation of CD8 α -dendritic cells, thereby impairing Th1 cell responses while enhancing Th2 cell responses (Kim et al., 2021). Other evidence from oncology has demonstrated that multiple lipid species can be sensed by innate immune cells including macrophages and dendritic cells. Dyslipidemia is a critical regulator of adaptive immunity, which in turn can regulate adaptive immune cells (Kim et al., 2021).

However, the concentration of lipid profiles in patients with COVID-19 was reported with variable values (Gao et al., 2020; Hu et al., 2020; Lei et al., 2020; Malik et al., 2021). A likely explanation is that the genetic phenotypes and underlying diseases are significantly different among patients with COVID-19. To extend the existing evidence regarding the

relationship between COVID-19 and lipid profile, a retrospective study and mechanism exploration by bioinformatics analyses were performed. We did extensive research about the actual relation between viral pathogenesis and lipid alteration through existing data. We attempted to elucidate the correlation between lipid profile and immunoreaction among patients with COVID-19, including lipid metabolism and profile, for example, dyslipidemia mechanism, cytokines, and T-cell-mediated immune response.

MATERIALS AND METHODS

Clinical Information

This retrospective study included three cohorts, 25 COVID-19 cases, 25 cases of the healthy examination population (control group, CG), and 25 cases with chronic obstructive pulmonary disease (COPD), recruited from the Huizhou Central People's Hospital. COPD and CG never went through a previous infection with COVID-19 or received the vaccination. The patients were diagnosed with COVID-19 in light of the World Health Organization (WHO) guidelines.¹ The nasopharyngeal swabs of patients with COVID-19 were collected for diagnosis. The laboratory-confirmed patient was defined as a positive result on the real-time reverse-transcriptase polymerase chain reaction (RT-PCR) assay of nasopharyngeal swab specimens. These cases were well balanced for gender, age, and primary disease. All the COVID-19 symptoms were mild, and no severe cases appeared. This study was performed according to the principles of the Declaration of Helsinki and approved by the Huizhou Central People's Hospital following its guidelines for the protection of individual privacy.

Biochemical Measurements

For three cohorts, serum lipid profiles of patients with COVID-19, patients with COPD, and the healthy examination population were tested by biochemical methods (Roche Cobas 8000), including triglycerides (TG), TC HDL-C, and LDL-C. The sera of patients with COVID-19 were collected on admission.

Dataset Collection

The data based on the initial screening are retrieved mainly from the National Center for Biotechnology Information. Proteomic and lipidomic data from the sera/plasma of patients with COVID-19 were acquired from the early studies (Shen et al., 2020; Wu et al., 2020) and GEO datasets (GEO accession number: GSE157103). R was used to screen for differential proteins and lipids (R Core Team, 2018). Glycolysis pathway data were acquired from Caccuri et al. (2021). Profiles of serum cytokines and chemokines in patients with COVID-19 were acquired from Zawawi et al. (2021). SARS-CoV-2 S, E, Nsp15, Nsp16 (Sharma et al., 2021), and Nsp2 (Davies et al., 2020) proteins were determined and compared with the host transcriptomic responses to key viral genes.

¹<https://extranet.who.int/pqweb/vitro-diagnostics/coronavirus-disease-covid-19-pandemic-%E2%80%94-emergency-use-listing-procedure-eul-open>

Bioinformatics Analyses

The Venn diagram was generated based on the datasets (Venn, 2022). Gene Ontology (GO) [involving biological process (BP), cell component (CC), molecular function (MF), and Kyoto Encyclopedia of Genes and Genomes (KEGG)] were utilized to analyze the expected signaling pathways and corresponding functions of differential proteins via the package of R or platform of Enrichr (Chen et al., 2013; R Core Team, 2018). The heatmap was also generated by the package of R (R Core Team, 2018). The proteins network was constructed *via* the STRING dataset (Szklarczyk et al., 2021). Lipidmap was produced by the analysis of KEGG (Fahy et al., 2007). CytoHubba, an app of Cytoscape, was screened for hub genes (Shannon et al., 2003). The immune cell infiltration analysis was performed by GEPIA2021 (Li et al., 2021).

Statistical Analysis

Values of serum lipid were shown as the mean (M) $\pm$ standard deviation (SD). The comparison for the lipid of three cohorts was made by one-way ANOVA using the SPSS 24.0 (IBM Corp.). The least significant difference (LSD) was further compared to show any significant difference between the two groups. Graphic plotting was generated using GraphPad Prism 8 (GraphPad Software, Inc.).

RESULTS

Baseline Characteristics of Coronavirus Disease 2019 Patients

The study included 75 cases, which consisted of 25 COVID-19 patients positive for SARS-CoV-2 RNA, 25 cases diagnosed with COPD that all the history and symptoms supported, and 25

healthy people. The mean ages of CG, patients with COVID-19, and patients with COPD were 51 ± 16.2 , 47 ± 15.4 , and 54 ± 17.6 years, respectively. The patients with COVID-19 did not use any statins according to medication guidelines. Other cohorts were similar. In all cases, previously diagnosed metabolic diseases (obesity, hypertension, and diabetes) were not incorporated based on self-report.

The Lipid Level Change During the Coronavirus Disease 2019 Courses

We found that there were significant differences in TG ($F = 3.506$, $P < 0.05$), TC ($F = 17.123$, $P < 0.0001$), and HDL-C ($F = 21.473$, $P < 0.0001$) levels between the three cohorts, but no significant difference was observed in LDL-C ($F = 0.97$, $P > 0.05$). In line with the biochemical data of the three cohorts, the statistical result displayed that the patients with COVID-19 were more likely to have a lower level of TC ($P < 0.001$) and HDL-C ($P < 0.001$) as compared with the healthy examination population. Patients with COPD had similar results ($P < 0.001$) (Figure 1).

Differential Proteins and Sub-Network Module Enrichment Analysis

Differential proteins in patients with COVID-19 versus CG were acquired in GEO datasets. To further study the role of differential proteins, GO and KEGG signaling pathway analysis indicated that some proteins were involved in lipid pathways (Supplementary Table 1), such as the PPAR signaling pathway, cholesterol metabolism, fatty acid biosynthesis, positive regulation of cholesterol esterification (GO:0010873), and high-density lipoprotein particle remodeling (GO:0034375). The proteome of the sera/plasma of patients with COVID-19 showed 21 common proteins (Figure 2A), which are ORM1,

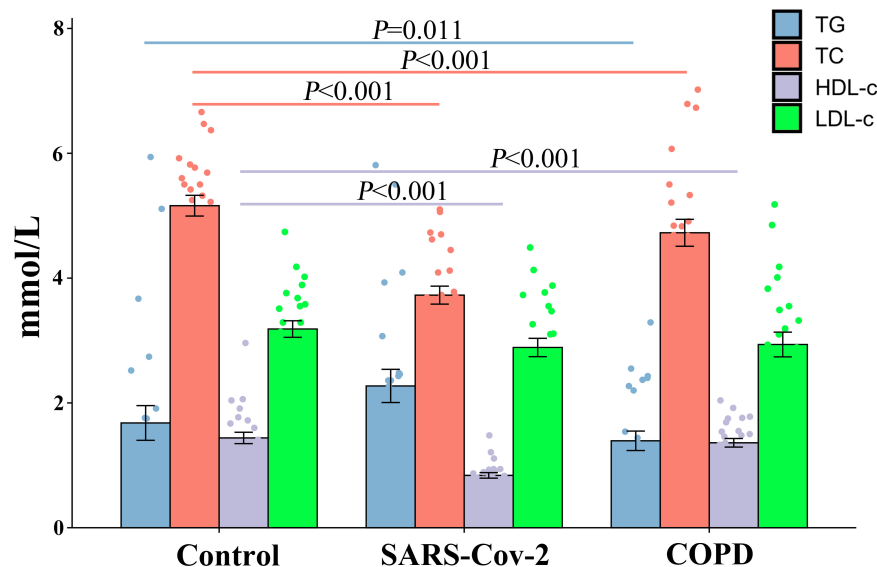


FIGURE 1 | Comparison of the lipid level between the COVID-19, COPD patients, and healthy population. In COVID-19 and COPD patients, TG and HDL-C expression was reduced, but not for TC and LDL-C.

ITIH3, ALB, SAA2, PGLYRP2, APOA1, NID1, GSN, CPN2, LGALS3BP, AGT, LCP1, C2, CLEC3B, ITIH4, APOM, CRTAC1, APOA2, ORM2, AHSG, and GPLD1. These 21 proteins had similar enrichment results that were associated with lipid pathways (Figure 2B and Supplementary Table 2). The lipid pathway included cholesterol metabolism, the PPAR signaling pathway, fat digestion and absorption, positive regulation of cholesterol esterification (GO:0010873), regulation of cholesterol esterification (GO:0010872), and so on. Conversely, lipid composition in patients with COVID-19 did not correspond with what other studies showed (data not shown). The enrichment analysis of lipidome in patients with COVID-19 showed that the blood lipid of humans was mainly involved in sphingolipid metabolism, cholesterol metabolism, fat digestion and absorption, and the sphingolipid signaling pathway (Supplementary Table 3).

Severe Acute Respiratory Syndrome Coronavirus 2 Infection and Viral Proteins Cause Metabolic Reprogramming

The heatmap analysis showed the expression and increment of LDHA, GAPDH, and PKM post-SARS-CoV-2 infection; UV-inactivated SARS-CoV-2 can increase LDHA (Figures 3A,B). The GO and KEGG signaling pathway analysis indicated that the differential proteins of SARS-CoV-2 in endothelial cells were involved in glycolysis (Figure 3C). The lipid pathway and glycolysis occurred showed the potential

for metabolic reprogramming post-SARS-CoV-2 infection (Figures 3D–F).

Protein Network for Targeting Cytokine and Chemokine Regulation

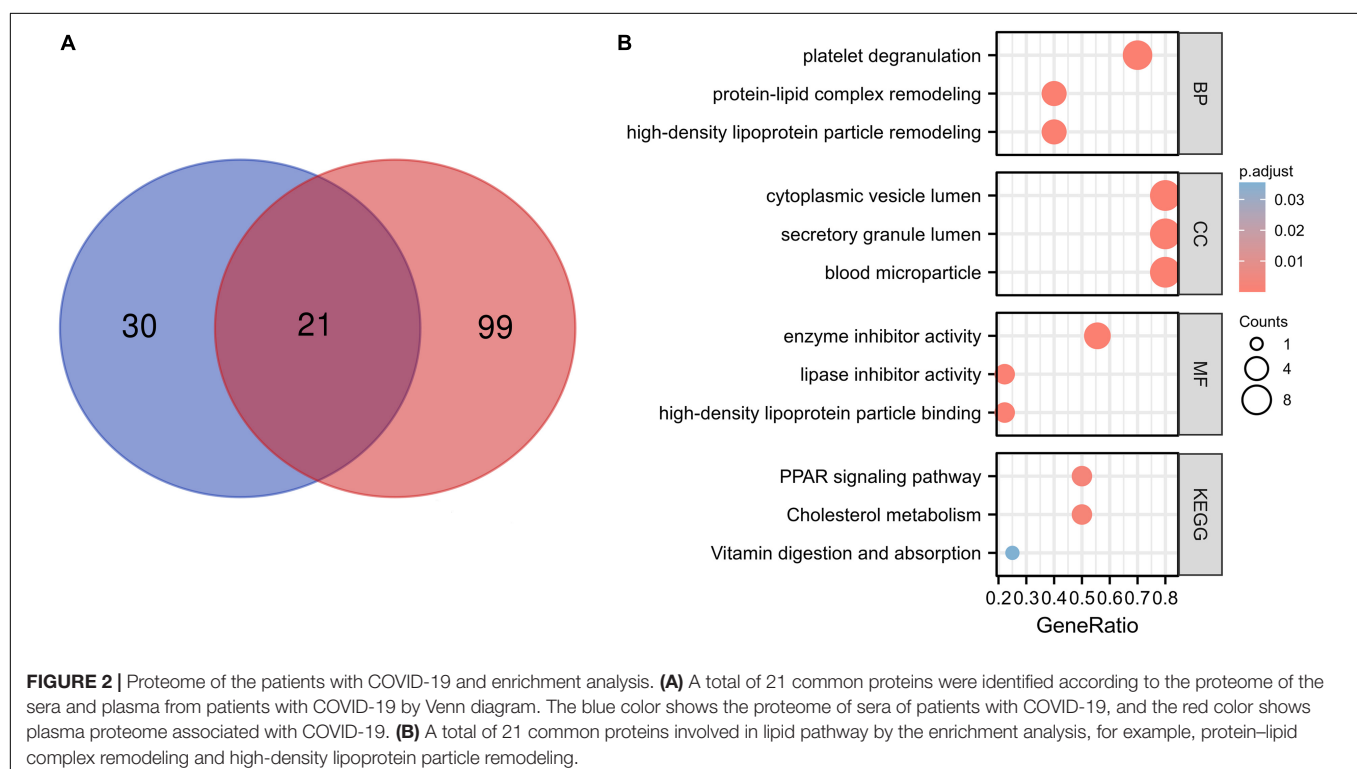
The first 10 nodes (subproteins) (APOA1, ALB, AHSG, APOA2, ITIH4, ITIH3, ORM1, GSN, ORM2, and APOM) with the highest values were screened as fibrin clot (clotting cascade) and lipoprotein particle (Figures 4A,B). A protein–protein interaction (PPI) network for the first 10 nodes was constructed using the STRING database. These subproteins may regulate cytokines and chemokines in patients with COVID-19 (Figure 4C).

Ten Sub-Proteins and Cell Type-Level Expression Analysis

GEPIA2021 analysis further confirmed the correlations between the 10 sub-protein levels and cell types. Regarding the 10 sub-protein expression levels, the analysis of immune infiltration revealed that the CD4⁺ cell has the highest median value in the lung and the CD8⁺ cell has the highest median value in blood except GSN. A component analysis of the immune cells showed that CD4⁺ T cells, CD8⁺ T cells, and NK cells were significantly related to 10 subproteins (Figure 5).

The Viral Proteins Correlate With Metabolic Reprogramming

The S and Nsp2 proteins may involve metabolic reprogramming (Figure 6), but N, Nsp15, and Nsp16 would not. S1 subunit



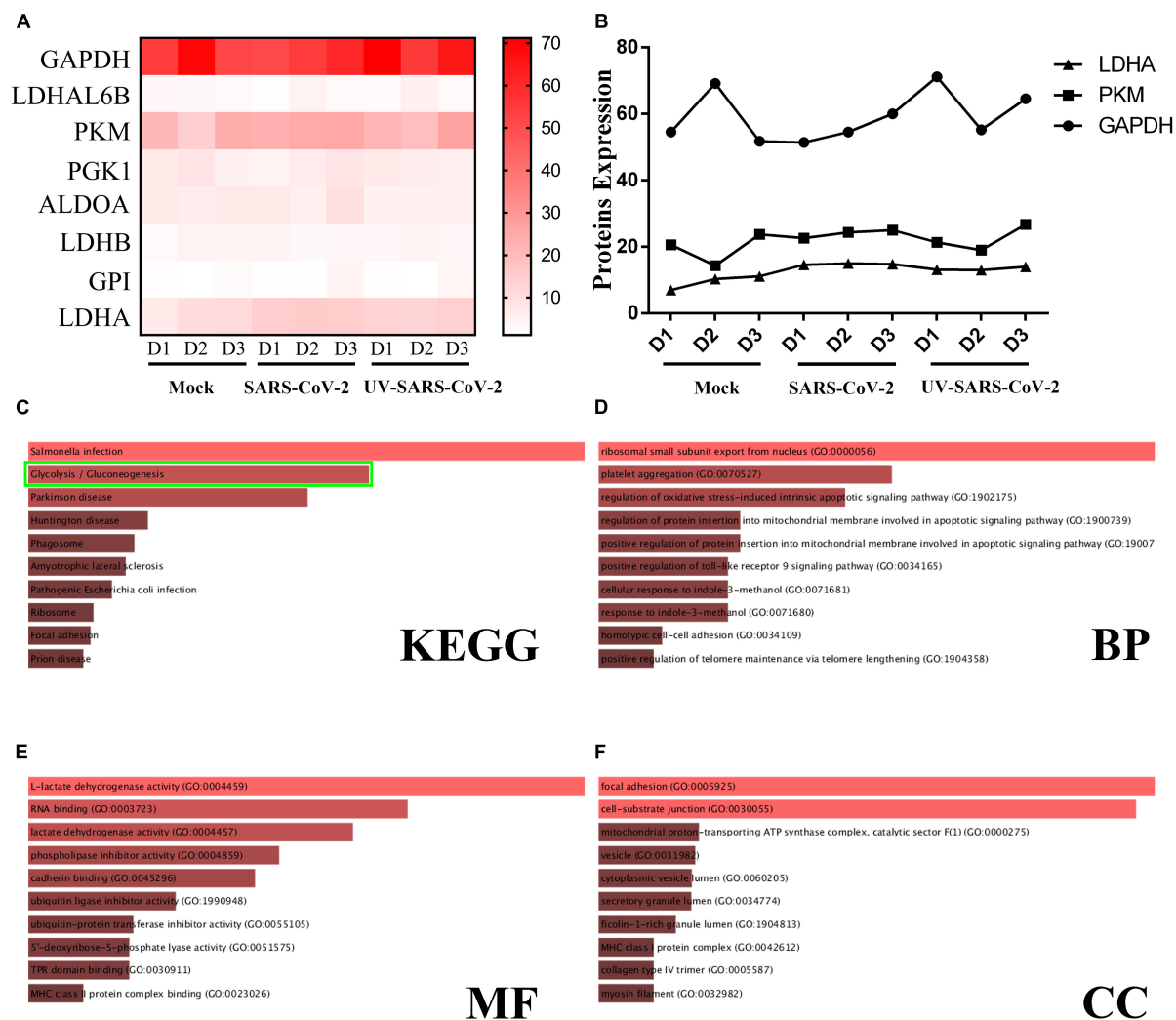


FIGURE 3 | SARS-CoV-2 infection upregulates the glycolysis pathway. (A) A heatmap was generated from the SARS-CoV-2 infection and UV-inactivated SARS-CoV-2 treatment. In both groups, LDHA level was increased. **(B)** The expression of LDHA, PKM, and GAPDH was significantly different among the healthy population, SARS-CoV-2, and UV-SARS-CoV-2 groups. **(C)** The KEGG analysis revealed the SARS-CoV-2 infection and protein correlation involved in the glycolysis pathway (the green bounding box). **(D–F)** The BP, MF, CC analysis revealed the SARS-CoV-2 infection involved in the oxidative stress, the lactate dehydrogenase activity, et al.

seems to regulate HSPA1A, HSPA6, HSPA1B, DDIT3, LDHB, HSP90B1, and EIF2AK3; S2 subunit seems to regulate HSPA1B, HSPA1A, HSPA6, and DDIT3; Nsp2 may regulate PLD3, VDAC2, HSPA8, HSPA5, ERLIN1, ERLIN2, and AGPAT2. These proteins are related to glycolysis and lipid pathways.

DISCUSSION

Lipid profile alteration was used as a potential biomarker to aid diagnostics via triggers of viral infection. Our findings indicate that TC and HDL-C were reduced in patients with COVID-19, but TG and LDL-C did not. This finding is consistent with previous studies (Li G. et al., 2020; Li J. et al., 2020; Lv et al., 2020; Sun et al., 2020; Tanaka et al., 2020; Xie et al., 2020; Xue et al., 2020; Zhang B. et al., 2020; Zhang Q. et al., 2020).

The serum/plasma concentrations of total TC and HDL-C were significantly lower in patients with COVID-19 with more severe diseases but were not for the TG. Significant changes in host lipidomes were observed in the cases of viral infection with severe disease, which induced changes in host immune function and benefited viral replication. On account of population and deviation, the results are possibly different in LDL-C. Distinctly, the heterogeneity between studies was generally large-to-extreme and multiple studies included small sample sizes. Interestingly, TC and HDL-C levels were associated with the clinical phenotype of SARS-CoV-2 infection. TC and HDL-C have beneficial effects on various pulmonary diseases and other diseases (Nie et al., 2020) and play a key role in modulating both innate and adaptive immune cell responses (Bietz et al., 2017). HDL has a function in inducing an anti-inflammatory or inflammatory profile (Van Lenten et al., 1995; Khovidhunkit et al., 2004). The reduced

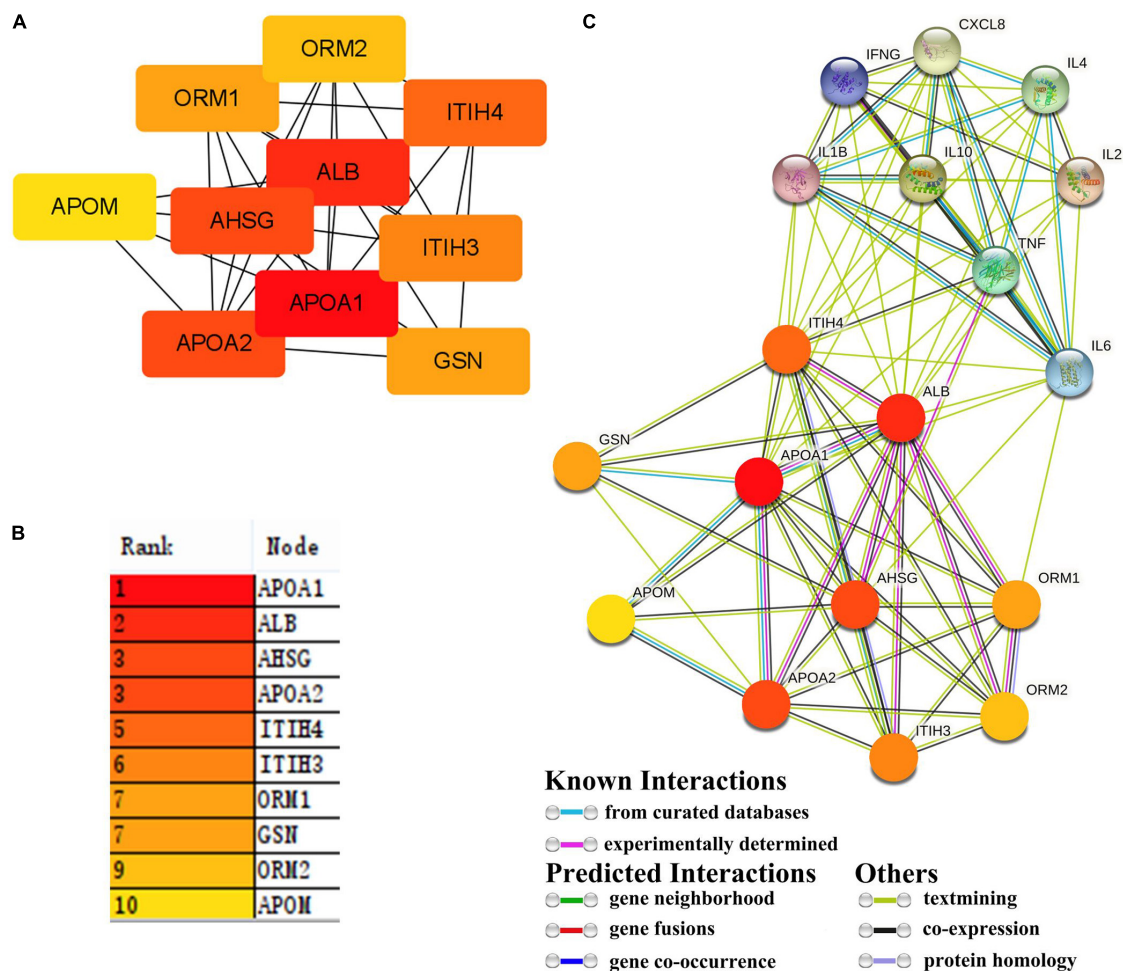


FIGURE 4 | Hub protein in 21 proteins and interaction between hub proteins and cytokines. **(A,B)** 21 proteins were screened for 10 hub proteins. **(C)** The interaction between hub proteins and cytokines.

plasma levels of HDL can be found in patients with infection and sepsis (Wu et al., 2004; Cirstea et al., 2017). Lipid profile alteration is a useful indicator for early warning of the severity of COVID-19 disease (mild or severe) (Nie et al., 2020).

Energy and metabolites are required for cell survival. SARS-CoV-2 infections lead to a hypoxic microenvironment (Bhattacharya et al., 2021). This process is akin to the tumor microenvironment (TME) that the feature of TME is hypoxic. This promotes the host to compensate for their metabolic profiles to sustain a reprogramming state. The heatmap analysis showed that the expression of LDHA, a protein involved in glycolysis, was increased in SARS-CoV-2 infection. Furthermore, the proteomic data in two studies were analogous (Shen et al., 2020; Wu et al., 2020). The interaction network and enrich analysis revealed that the related pathways of lipid were located in the central node of all patient groups. There were significant similarities in lipid pathways among patients with COVID-19 from different regions. The 21 common proteins in this study supported this view. Enrichment analysis showed that the proteins were mainly involved in cholesterol metabolism, the PPAR signaling pathway, and so on. Anyhow,

the hypoxic microenvironment in patients with COVID-19 increases the metabolic reprogramming for local nutrients and oxygen. However, the exact role or influence of metabolism reprogramming in SARS-CoV-2 immune response remains unclear, and lipids may regulate SARS-CoV-2 infection by multiple mechanisms.

Mechanisms derived from the previous study may also shed light on factors contributing to SARS-CoV-2 infectivity, where cholesterol is important either through immune regulator or by mediating signal pathway. The effects of lipids on infection development play a pivotal role. The function of lipids was gradually decrypted, which was used as an alternative source in pathologic conditions (Olsen et al., 2021), and was involved in the virus infection, was involved in transport of cell membrane, and activated intricate signaling pathways related to the immune system (Yu et al., 2021). Lipid metabolism dysfunction in the host has extensive effects on immune cells. The hub proteins were correlated with cytokines and chemokines in patients with COVID-19, and a distinct connection with immune cells was identified. However, the explanations for the lipid phenotype of patients are complex. In addition, individuals

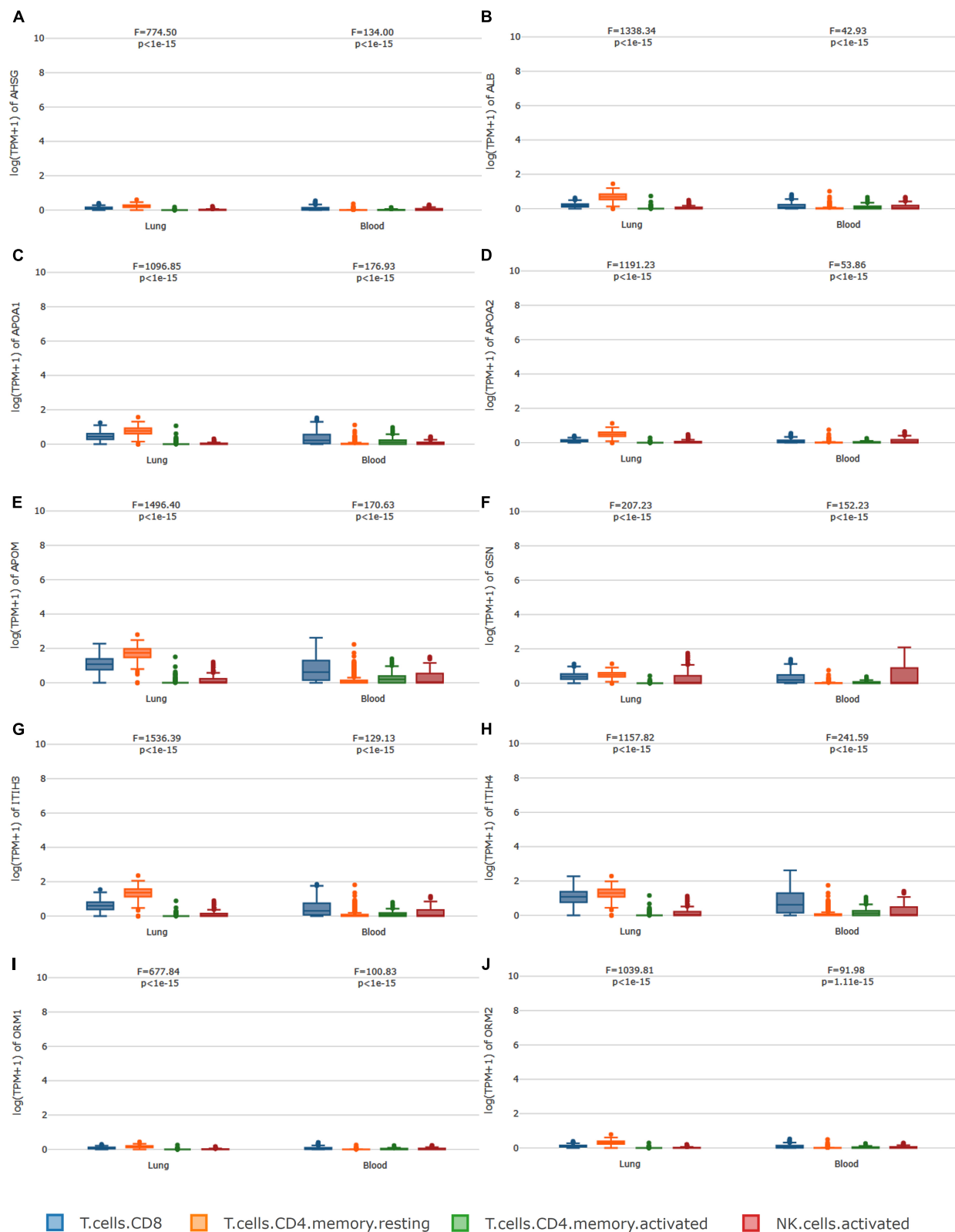
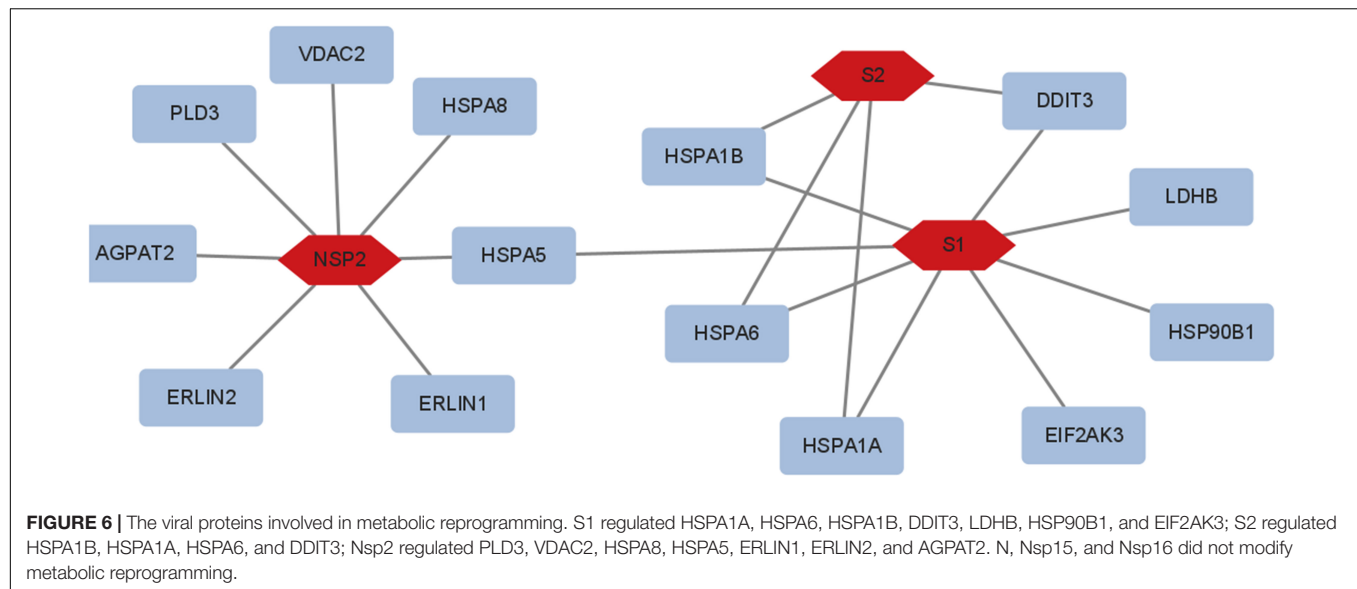


FIGURE 5 | Immune cell type-level expression analysis. In regard to (A–J), the analysis of immune infiltration revealed that the CD4⁺ cell has the highest median value in the lung, and the CD8⁺ cell has the highest median value in blood except for GSN. A component analysis of the immune cells showed that CD4⁺ T cells, CD8⁺ T cells, and NK cells were significantly related to the 10 subproteins.



with underlying comorbidities (primary disease and metabolic disturbance in patients) will have more dramatic changes such that cholesterol provides a more complicated explanation and elaborate medical regimen. Due to the complex composition of lipids and a dynamically anabolic process, different points-in-time may respond very diversely to changes in lipid metabolism and give rise to ambiguous phenotypes. Enrichment analysis of lipids showed blood lipids of humans mainly involved in the sphingolipid metabolism, the cholesterol metabolism, fat digestion and absorption, and the sphingolipid signaling pathway, which suggested that the pathway was mainly for lipid-controlled biosynthesis or signaling. However, the studies of blood lipid were very heterogeneous. The quantification of blood lipids is still non-determined because of the complex component and much fluctuation of lipid quality and quantity in different space and time.

Interestingly, the lipid levels in patients with COPD changed and compared with the healthy population, but it was similar to patients with COVID-19. In addition, hypoxia is a common characteristic of patients that can change the metabolism (Grieb et al., 2021; Palm and Ekström, 2021). Hence, the patients were artificially ventilated, a procedure that can cause intraoperative complications but also can remit glycolysis or further metabolic reprogramming. So, the external reason was partially confirmed. The glycolysis suppression may be taken as a strategy for COVID-19 therapy and has profound therapeutic implications and significance. On the other hand, the lipid metabolism in patients with COVID-19 as a major altered function is highly similar to infection and sepsis, which is in accordance with a reply for multiple pathogens infection and in modulating inflammatory responses by the lipid moieties. These results indicate that metabolism plays a key role in SARS-CoV-2 pathogenesis and is a possible therapeutic target.

Meanwhile, the data showed that LDHA expression is increased in UV-SARS-CoV-2 infection. Besides anoxia, metabolic reprogramming was induced by the viral proteins as

well. Viral structural proteins are involved in such processes. Numerous viruses (Negro, 2010; Funderburg and Mehta, 2016; Melo et al., 2016; Tisoncik-Go et al., 2016; Einfeld et al., 2017; Kyle et al., 2019), such as Ebola virus, HIV, HBV, HCV, and homologous SARS-CoV (Wu et al., 2017) and MERS (Yan et al., 2019), can dramatically alter the human plasma lipidome. Even in the 12 years since the SARS-CoV infection, lipidome had been significantly changed (Wu et al., 2017). Therefore, viral proteins are involved similarly in metabolic reprogramming.

The viral proteins (Nsp2 and S) also affect lipid synthesis and modification (Díaz, 2020). The S protein of SARS-CoV-2 is a key protein. Numerous studies have confirmed that the S protein binds to ACE2 receptors on the surface of host cells to facilitate viral entry (Du et al., 2009; Walls et al., 2020). The S protein comprises S1 and S2 subunits in the virus replication cycle, binding the host cell receptor or fusing the viral envelope with host cell membranes. S1 plays an important role in protein processing in the endoplasmic reticulum, lipid, atherosclerosis, and so on. S2 was concerned with protein processing in the endoplasmic reticulum, the lipid, and atherosclerosis. Therefore, both S1 and S2 also modify lipid synthesis. Nsp2, a non-structural protein of SARS-CoV-2, disrupts host cell cycle and has similar functionality, which was concerned with protein processing in the endoplasmic reticulum, the lipid, and atherosclerosis. Our analysis revealed that S and Nsp2 proteins are associated with HSPA5, HSPA6, and LDHB in metabolic reprogramming. The hub proteins do not overlap, so SARS-CoV-2 pathogenesis is complicated. These findings also suggest an unknown potential protein inducing lipid synthesis and modification. What is driving the metabolic reprogramming is not clear.

In this study, essential baseline data, such as primary disease and statin use or not, might eliminate the observed heterogeneity. However, the exact timing of the blood collection for lipid profile remains uncertain. However, this can be ignored, as lipid metabolism of SARS-CoV-2 infection is a lengthy process as stated earlier. To eliminate the large between-study

heterogeneity, population experiments have been incorporated in this study. This study mainly discussed pivotal lipids (TC and HDL-C) and glycolysis in metabolic reprogramming but did not mention other lipid species, such as the sphingolipids, and their related pathways, or amino acids, organic acids, and nucleotides. In addition, the full impact of metabolic reprogramming in SARS-CoV-2 infection cannot be confirmed by cell culture without pressures imposed by the immune microenvironment.

CONCLUSION

In conclusion, viral infection induces the alteration of host metabolic reprogramming, which is a remarkable feature. This alteration not only changes the immune and clinical phenotype of patients but is also involved in viral pathogenesis. So the virus–host interaction is figured thoroughly out. Therefore, antivirals may be developed *via* further study of the metabolic reprogramming mechanism along with the key proteins.

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 Huizhou Central People's Hospital. Written

informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

TZ wrote the manuscript and created the figures. CW provided the data of serum of patients with COVID-19. BD and PY edited the manuscript. JW and QZ reviewed the data 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/fmicb.2022.863802/full#supplementary-material>

REFERENCES

- Bhattacharya, S., Agarwal, S., Shrimali, N. M., and Guchhait, P. (2021). Interplay between hypoxia and inflammation contributes to the progression and severity of respiratory viral diseases. *Mol. Aspects Med.* 81:101000. doi: 10.1016/j.mam.2021.101000
- Bietz, A., Zhu, H., Xue, M., and Xu, C. (2017). Cholesterol metabolism in T cells. *Front. Immunol.* 8:664. doi: 10.3389/fimmu.2017.01664
- Caccuri, F., Bugatti, A., Zani, A., De Palma, A., Di Silvestre, D., Manocha, E., et al. (2021). SARS-CoV-2 infection remodels the phenotype and promotes angiogenesis of primary human lung endothelial cells. *Microorganisms* 9:1438. doi: 10.3390/microorganisms9071438
- Chen, E. Y., Tan, C. M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G. V., et al. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics* 14:128. doi: 10.1186/1471-2105-14-128
- Cirstea, M., Walley, K. R., Russell, J. A., Brunham, L. R., Genga, K. R., and Boyd, J. H. (2017). Decreased high-density lipoprotein cholesterol level is an early prognostic marker for organ dysfunction and death in patients with suspected sepsis. *J. Crit. Care* 38, 289–294. doi: 10.1016/j.jcrc.2016.11.041
- Davies, J. P., Almasy, K. M., McDonald, E. F., and Plate, L. (2020). Comparative multiplexed interactomics of SARS-CoV-2 and homologous coronavirus non-structural proteins identifies unique and shared host-cell dependencies. *ACS Infect. Dis.* 6, 3174–3189. doi: 10.1021/acsinfectdis.0c00500
- Díaz, J. (2020). SARS-CoV-2 Molecular Network Structure. *Front. Physiol.* 11:870. doi: 10.3389/fphys.2020.00870
- Du, L., He, Y., Zhou, Y., Liu, S., Zheng, B.-J., and Jiang, S. (2009). The spike protein of SARS-CoV target for vaccine and therapeutic development. *Nat. Rev. Microbiol.* 7, 226–236. doi: 10.1038/nrmicro2090
- Eisfeld, A. J., Halfmann, P. J., Wendler, J. P., Kyle, J. E., Burnum-Johnson, K. E., Peralta, Z., et al. (2017). Multi-Platform 'omics analysis of human ebola virus disease pathogenesis. *Cell Host Microbe* 22, 817–829. doi: 10.1016/j.chom.2017.10.011
- Fahy, E., Sud, M., Cotter, D., and Subramaniam, S. (2007). LIPID MAPS online tools for lipid research. *Nucleic Acids Res.* 35, 606–612. doi: 10.1093/nar/gkm324
- Funderburg, N. T., and Mehta, N. N. (2016). Lipid abnormalities and inflammation in HIV infection. *Curr. HIV/AIDS Rep.* 13, 218–225. doi: 10.1007/s11904-016-0321-0
- Gao, S., Jiang, F., Jin, W., Shi, Y., Yang, L., Xia, Y., et al. (2020). Risk factors influencing the prognosis of elderly patients infected with COVID-19: a clinical retrospective study in Wuhan, China. *Aging* 12, 12504–12516. doi: 10.18632/aging.103631
- Grieb, P., Swiatkiewicz, M., Prus, K., and Rejdak, K. (2021). Hypoxia may be a determinative factor in COVID-19 progression. *Curr. Res. Pharmacol. Drug Discov.* 2:100030. doi: 10.1016/j.crphar.2021.100030
- Hu, X., Chen, D., Wu, L., He, G., and Ye, W. (2020). Declined serum high density lipoprotein cholesterol is associated with the severity of COVID-19 infection. *Clin. Chim. Acta* 510, 105–110. doi: 10.1016/j.cca.2020.07.015
- Jin, H., He, J., Dong, C., Li, B., Ma, Z., Li, B., et al. (2021). Altered lipid profile is a risk factor for the poor progression of COVID-19: from two retrospective cohorts. *Front. Cell. Infect. Microbiol.* 11:712530. doi: 10.3389/fcimb.2021.712530
- Khovidhunkit, W., Kim, M. S., Memon, R. A., Shigenaga, J. K., Moser, A. H., Feingold, K. R., et al. (2004). Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. *J. Lipid Res.* 45, 1169–1196. doi: 10.1194/jlr.R300019-JLR200

- Kim, D., Chung, H., Lee, J. E., Kim, J., Hwang, J., and Chung, Y. (2021). Immunologic aspects of dyslipidemia: a critical regulator of adaptive immunity and immune disorders. *J. Lipid Atheroscler.* 10, 184–201. doi: 10.12997/jla.2021.10.2.184
- Kyle, J. E., Burnum-Johnson, K. E., Wendler, J. P., Eisfeld, A. J., Halfmann, P. J., Watanabe, T., et al. (2019). Plasma lipidome reveals critical illness and recovery from human ebola virus disease. *Proc. Natl. Acad. Sci. U.S.A.* 116, 3919–3928. doi: 10.1073/pnas.1815356116
- Lei, P., Zhang, L., Han, P., Zheng, C., Tong, Q., Shang, H., et al. (2020). Liver injury in patients with COVID-19: clinical profiles, CT findings, the correlation of the severity with liver injury. *Hepatol. Int.* 14, 733–742. doi: 10.1007/s12072-020-10087-1
- Li, C., Tang, Z., Zhang, W., Ye, Z., and Liu, F. (2021). GEPIA2021: integrating multiple deconvolution-based analysis into GEPIA. *Nucleic Acids Res.* 49, W242–W246. doi: 10.1093/nar/gkab418
- Li, G., Zhou, C. L., Ba, Y. M., Wang, Y. M., Song, B., Cheng, X. B., et al. (2020). Nutritional risk and therapy for severe and critical COVID-19 patients: a multicenter retrospective observational study. *Clin. Nutr.* 40, 2154–2161. doi: 10.1016/j.clnu.2020.09.040
- Li, J., Li, M., Zheng, S., Li, M., Zhang, M., Sun, M., et al. (2020). Plasma albumin levels predict risk for nonsurvivors in critically ill patients with COVID-19. *Biomark. Med.* 14, 827–837. doi: 10.2217/bmm-2020-0254
- Ludewig, B., Jäggi, M., Dumrese, T., Brduscha-Riem, K., Odermatt, B., Hengartner, H., et al. (2001). Hypercholesterolemia exacerbates virus-induced immunopathologic liver disease via suppression of antiviral cytotoxic T cell responses. *J. Immunol.* 166, 3369–3376. doi: 10.4049/jimmunol.166.5.3369
- Lv, Z., Wang, W., Qiao, B., Cui, X., Feng, Y., Chen, L., et al. (2020). The prognostic value of general laboratory testing in patients with COVID-19. *J. Clin. Lab. Anal.* 35:e23668. doi: 10.1002/jcla.23668
- Mahat, R. K., Rathore, V., Singh, N., Singh, N., Singh, S. K., Shah, R. K., et al. (2021). Lipid profile as an indicator of COVID-19 severity: a systematic review and meta-analysis. *Clin. Nutr. ESPEN* 45, 91–101. doi: 10.1016/j.clnesp.2021.07.023
- Malik, J., Laique, T., Ishaq, U., Ashraf, A., Malik, A., Ali, M., et al. (2021). Effect of COVID-19 on lipid profile and its correlation with acute phase reactants. *MedRxiv [Preprint]*. doi: 10.1101/2021.04.13.21255142
- Melo, C. F., de Oliveira, D. N., Lima, E. O., Guerreiro, T. M., Esteves, C. Z., Beck, R. M., et al. (2016). A Lipidomics approach in the characterization of zika-infected mosquito cells: potential targets for breaking the transmission cycle. *PLoS One* 11:e0164377. doi: 10.1371/journal.pone.0164377
- Negro, F. (2010). Abnormalities of lipid metabolism in hepatitis C virus infection. *Gut* 59, 1279–1287. doi: 10.1136/gut.2009.192732
- Nie, S., Zhao, X., Zhao, K., Zhang, Z., Zhentao, Z., Zhang, Z., et al. (2020). Metabolic disturbances and inflammatory dysfunction predict severity of coronavirus disease 2019 (COVID-19): a retrospective study. *MedRxiv [Preprint]*. doi: 10.1101/2020.03.24.20042283
- Olsen, L., Thum, E., and Rohner, N. (2021). Lipid metabolism in adaptation to extreme nutritional challenges. *Dev. Cell* 56, 1417–1429. doi: 10.1016/j.devcel.2021.02.024
- Palm, A., and Ekström, M. (2021). Hypoxemia severity and survival in ILD and COPD on long-term oxygen therapy-The population-based DISCOVERY study. *Respir. Med.* 189:106659. doi: 10.1016/j.rmed.2021.106659
- Puleston, D. J., Villa, M., and Pearce, E. L. (2020). Ancillary activity: beyond core metabolism in immune cells. *Cell Metab.* 26, 131–141. doi: 10.1016/j.cmet.2017.06.019
- R Core Team (2018). *R: A language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.
- Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303
- Sharma, A., Ong, J. W., Loke, M. F., Chua, E. G., Lee, J. J., Choi, H. W., et al. (2021). Comparative transcriptomic and molecular pathway analyses of HL-CZ human pro-monocytic cells expressing SARS-CoV-2 Spike S1, S2, NP, NSP15 and NSP16 Genes. *Microorganisms* 9:1193. doi: 10.3390/microorganisms9061193
- Shen, B., Yi, X., Sun, Y., Bi, X., Du, J., Zhang, C., et al. (2020). Proteomic and metabolomic characterization of COVID-19 patient sera. *Cell* 182, 59–72.e15. doi: 10.1016/j.cell.2020.05.032
- Sun, J. T., Chen, Z., Nie, P., Ge, H., Shen, L., Yang, F., et al. (2020). Lipid profile features and their associations with disease severity and mortality in patients with COVID-19. *Front. Cardiovasc. Med.* 7:584987. doi: 10.3389/fcvm.2020.584987
- Szklarczyk, D., Gable, A. L., Nastou, K. C., Lyon, D., Kirsch, R., Pyysalo, S., et al. (2021). The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Res.* 49, D605–D612. doi: 10.1093/nar/gkaa1074
- Tanaka, S., De Tymowski, C., Assadi, M., Zappella, N., Jean-Baptiste, S., Robert, T., et al. (2020). Lipoprotein concentrations over time in the intensive care unit COVID-19 patients: results from the ApoCOVID study. *PLoS One* 15:e0239573. doi: 10.1371/journal.pone.0239573
- Theken, K. N., Tang, S. Y., Sengupta, S., and FitzGerald, G. A. (2021). The roles of lipids in SARS-CoV-2 viral replication and the host immune response. *J. Lipid Res.* 62:100129. doi: 10.1016/j.jlr.2021.100129
- Tisoncik-Go, J., Gasper, D. J., Kyle, J. E., Eisfeld, A. J., Selinger, C., Hatta, M., et al. (2016). Integrated omics analysis of pathogenic host responses during pandemic H1N1 influenza virus infection: the crucial role of lipid metabolism. *Cell Host Microbe* 19, 254–266. doi: 10.1016/j.chom.2016.01.002
- Van Lenten, B. J., Hama, S. Y., de Beer, F. C., Stafforini, D. M., McIntyre, T. M., Prescott, S. M., et al. (1995). Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. *J. Clin. Invest.* 96, 2758–2767. doi: 10.1172/JCI118345
- Venn (2022). *Calculate and Draw Custom Venn Diagrams*. Available online at: <http://bioinformatics.psb.ugent.be/webtools/Venn/>
- Walls, A. C., Park, Y.-J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 181, 281–292. doi: 10.1016/j.cell.2020.02.058
- Wei, X., Zeng, W., Su, J., Wan, H., Yu, X., Cao, X., et al. (2020). Hypolipidemia is associated with the severity of COVID-19. *J. Clin. Lipidol.* 14, 297–304. doi: 10.1016/j.jacl.2020.04.008
- World Health Organization [WHO] (2022). *Coronavirus Disease (COVID-19) Pandemic*. Geneva: WHO.
- Wu, A., Hinds, C. J., and Thiemermann, C. (2004). High-density lipoproteins in sepsis and septic shock: metabolism, actions, and therapeutic applications. *Shock* 21, 210–221. doi: 10.1097/01.shk.0000111661.09279.82
- Wu, D., Shu, T., Yang, X., Song, J. X., Zhang, M., Yao, C., et al. (2020). Plasma metabolomic and lipidomic alterations associated with COVID-19. *Natl. Sci. Rev.* 7, 1157–1168. doi: 10.1093/nsr/nwaa086
- Wu, Q., Zhou, L., Sun, X., Yan, Z., Hu, C., Wu, J., et al. (2017). Altered lipid metabolism in recovered SARS patients twelve years after infection. *Sci. Rep.* 7:9110. doi: 10.1038/s41598-017-09536-z
- Xie, Y., You, Q., Wu, C., Cao, S., Qu, G., Yan, X., et al. (2020). Impact of cardiovascular disease on clinical characteristics and outcomes of coronavirus disease 2019 (COVID-19). *Circ. J.* 84, 1277–1283. doi: 10.1253/circ.CJ-20-0348
- Xue, G., Gan, X., Wu, Z., Xie, D., Xiong, Y., Hua, L., et al. (2020). Novel serological biomarkers for inflammation in predicting disease severity in patients with COVID-19. *Int. Immunopharmacol.* 89:107065. doi: 10.1016/j.intimp.2020.107065
- Yan, B., Chu, H., Yang, D., Sze, K.-H., Lai, P.-M., Yuan, S., et al. (2019). Characterization of the Lipidomic profile of human coronavirus-infected cells: implications for lipid metabolism remodeling upon coronavirus replication. *Viruses* 11:73. doi: 10.3390/v11010073
- Yu, Y., Gao, L., Wang, Y., Xu, B., Maswikiti, E. P., Li, H., et al. (2021). A forgotten corner in cancer immunotherapy: the role of lipids. *Front. Oncol.* 11:751086. doi: 10.3389/fonc.2021.751086
- Zawawi, A., Naser, A. Y., Alwafi, H., and Minshaw, F. (2021). Profile of circulatory cytokines and chemokines in human coronaviruses: a systematic review and meta-analysis. *Front. Immunol.* 12:666223. doi: 10.3389/fimmu.2021.666223
- Zhang, B., Dong, C., Li, S., Song, X., Wei, W., and Liu, L. (2020). Triglyceride to high-density lipoprotein cholesterol ratio is an important determinant of cardiovascular risk and poor prognosis in coronavirus disease-19: a retrospective case series study. *Diabetes Metab. Syndr. Obes.* 13, 3925–3936. doi: 10.2147/DMSO.S268992

Zhang, Q., Wei, Y., Chen, M., Wan, Q., and Chen, X. (2020). Clinical analysis of risk factors for severe COVID-19 patients with type 2 diabetes. *J. Diabetes Complicat.* 34:107666. doi: 10.1016/j.jdiacomp.2020.107666

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Harmonization of Multiple SARS-CoV-2 Reference Materials Using the WHO IS (NIBSC 20/136): Results and Implications

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Background: There is an urgent need for harmonization between severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) serology platforms and assays prior to defining appropriate correlates of protection and as well inform the development of new rapid diagnostic tests that can be used for serosurveillance as new variants of concern (VOC) emerge. We compared multiple SARS-CoV-2 serology reference materials to the WHO International Standard (WHO IS) to determine their utility as secondary standards, using an international network of laboratories with high-throughput quantitative serology assays. This enabled the comparison of quantitative results between multiple serology platforms.

Methods: Between April and December 2020, 13 well-characterized and validated SARS-CoV-2 serology reference materials were recruited from six different providers to qualify as secondary standards to the WHO IS. All the samples were tested in parallel with the National Institute for Biological Standards and Control (NIBSC) 20/136 and parallel-line assays were used to calculate the relevant potency and binding antibody units.

Results: All the samples saw varying levels of concordance between diagnostic methods at specific antigen-antibody combinations. Seven of the 12 candidate materials had high concordance for the spike-immunoglobulin G (IgG) analyte [percent coefficient of variation (%CV) between 5 and 44%].

Conclusion: Despite some concordance between laboratories, qualification of secondary materials to the WHO IS using arbitrary international units or binding antibody

units per milliliter (BAU/ml) does not provide any benefit to the reference materials overall, due to the lack of consistent agreeable international unit (IU) or BAU/ml conversions between laboratories. Secondary standards should be qualified to well-characterized reference materials, such as the WHO IS, using serology assays that are similar to the ones used for the original characterization of the WHO IS.

Keywords: SARS-CoV-2, serology, International Standards, concordance, immunology, harmonization, parallel-line assay

INTRODUCTION

There is an urgent need for harmonization between severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) serology platforms and assays prior to defining appropriate correlates of protection and as well inform the development of new rapid diagnostic tests that can be used for serosurveillance as new variants of concern (VOC) emerge (Berry et al., 2020; Ciotti et al., 2021; Giavarina and Carta, 2021; Infantino et al., 2021; Perkmann et al., 2021; Petrone et al., 2021; Knezevic et al., 2022).

Conversion of results from different laboratory methods to a harmonized international unit reduces the interlaboratory/method variability (Cooper et al., 2018; McDonald et al., 2018; Mattiuzzo et al., 2019, 2020; Ciotti et al., 2021; Knezevic et al., 2022). The WHO International Standards (ISs) are considered the highest quality materials to use for comparison between diagnostic methods using international units (Mattiuzzo et al., 2020). The WHO IS for SARS-CoV-2 serology standard is the National Institute for Biological Standards and Control (NIBSC) 20/136 (United Kingdom, 2020). This standard, as most biological standards, was produced in limited quantities, making it difficult to be used exclusively as a calibrant to compare results between multiple SARS-CoV-2 serology assays on a global scale. Therefore, there is a pressing need to increase the availability of appropriate reference materials that are considered equivalent to the WHO IS. Other well-characterized reference samples can be evaluated against the WHO IS to obtain a valid measurement and calibrated to the arbitrary WHO IS values of 1,000 international units per milliliter (IU/ml) for neutralization assays and 1,000 binding antibody units per milliliter (BAU/ml) (National Institute for Biological Standards Control, 2020).

We compared multiple SARS-CoV-2 serology reference materials to the WHO IS to determine their utility as secondary standards, using an international network of laboratories with high-throughput quantitative serology assays. This enabled the comparison of quantitative results between multiple serology platforms. Furthermore, each serology method can derive a BAU/ml (or IU/ml as appropriate) conversion for multiple antigen–antibody combinations within each sample that are scaled to the arbitrary 1,000 BAU/ml value assigned to the WHO IS. We also note that neutralization assays that report IU/ml may additionally be calibrated to the WHO IS.

MATERIALS AND METHODS

Recruitment of Severe Acute Respiratory Syndrome Coronavirus 2 Serology Reference Materials

Between April and December 2020, 13 well-characterized and validated SARS-CoV-2 serology reference materials were recruited from six different providers (**Table 1**) (National Institute for Biological Standards Control, 2020; Frederick National Laboratory for Cancer Research, 2021; Oneworld Accuracy, 2021; Thermo Fisher Scientific, 2021; Windsor et al., 2021; Zeichhardt and Kammel, 2021). Reference materials were selected based on the following criteria: originally characterized by the suppliers with the relevant test's thresholds for positive and negative results, are readily available, enough panels will exist after this study to distribute for widespread use, and the providers intend to distribute their reference materials to other (primarily low-resource) laboratories. All the materials were individually evaluated against the WHO IS using previously validated diagnostic tests given in **Table 2** and characterized according to the anticipated results shown in **Table 1**. All the reference materials and diagnostic tests were handled according to manufacturers' and the respective Clinical Laboratory Improvement Amendments (CLIA) laboratory developed test instructions.

Neutralization Assays

Severe Acute Respiratory Syndrome Coronavirus 2 Focus Reduction Neutralization Test

Vero E6 cells (ATCC, CRL-1586; Manassas, Virginia, USA) were maintained at 37°C in Dulbecco's Modified Eagle Medium (DMEM) (HyClone 11965-084; Logan, Utah, USA) supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin. SARS-CoV-2 strain 2019 n-CoV/USA-WA1/2020 was obtained from ATCC. The virus was passaged once in Vero E6 cells and titrated by the focus reduction neutralization test (FRNT) on Vero E6 cells. All the work with infectious SARS-CoV-2 was performed in Biosafety Level 3 (BSL3) facilities at the University of Colorado School of Medicine.

The focus reduction neutralization test (FRNT) was performed as previously described (Annen et al., 2021; Schultz et al., 2021; Taylor et al., 2021). Vero E6 cells were seeded in 96-well plates at 10^4 cells/well. On the next day, serum samples were heat inactivated at 56°C for 30 min and then serially

TABLE 1 | Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) serology harmonization reference material providers.

Institution	Type of provider	SARS-CoV-2 serology panel name	Sample IDs	Material type	Anticipated results from development
University of Colorado	Academic/Research	COVID-19 Serology Control Panel (Windsor et al., 2021)	CSCP-HR	Pooled Convalescent Plasma	N-IgG, Total = Reactive; RBD-IgG, Total = Highly Reactive; S-IgG, Total = Reactive
			CSCP-WR	Pooled Convalescent Plasma, 1:4 dilution of the CSCP_HR	N-IgG, Total = Reactive; RBD-IgG, Total = Reactive; S-IgG, Total = Reactive
			CSCP-NR	Pre-2019 Donor Plasma	Non-Reactive
NCI Frederick Lab	Government	Human SARS-COV-2 Serology Standard (Frederick National Laboratory for Cancer Research, n.d.)	NCI Frederick	Pooled Convalescent Plasma	N-IgG = Reactive; N-IgM = Reactive; S-IgG = Reactive; S-IgM = Reactive
Oneworld Accuracy	Commercial	COVS434 SARS-CoV-2 Serology (Oneworld Accuracy, 2021)	1WA-A	Single Donor Human Plasma	No Ag indication, IgG against SARS-CoV-2, Total = Reactive
			1WA-B	Single Donor Human Plasma	No Ag indication, IgG against SARS-CoV-2, Total = Reactive
			1WA-C	Single Donor Human Plasma	No Ag indication, IgG+IgM against SARS-CoV-2, Total = Reactive
			1WA-D	Pre-2019 Donor Plasma	Non-Reactive
INSTAND	Commercial	Samples from EQA scheme (416) SARS-CoV-2 (Ak) (Zeichhardt and Kammel, n.d.)	416006	Convalescent Serum of a single donor after infection with human coronaviruses OC43 and HKU1 (single donation, blood collected 2 years after last infection)	Non-Reactive
			416029	Convalescent Serum of a donor after SARS-CoV-2 infection (single donation, blood collected 154 day after onset of disease)	N-IgG, Total = Reactive; RBD/S-IgG, Total = Reactive
			416048	Post Pfizer-BioNTech COVID-19 Vaccine donor Serum (single donation, blood collected 63 days after 2 nd vaccination; no prior evidence of infection)	N-IgG, Total = Non-Reactive; RBD/S-IgG, Total = Reactive
Thermo Fisher	Commercial	MAST TM SARS-CoV-2 IgG Positive Control Kit (Cat# 10028305) (Thermo Fisher Scientific, n.d.)	ThermoFisher	Pooled COVID-19 positive human plasma added to defibrinated plasma with ProClin 950 and Sodium azide	N-IgG, Total = Reactive; RBD-IgG, Total = Reactive; S-IgG, Total = Reactive
National Institute for Biological Standards and Controls	Government	NIBSC 20/136 (National Institute for Biological Standards Control, 2020)	WHO IS	Pooled Convalescent Plasma	1000 BAU/mL for IgM, IgG, and IgA subtypes

diluted (2-fold, starting at 1:10) in DMEM supplemented with 1% fetal bovine serum (FBS) and 10 mM 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) (Merck, 7365-45-9, Darmstadt, Germany). Approximately, 100 focus-forming units (FFUs) of virus were added to each well and the serum/virus mixture was incubated for 1 h at 37°C. Following co-incubation of serum and virus, medium was removed from cells and the serum/virus mixture was added to the cells for 1 h at 37°C. Serum/virus mixture was removed and cells overlaid with 1% methylcellulose (MilliporeSigma, M0512; Burlington, Massachusetts, USA) in DMEM plus 2% FBS and incubated for 24 h at 37°C. Cells were fixed with 1% paraformaldehyde (PFA) (Acros Organics, 416780030; Morris Plains, New Jersey, USA)

for 1 h, washed six times with phosphate-buffered saline-0.05% Tween 20 (PBS-T), and probed with 1 µg/ml of chimeric human anti-SARS-CoV spike antibody (CR3022, Absolute Antibody, Ab01680; Oxford, UK) in Perm Wash Buffer [1X PBS/0.1% saponin/0.1% bovine serum albumin (BSA)] for 2 h at 25°C. After three washes with PBS-T, cells were incubated with goat antihuman immunoglobulin G (IgG) Fc-horseradish peroxidase (HRP) (Southern Biotech, 2014-05; Birmingham, Alabama, USA) diluted at 1:1,000 in Perm Wash Buffer for 1.5 h at 25°C. SARS-CoV-2-positive foci were visualized with TrueBlue substrate (SeraCare, 5510-0030, Milford, Massachusetts, USA) and counted using the CTL BioSpot analyzer and BioSpot software (Cellular Technology Ltd.,

TABLE 2 | SARS-CoV-2 serology harmonization testing laboratories and methods.

Institution	Type of lab	Platform	Method	Antigen targets	Antibodies
University of Colorado	Academic/ Research	Lab-Developed Test	SARS-CoV Focus Reduction Neutralization Titer (FRNT)	2019 n-CoV/USA-WA1/2020	Total Ig
Biodesix, Inc.	Commercial	GenScript cPass Nab Bio-Rad Platelia	Neutralization(Nab) ELISA ELISA	RBD N	Total Ig IgG, IgM, IgA
Brigham and Women's Hospital	Academic/ Clinical	Laboratory Developed Test upon Quanterix Simoa HD-X platform	Multiplexed Single Molecule Array (MSMA)	S, RBD, N, S1	IgG, IgM, IgA
Wadsworth Center, New York State Department of Health	Reference/ Public Health	Lab-Developed Test upon Luminex Platform	Multiplexed microsphere assay (MMA)	S, RBD, N, S1, S2	IgG, IgM, IgA, Total Ig
University of Colorado	Academic/ Research	Lab-Developed Test	Multiplex microsphere immunoarray (MIA)	N, RBD, S1, S2	IgG

Shaker Heights, Ohio, USA). The FRNT50 titers were calculated relative to a virus only control (no serum) set at 100%, using GraphPad Prism 9.1.2 default nonlinear curve fit constrained between 0 and 100%.

CPass α -Receptor-Binding Domain (GenScript) Neutralization Antibody Test

The cPass α -receptor-binding domain (RBD) neutralization antibody (nAb) test is a quantitative assay that specifically measures a subset of spike-binding antibodies that can block the interaction between the RBD on the SARS-CoV-2 spike protein and the human host receptor angiotensin-converting enzyme 2 (ACE2) (GenScript, 2021). The assay is performed as a blocking ELISA as described in the Food and Drug Administration (FDA) Emergency Use Authorization (EUA) instructions for use in the cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit. The surrogate virus neutralization test (SVNT) cPass assay was clinically validated and shown to be 100% sensitive and specific when compared to a gold standard plaque reduction neutralization test (PRNT), with qualitative analysis results 100% in agreement (GenScript, 2021). The reference materials were diluted and preincubated 1:1 with RBD protein conjugated to HRP at 37°C for 30 min. The mixture (100 μ l) was then added to a 96-well plate coated with human ACE2 receptor protein; the plate was sealed and incubated for an additional 15 min at 37°C. The plate was washed four times with 260 μ l/well Wash Solution provided in the kit before addition of 100 μ l per well 3,3',5,5'-Tetramethylbenzidine (TMB) substrate for 15 min at room temperature. 50 μ l of 1 N sulfuric acid solution was added to each well and the optical density (OD) was measured at 450 nm using a spectrophotometer. The nAb assay readout was percent signal inhibition by neutralizing antibodies, which was calculated to be the OD value of the sample relative to the OD of the negative control subtracted from one (Tan et al., 2020; Petrone et al., 2021; Taylor et al., 2021). The positive cutoff results are $\geq 30\%$ signal inhibition and results $< 30\%$ are reported negative based on previously conducted clinical validation studies (Petrone et al., 2021).

Binding Antibody Assays

Platelia α -Nucleocapsid Total Antibody Test

The Platelia α -nucleocapsid (anti-N) total antibody test detects antibodies [IgG, immunoglobulin M (IgM), and immunoglobulin A (IgA) combined; Bio-Rad Incorporation] to the nucleocapsid protein. The assay is performed as a one-step antigen capture ELISA as described in the FDA EUA instructions for use for the Platelia SARS-CoV-2 Total Antibody Test Kit (Bio-Rad, 2021). The diluted plasma (1:5) and the WHO IS (1.5-fold serial dilution series up to 8 times, starting at 1:90 dilution) were mixed with SARS-CoV-2 nucleocapsid protein coupled with horseradish peroxidase (HRP) enzyme at a 1:1 ratio and 100 μ l added to a 96-well plate coated with the nucleocapsid protein. The plate was covered with an adhesive plate sealer and incubated at 37°C for 1 h. The plate was then washed five times with the Working Washing Solution provided in the kit and 200 μ l of the Enzyme Development Solution was added to each well. After a 30-min incubation in the dark at room temperature (18–30°C), the reaction was stopped by adding 100 μ l per well of an acidic stopping solution and mixing thoroughly before measuring the OD at 450 nm using a spectrophotometer. The assay readout was a ratio of the specimen OD to cutoff control OD. A positive specimen-to-cutoff ratio ≥ 1.0 and < 0.8 is negative and in between is reported equivocal with the recommendation of another specimen collected 3 days later. The Platelia assay has FDA EUA clearance for a qualitative interpretation of results (Bio-Rad, 2021).

Simoa Serology Assay

Simoa assays for IgG, IgA, and IgM against four SARS-CoV-2 targets (spike, S1, nucleocapsid, and RBD) were performed as previously described (Norman et al., 2020). Reference materials were diluted 1:250-, 1:1,000-, 1:4,000-, and 1:16,000-fold in Homebrew Detector/Sample Diluent (Quanterix Corporation, Product code: 101359, Billerica, Massachusetts, USA). Four antigen-conjugated capture beads were mixed and diluted in Bead Diluent (Quanterix Corporation, Product code: 101362, Billerica, Massachusetts, USA), with a total of 500,000 beads per reaction (125,000 of each bead type). Biotinylated

antibodies were diluted in Homebrew Detector/Sample Diluent to final concentrations of IgG (Bethyl Laboratories A80-148B; Montgomery, Texas, USA): 7.73 ng/ml, IgA (Abcam ab214003, Waltham, Massachusetts, USA): 150 ng/ml, and IgM (Thermo Fisher Scientific, MII0401, Pittsburgh, Pennsylvania, USA): 216 ng/ml: Streptavidin- β -galactosidase (S β G) concentrate (Quanterix Corporation, Product code: 1013397, Billerica, Massachusetts, USA) was diluted to 30 pM in S β G Diluent (Quanterix Corporation, Product code: 100376, Billerica, Massachusetts, USA). The serology assay was performed on the HD-X Analyzer (Quanterix) in an automated three-step assay. Average enzymes per bead (AEB) values were calculated by the HD-X Analyzer software (Norman et al., 2020).

Multiplexed Microsphere Assay

Specimens were assessed for the presence of antibodies reactive with SARS-CoV-2 using a multiplexed microsphere assay (MMA). Recombinant SARS-CoV-2 full-length spike, nucleocapsid, S2 (The Native Antigen Company, REC31868, REC31812, and REC31807, respectively, Kidlington, Oxfordshire, UK), RBD, and S1 (Mass Biologics, <https://www.umassmed.edu/massbiologics>, Boston, Massachusetts, USA) subunits were covalently linked to the surface of fluorescent microspheres (Luminex Corporation, LC10047, LC10006, LC10071, LC10061, and LC10023, respectively, Austin, Texas, USA). Serum samples (25 μ l at doubling dilutions from 1:50 to 1:102,400) and antigen-coupled microspheres (25 μ l at 5×10^4 microspheres/ml) were mixed and incubated 30 min at 37°C. Serum-bound microspheres were washed and incubated with phycoerythrin (PE)-conjugated secondary antibody. The PE-conjugated antibodies were chosen to specifically recognize total Ig (Pan-Ig), IgM, IgA, and IgG (Southern Biotechnology Associates Incorporation, 2010–2009, 2020–2009, 2050–2009, and 2040–2009, respectively, Birmingham, Alabama, USA). After washing and final resuspension in buffer, the samples were analyzed on the FlexMap 3D analyzer using xPONENT software (Luminex Corporation, version 4.3, Austin, Texas, USA).

Multiplexed Microsphere Immunoassay (MIA)

A multiplexed microsphere immunoassay (MIA) was developed using BioLegend carboxylated LEGENDplex microbeads to simultaneously quantify IgG and IgA against the spike RBD and nucleocapsid of the Wuhan strain of SARS-CoV-2 (BEIresources.org, NR-52366, North Bethesda, Maryland, USA), three variants of concern beta gamma, delta (BEIresources.org, NR-54004/54005, North Bethesda, Maryland, USA), three season coronavirus strains (OC43, 229E, and HKU1) (BEIresources.org, NR-53713, North Bethesda, Maryland, USA), and tetanus toxoid (TT) (MilliporeSigma, #582231-25UG, St. Louis, Mosby, USA) as a positive control. Bovine serum albumin (BSA) (10%) (MilliporeSigma, #A7030, St. Louis, Mosby, USA) conjugated beads were used as a negative control. Multiplex bead protein conjugation, sample incubation, and flow cytometric analysis were performed as previously described (Schultz et al., 2021). Geometric mean fluorescence intensity (gMFI) of the IgG/IgA

for each sample and dilution was captured with the CytoFLEX S Flow Cytometer (Beckman Coulter, Indianapolis, Indiana, USA) and analyzed with FlowJo (version 10.7.1; BD Biosciences, San Jose, California, USA). Prism (version 8.4.3, GraphPad) was used to plot data (Schultz et al., 2021).

Statistical Analysis

Parallel-line assay (PLA) was used to compare all the secondary standard candidate samples to the WHO IS; all the analytes were set at 1,000 IU or BAU/ml (Finney and Schild, 1966). All the samples were tested in triplicate with each diagnostic test at dilutions within each assay's given linear range for the WHO IS. Data were analyzed using PLA analysis using R 3.5.0 that we created (R Core Team, 2021). Sample results and their corresponding dilutions were log-transformed and assessed for parallelism using the relative slope calculated individually between each sample and the WHO IS. To ensure the assumption of parallelism for PLA analysis to occur, a relative slope between 0.8 and 1.2 was considered parallel and samples with relative slopes outside the range were excluded from further analysis because they violated the PLA assumption of parallel lines (Mattiuzzo et al., 2020). The relative potency was calculated for each sample whose slope was within 20% of the WHO IS slope. Relative potencies were then converted to IU or BAU/ml based on the assay used (Finney and Schild, 1966) and parametric bootstrapping was used to calculate CIs for each sample (B. Efron, 1979; Landes et al., 2019). The full reproducible code and readme file are both available at: github.com/yroell/pla. and the overview of our created PLA analysis is shown in **Supplementary Figure 1** showing an overview used for each sample. IU and BAU/ml conversions were then compared for interassay variability using percent coefficient of variation (%CV) (Reed et al., 2002; Wood et al., 2012).

RESULTS

Analysis of Samples and Binding Antibody Unit Conversions

Thirteen samples (including the WHO IS) from six different providers (**Table 1**) were tested using six different SARS-CoV-2 serology diagnostic platforms. Twenty-one total antigen-antibody (Ag-Ab) combinations were evaluated. Three of the platforms were multiplexed platforms targeting multiple Ag-Ab combinations. The remaining three platforms consisted of two SARS-CoV-2 neutralization tests and one nucleocapsid-specific ELISA (**Table 2**). Each laboratory performed serial dilutions of the WHO IS to establish the linear range of the WHO within each testing platform. All the reference samples were then serially diluted within the WHO IS linear range and tested in triplicate.

Results from each laboratory were compiled and evaluated using PLA. Reference material samples were considered "parallel" if their relative slope against the WHO IS was between 0.8 and 1.2. Samples that failed to fall within the range were excluded from further analysis. For each sample at each Ag-Ab combination, BAUs (or IUs for neutralization tests) were calculated using sample relative potency. BAU conversions for each sample are shown in **Table 3** and **Figure 1** summarizes

TABLE 3 | Binding antibody unit conversions for serology harmonization samples.

Ab	Ag	Method	416006		416029		416048		1WA-A		1WA-B		1WA-C		1WA-D		CSCP-HR		CSCP-NR		CSCP-WR		NCI Frederick		Thermo Fisher	
			BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI
Total Ig	N	ELISA	NA	NA	NA	NA	NA	NA	1986	1958–2014	NA	NA	NA	NA	NA	NA	497	594–500	NA	NA	NA	NA	1116	1113–1119	NA	NA
		MMA	NA	NA	109	0	0	0	658	656–660	126	125–127	496	495–497	NA	NA	579	577–581	NA	NA	160	159–161	783	781–785	82	0
	RBD	MMA	NA	NA	49	0	1542	1,538–1,548	167	0	204	203–205	851	849–853	NA	NA	615	613–617	NA	NA	170	169–171	585	583–587	39	0
		Nab	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	S	MMA	NA	NA	93	0	1860	1,854–1,866	315	314–316	260	259–261	972	969–975	NA	NA	474	473–475	NA	NA	129	0	980	977–983	45	0
		MMA	NA	NA	68	0	1865	1,859–1,871	294	293–295	266	265–267	936	934–938	NA	NA	507	506–508	NA	NA	136	0	765	763–767	36	0
	S2	MMA	NA	NA	68	0	115	0	175	0	36	0	237	236–238	NA	NA	230	229–231	NA	NA	46	0	718	716–720	25	0
IgG	N	FRNT	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1054	1045–1063	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		MIA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	745	723–767	NA	NA
		MMA	NA	NA	96	0	0	0	585	583–587	125	124–126	373	372–374	NA	NA	616	614–618	NA	NA	161	160–162	792	2	62	0
	RBD	MSMA	NA	NA	35	34–36	156	149–173	251	248–254	110	106–114	419	406–432	NA	NA	135	132–138	NA	NA	40	39–41	856	843–869	36	35–37
		MIA	NA	NA	NA	NA	NA	NA	82	79–85	129	126–132	846	832–860	0	0	69	67–71	NA	NA	7	0	489	480–498	11	12–Oct
		MMA	NA	NA	54	0	2306	2,294–2,316	124	0	189	188–190	917	914–920	NA	NA	815	813–817	NA	NA	224	223–225	768	766–770	48	0
	S	MSMA	NA	NA	58	56–60	2060	2,020–2,100	142	139–145	167	160–174	924	905–943	NA	NA	589	576–602	NA	NA	123	119–127	691	676–706	58	56–60
		MMA	NA	NA	97	0	2115	2,109–2,121	281	280–282	243	242–244	938	936–940	NA	NA	507	506–508	NA	NA	141	0	1090	1088–1092	43	0
		MSMA	NA	NA	307	301–313	2749	2,703–2,792	299	294–304	424	415–433	1160	1,142–1,178	NA	NA	477	469–485	NA	NA	120	118–122	2067	2032–2102	120	118–122
	S1	MIA	NA	NA	NA	NA	NA	NA	36	31–41	81	75–87	703	781–725	NA	NA	24	23–25	NA	NA	NA	NA	463	448–478	NA	NA
		MMA	NA	NA	74	0	2453	2,441–2,465	271	270–272	260	259–261	883	881–885	NA	NA	609	608–610	NA	NA	167	0	925	923–927	38	0
		MSMA	NA	NA	75	73–77	2411	2,373–2,450	108	106–110	149	146–152	783	770–796	NA	NA	393	386–400	NA	NA	97	95–99	647	636–658	35	34–36
	S2	MIA	NA	NA	NA	NA	NA	NA	29	27–31	20	19–21	92	90–94	NA	NA	10	0	NA	NA	1	0	443	436–450	1	0
		MMA	NA	NA	61	0	113	0	160	159–161	18	0	210	0	NA	NA	247	246–248	NA	NA	43	0	1580	1576–1584	20	0
IgM	N	MMA	NA	NA	288	287–289	89	0	603	601–605	384	382–386	8360	8336–8384	NA	NA	1387	1381–1393	NA	NA	349	349–351	531	529–533	118	117–119
		MSMA	120	112–128	417	497–437	228	211–245	NA	NA	NA	NA	1383	1,314–1,452	18	16–20	1894	1702–2086	24	22–26	352	325–379	NA	NA	NA	NA
	RBD	MMA	NA	NA	25	0	10	0	232	231–233	202	0	436	435–437	NA	NA	375	374–376	NA	NA	94	0	273	272–274	3	0
		MSMA	6	0	24	0	27	0	200	198–202	174	172–176	507	503–511	4	0	372	368–376	NA	NA	94	92–96	279	276–282	NA	NA
	S	MMA	NA	NA	17	0	13	0	236	235–237	261	0	595	594–596	NA	NA	583	582–584	NA	NA	139	0	215	214–216	4	0
		MSMA	13	0	21	20–22	160	158–162	333	330–336	313	308–318	819	811–827	NA	NA	1280	1,268–1,292	NA	NA	267	264–270	365	362–368	NA	NA
	S1	MMA	NA	NA	22	0	13	0	217	216–218	221	220–222	556	555–557	NA	NA	459	458–460	NA	NA	111	0	224	223–225	3	0
		MSMA	4	0	14	13–15	63	61–65	310	307–313	266	262–270	299	296–302	NA	NA	723	716–730	NA	NA	152	151–153	213	210–216	NA	NA

(Continued)

TABLE 3 | Continued

Ab	Ag	Method	416006	416029	416048	1WA-A	1WA-B	1WA-C	1WA-D	CSCP-HR	CSCP-NR	CSCP-WR	NCI Frederick	Thermo Fisher
			BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI	BAU	95% CI
IgA	N	S2	MMA	NA	NA	NA	541	533–549	198	187–209	NA	NA	NA	NA
			BAU	NA	NA	NA	41	40–42	290	289–291	NA	NA	2003	1978–2028
			95% CI	NA	NA	NA	347	338–356	900	891–909	74	72–76	NA	NA
RBD			MSMA	NA	NA	NA	141	0	437	436–438	NA	NA	274	273–275
			BAU	NA	NA	NA	211	210–212	557	552–562	0	0	NA	NA
			95% CI	NA	NA	NA	332	331–333	465	463–467	NA	NA	432	430–434
S			MMA	NA	NA	NA	305	304–306	564	561–567	NA	NA	NA	NA
			BAU	NA	NA	NA	473	470–476	559	554–564	NA	NA	392	389–395
			95% CI	NA	NA	NA	56	0	10	0	NA	NA	NA	NA
S1			MMA	NA	NA	NA	305	304–306	564	561–567	NA	NA	NA	NA
			BAU	NA	NA	NA	473	470–476	559	554–564	NA	NA	NA	NA
			95% CI	NA	NA	NA	56	0	10	0	NA	NA	NA	NA
S2			MMA	NA	NA	NA	305	304–306	564	561–567	NA	NA	NA	NA
			BAU	NA	NA	NA	473	470–476	559	554–564	NA	NA	NA	NA
			95% CI	NA	NA	NA	56	0	10	0	NA	NA	NA	NA

Table 3 breaks down the computed BAU or IU/mL conversions and bootstrapped 95% Confidence Intervals of each method by their measured antibody and antigen analyte combination. *NA indicates the result was either not measured by the particular assay, or the relative slope fell outside of 20% and therefore not possible to convert to International Units or Binding Antibody Units/mL (BAU); Ab, antibody; Ag, antigen; N, Nucleocapsid; S, Spike; RBD, Receptor Binding Domain; WW, Whole Virus; MSMA, multiplexed single molecule array; MMA, multiplexed microsphere immunoarray; MMA, multiplexed microsphere assay; Nab, neutralization; ELISA, enzyme-linked immunosorbent assay; FRNT, focus-reduction neutralization titer. A more comprehensive breakdown of the individual samples and test methods are found in (Tables 1, 2) respectively.

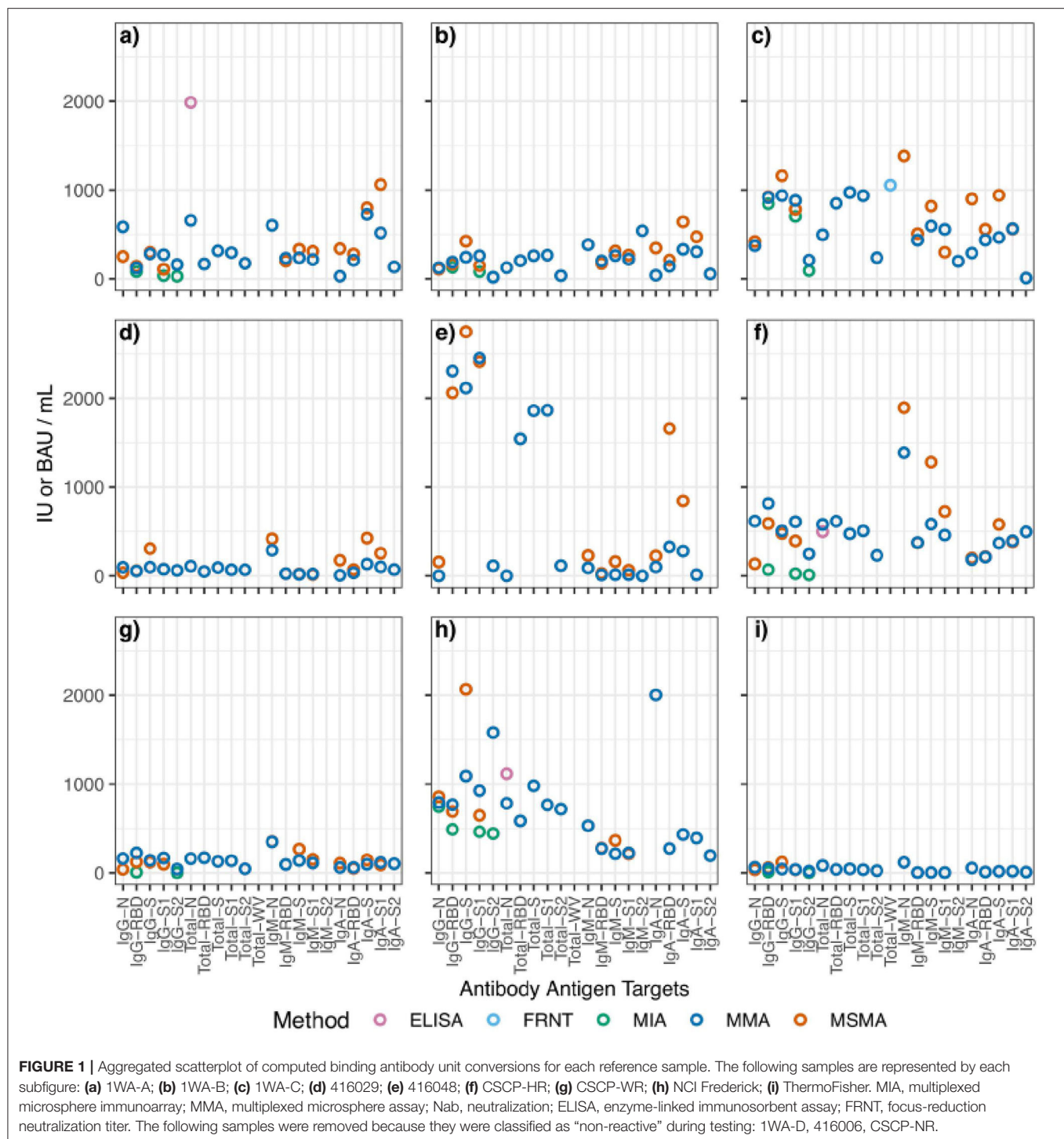
the BAU or IU conversions for each sample at each analyte. The IgG-spike analyte had more consistent BAU or IU/ml conversions between methods, regardless of the sample type. For example, the multiplexed microsphere assay (MMA) and MSMA results for the oneworld accuracy a(b,c,d) (1WA-A) sample were 281 (95% CI = 280–282) and 299 (95% CI = 294–304) BAU/ml, respectively; for the 1WA-C sample, the MMA and MSMA results were 938 (95% CI = 936–940) and 1,150 (95% CI = 1,142–1,178) BAU/ml, respectively. Further, for the covid-19 serology control panel-high reactive (CSCP-HR) sample, the MMA and MSMA results were 507 (95% CI = 506–508) and 477 (95% CI = 469–485) BAU/ml, respectively, and for the covid-19 serology control panel-weak reactive (CSCP-WR) sample, the MMA and MSMA results were 141 (95% CI = 0) and 120 (95% CI = 118–122) BAU/ml, respectively. Oppositely, the IgG-nucleocapsid analytes saw wider differences in BAU or IU/ml conversions between methods within the CSCP-HR, covid-19 serology control panel-non reactive (CSCP-NR), Thermo Fisher Scientific, 416,026, and 416,048 samples.

Interlaboratory/Method Binding Antibody Units Concordance

Once BAUs were calculated, we evaluated results for overall intermethod concordance if multiple laboratories yielded results for each Ag–Ab combination using percent coefficient of variation (%CV). Lower %CV values (<21%) indicate that results are highly agreeable between laboratories. None of the samples tested yielded universally high concordance between methods (regardless of Ag–Ab combination). For specific Ag–Ab combinations, there was no universal concordance between methods regardless of the sample tested. Samples 1WA-A, 1WA-B, and 1WA-C saw high concordance between laboratories for both the IgG and IgM bound to S, RBD, and N antigens (%CV range between 5 and 57%). CSCP-HR and CSCP-WR were highly concordant within the IgG-S combination (5 and 12%, respectively) and IgA and IgM bound to S, RBD, and N antigens (%CV range between 2 and 53%). Sample 416,029 was highly concordant between laboratories for IgG-RBD and IgG-S1 combinations (%CV 6 and 1%, respectively). Sample 416,048 saw high concordance with IgG S, S1, and RBD combinations (%CV = 19, 2, and 8%, respectively). The highest %CV value in **Figure 2** was found in sample 416,006 at the IgG-N analyte, which is likely because that particular sample was acquired from a postvaccinee individual and was not highly reactive to IgG-N during its characterization (**Table 1**). The National Cancer Institute (NCI) Frederick sample saw good overall concordance between laboratories for all the measured analytes. The IgG-S1 of the Thermo Fisher Scientific sample was highly concordant between laboratories (%CV = 6%). Result concordance between testing methods at each Ag–Ab combination is shown in **Figure 2**.

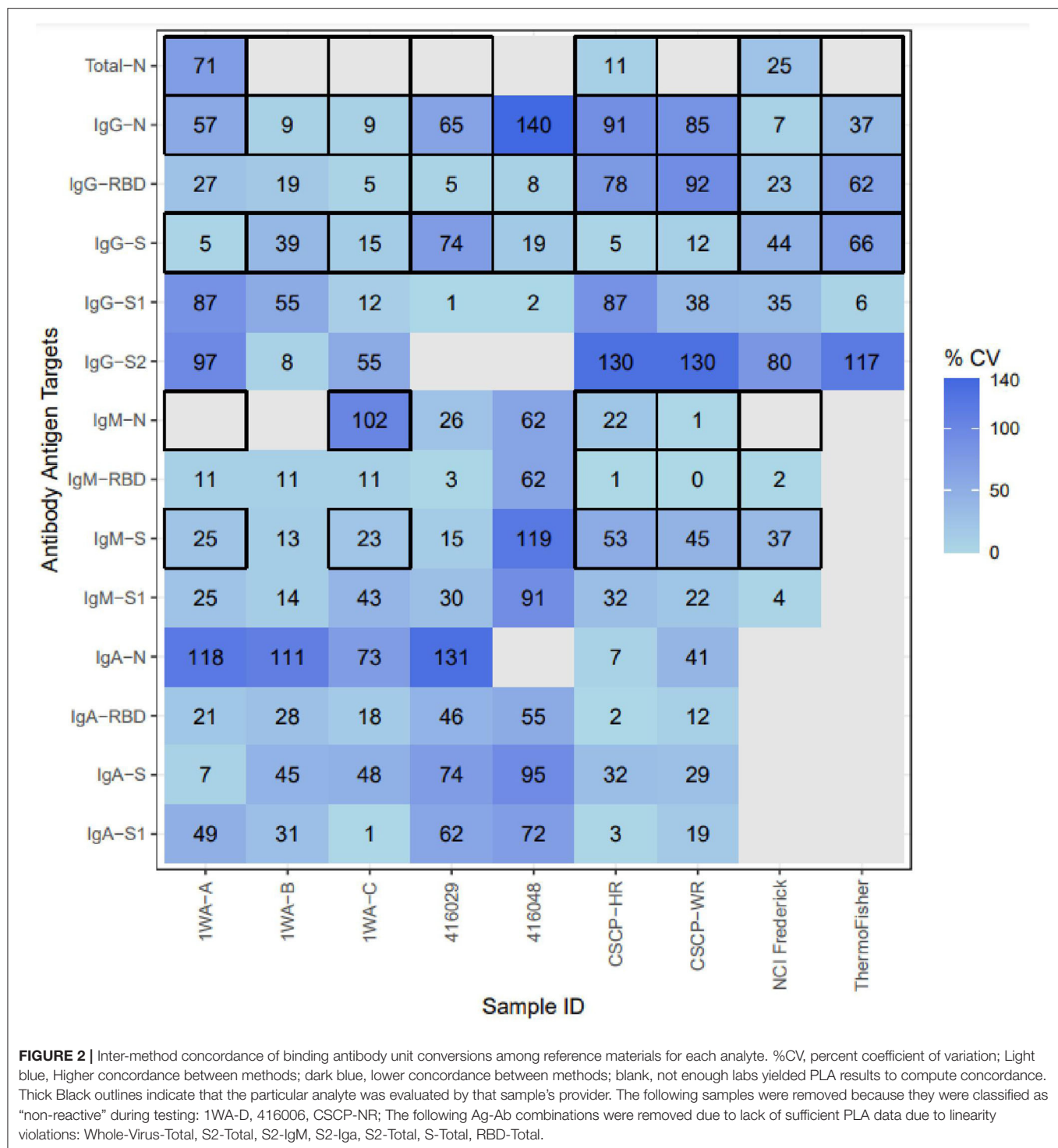
DISCUSSION

We evaluated multiple candidate reference materials against the WHO IS (NIBSC 20/136) to determine whether secondary



standards could be established. We then evaluated the applicability of using arbitrary BAU conversions to compare results between laboratories and serology diagnostic methods. Many seroprevalence studies use different serology assays to estimate transmission and/or herd immunity. The differences between assays make it nearly impossible to harmonize and establish a reliable limit of detection. A reference standard would theoretically allow for comparison between such studies.

A number of studies have determined that internal standards provided by the WHO for various pathogens may be useful and should be used to compare results across laboratories and diagnostic methods to help establish correlates of protection for SARS-CoV-2 and other high-threat pathogens (Cooper et al., 2018; McDonald et al., 2018; Mattiuzzo et al., 2019, 2020; Ciotti et al., 2021; Knezevic et al., 2022). For example, when assessing candidate reference materials for enterovirus serology,



one study evaluating the interassay variability for both the raw neutralization titer and the calculated relative potencies found a marked decrease in interassay variability. Their calculated percent geometric coefficient of variation (%GCV) was between 30 and 94% (Cooper et al., 2018), indicating that although their candidate materials had decreased interassay variability

after the results were converted to a harmonized metric, it is difficult to know what is considered an acceptable coefficient of variation across methods in this context. Two additional studies that evaluated candidate reference materials for Zika virus found similar improvements to intermethod concordance with the reference material, yet GCVs remained exceptionally

high, suggesting that a threshold for acceptable intermethod concordance may be difficult, if not impossible, to establish in these contexts (Mattiuzzo et al., 2019; Berry et al., 2020).

Finally, the developers of the WHO IS conducted a robust evaluation of the candidate standard that included 125 different SARS-CoV-2 serology assays (Mattiuzzo et al., 2020; Knezevic et al., 2022). When evaluating the interassay variability of results, they stratified their comparisons into neutralization assays, ELISAs, and “other” assays relative to what is now the WHO IS. Interassay variability between neutralization assays for samples tested relative to the WHO IS did not fall below 67% (%GCV range 67–250%). The interassay variability for the WHO IS itself was 241% (Mattiuzzo et al., 2020). Similar results were found when comparing ELISA methods and there were no data that evaluated the “other” methods included in the characterization. The assignment of an arbitrary 1,000 IU for neutralization assays and 1,000 BAU/ml for other assays—despite the large interassay variability relevant to the WHO IS—does not account for the vast differences between assays. Additionally, the interassay variability between all the methods used was not presented, which, therefore, makes it difficult to fully understand how best to harmonize results between multiple laboratories in order to assess correlates of protection. This study evaluated the interassay variability relative to the WHO IS across all the methods used. We also present the variability between laboratories for multiple Ag–Ab combinations to differentiate which ones are more likely to remain consistent or be highly variable within each sample.

Other studies also suggest that SARS-CoV-2 serology tests cannot be calibrated to the same measurement “ruler” and results compared between assays (Cooper et al., 2018; Bradley et al., 2021; Castillo-Olivares et al., 2021; Giavarina and Carta, 2021; Infantino et al., 2021; Perkmann et al., 2021; Solastie et al., 2021; Knezevic et al., 2022). It is also important to note that the IU or BAU assigned to the WHO IS is arbitrary and not based on an analytical concentration measurement. Additionally, results attained using the WHO IS are highly variable between assays. Our results demonstrate that any reference material should be characterized independently for each assay and it is not advisable to compare quantitative IU or BAUs between different assays. Therefore, arbitrary BAUs that were not calculated should not be used to benchmark any characterizations made for other reference materials, especially candidate secondary standards (Bradley et al., 2021; Giavarina and Carta, 2021; Perkmann et al., 2021). International Standards are not able to account for the wide variety of reagent formulations and nuances between testing methods using a universal metric such as an IU or BAU conversion. Finally, our findings show the qualification of secondary standards using the WHO IS using the 1,000 IU or BAU as a baseline metric that does not yield consistent IU or BAU conversions between assays.

Regardless of the pathogen, many other evaluations of “candidate” reference materials from the WHO have revealed a high degree of interassay and interlaboratory variability during characterization (Bozsoky, 1963; Holder et al., 1995; Wood et al., 2012; Dimech et al., 2013; Cooper et al., 2018; McDonald et al., 2018; Mattiuzzo et al., 2019; Kempster et al., 2020; Timiryasova et al., 2020). Although these findings cannot be

verified within the context of this study, our findings reinforce that SARS-CoV-2 serology reference materials face the same challenges and interpretation issues that other groups have seen (Mattiuzzo et al., 2020; Castillo-Olivares et al., 2021; Ciotti et al., 2021; Giavarina and Carta, 2021; Infantino et al., 2021; Kristiansen et al., 2021). Standardization of IU or BAU values for candidate secondary standards relative to the WHO IS could not be achieved across different laboratory assays using methods consistent with the NIBSC characterization of the WHO IS (Mattiuzzo et al., 2020). This calls into question the feasibility of standardizing different serology assays in the future and what this means when interpreting seroprevalence or distinguishing between natural infections and vaccine-induced responses.

Limitations

Some limitations are noted for this study. Among our laboratories, some were unable to yield relative potency values to use for a BAU/ml conversion for certain Ag–Ab combinations. Our criteria for PLA parallelism were more strict (relative slope = 0.8–1.2) than the standards set by the NIBSC (relative slope = 0.8–1.25) during the initial characterization of the NIBSC 20/136 because we wanted to set a more consistent range for relative slopes on either end (Mattiuzzo et al., 2020). Furthermore, the NIBSC does not clarify why they established an acceptable relative slope range of 0.8–1.25 was chosen. Manufacturing convalescent plasma/serum samples at scale is not common practice due to low volume donations and lot-to-lot differences. So, unlike molecular standards, it is difficult to generate large batches and consistent lots for harmonization or even for testing (in a postharmonization world). Two of the six methods used were neutralization assays; one did not yield relative potency for any samples tested and the other only yielded a relative potency for a single sample. Even after log, the raw candidate sample neutralization results failed to fall within the parameters to accurately perform PLA (Taylor et al., 2021).

Similar studies have used a variety of different interassay comparability methods that include, but are not limited to the Spearman’s rank correlation coefficient, the Mann–Whitney *U* tests, and Bablok regression (McDonald et al., 2018; Castillo-Olivares et al., 2021; Giavarina and Carta, 2021; Perkmann et al., 2021). Percent coefficient of variation (%CV) is a flexible metric commonly used in clinical laboratories and the developers of International Standards to evaluate interassay, intralaboratory, and lot-to-lot variations (Reed et al., 2002; Mattiuzzo et al., 2020). Furthermore, each of the example of alternative comparison methods exclude outlier results from analysis, which biases comparisons to appear erroneously “better” in a study context where outlier laboratory results are important to consider when determining the effectiveness of candidate reference materials.

The MMA method tested the WHO standard as nonreactive (no reaction present) for IgM against the nucleocapsid and spike S2 and indeterminate (no result due to PLA violation) for IgA against the nucleocapsid. Even though the assay was sensitive enough to give values for these analytes, these numbers are below what was considered reactive. Because the standard was so low and set to 1,000 BAU/ml, any sample with detectable but similarly

TABLE 4 | Recommendations for future development, use, and interpretation of International and Secondary Standards.

Topic	Recommendation(s)
Regulatory Bodies	• Replace the process that qualifies candidate secondary materials to an international standard with standards or best practices set for the “characterization” process of any potential reference materials using historical development of WHO IS’ as a framework. *This will elevate the quality standards for characterization of samples.* • Regulatory bodies must also require more precise interpretation of how to use particular reference materials based on the results from their characterization. *These interpretations must take into account the nuances of reagent formulation, testing platform, and the results interpretation in a clinical setting. * • Once these interpretations are more precise, future studies can then appropriately compare the results between seroprevalence studies for SARS-CoV-2 and potentially other incoming pathogens of interest.
Reference Material Characterization	• When characterizing reference materials, the methodology, reagent formulation, and validation information must be shown and included in the interpretation of reference material testing results. Different assays with different reagent formulations might yield slightly different results. • Establish a minimum number of laboratory methods to include when characterizing potential reference materials. • Require that the development, manufacturing, and distribution of secondary standards align with Good Manufacturing Practices. • Establish a minimum list of pathogens to test for when determining sample microbial bioburden. • Establish a list of minimum requirements for “suitable assay” used to demonstrate reference material expected immunological activity. • Establish an acceptable level of concordance (%GCV or % CV) between laboratories for the average BAU IU conversion to be considered “reliable.”
Interpretation	• Clarify that reference material (international standards and secondary standards) characterization is extremely assay and context dependent, which can affect accuracy of result interpretations. Similar tests with similar reagents must be used when comparing BAU conversions, and seroprevalence study results. • Revoke the encouraged removal of outlier method results during sample characterization. Exclusion of outlier laboratory data that fall within the PLA assumptions makes reference materials less comparable between methods which might remove the ability to adequately compare results between seroprevalence studies. • In order to continue using any WHO IS after their supply runs out, consider the development artificial IS for serology. • Clarify and establish that the intended use of standard reference materials is for external quality assurance schemes, comparing results between studies using similar assays or reagents, and be used as “anchors” by testing the same standards in the beginning and the end of a longitudinal research study. Which will attest to the quality of the results presented by that research study.

low quantities of an analyte will give a misleadingly high BAU/ml value and should be interpreted with caution.

Finally, each method in this study used different formulations of commercial reagents as noted in the Materials and Methods section. For coronavirus disease 2019 (COVID-19) and detection of anti-SARS-CoV-2 antibodies, the field is complicated by multiple antigen sources, multiple host experiences (one or more natural infections and/or vaccines and boosters), multiple variants, and multiple test platforms. This makes it very difficult to achieve harmony. The nuanced differences between these reagent, platforms, and host experiences might contribute to the differences between IU and BAU conversions. Serology is extremely dense with methods and tests, regardless of the pathogen, which highlights the difficulty of applying the same standards for interpretation because it does not account for the nuances that accompany a wide range of assays. This highlights the need for a more precise interpretation of reference material characterizations, so these differences can be accounted in future studies and allow for better harmonization of results between methods.

CONCLUSION

Harmonization of serology reference materials will increase the accessibility of reference materials—particularly in low-resource

settings, provided the methods used for comparison are accurate and reliable. Our findings indicate that the arbitrary units of the WHO IS are not an accurate means to compare SARS-CoV-2 serology results between different laboratories or methods. This study also shows that even after IU or BAU conversion, candidate secondary material results are still drastically different between laboratory methods. Both the International Standards and candidate secondary standards should only be used to compare the results within the same laboratory methods, provided they are using identical testing platforms, protocols, and reagent formulations (Bradley et al., 2021; Giavarina and Carta, 2021; Perkmann et al., 2021). This must be highlighted by regulatory bodies to accurately portray the use of the WHO IS as an assay calibrator during development or external quality assurance material for intramethod comparison, not as a universal comparator (Holder et al., 1995; Infantino et al., 2021).

Finally, despite some concordance between laboratories, qualification of secondary materials to the WHO IS using arbitrary IU or BAU/ml does not provide any benefit to the reference materials overall, due to the lack of consistent agreeable IU or BAU/ml conversions between laboratories. Secondary standards should be qualified to well-characterized reference materials, such as the WHO IS, using serology assays that are similar to the ones used for the original characterization of the WHO IS. However, secondary standards are useful if qualified

using similar assays as the original characterization as source traceability for they can be used for intraassay adjustments and can be used in external quality assessment to identify binding to antigen(s) presented in an assay to a reference, thereby providing intralaboratory operations (Table 4).

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

WW oversaw the entire study operation, conducted the analyses, constructed the tables, and drafted the manuscript. YR conducted the data analyses in R, built an open source R script for the parallel-line assay (github.com/yroell/pla), and built all the figures (both the manuscript and supplementary). ML provided data analysis oversight. MC assisted WW with the overall study design, execution, and result interpretation. HT and WL provided their open source parallel-line assay analysis program to use as a comparison to the R script developed by YR. WW, HZ, MK, HW, AD, and DT contributed SARS-CoV-2 serology reference materials. The remaining authors

tested the samples in their respective laboratories. All authors contributed insights, edits, revisions, and interpretations to the final 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/fmicb.2022.893801/full#supplementary-material>

REFERENCES

- Annen, K., Morrison, T. E., DomBourian, M. G., McCarthy, M. K., Huey, L., Merkel, P. A., et al. (2021). Presence and short-term persistence of SARS-CoV-2 neutralizing antibodies in COVID-19 convalescent plasma donors. *Transfusion*. 61, 1148–1159. doi: 10.1111/trf.16261
- B. Efron. (1979). Bootstrap methods: another look at the jackknife. *Ann. Stat.* 7, 1–26. doi: 10.1214/aos/1176344552
- Berry, N., Kempster, S., Ham, C., Jenkins, A., Hall, J., Page, M., et al. (2020). Passive immunisation of convalescent human anti-Zika plasma protects against challenge with New World Zika virus in cynomolgus macaques. *NPJ Vaccines*. 5, 86. doi: 10.1038/s41541-020-00234-y
- Bio-Rad. (2021). *Platelia SARS-CoV-2 Total Ab Instructions for Use*. Available online at: <https://www.fda.gov/media/137493/download>
- Bozsok, S. (1963). The problem of standardization in rheumatoid arthritis serology. *Arthritis Rheumat.* 6, 641–649. doi: 10.1002/art.1780060508
- Bradley, B. T., Bryan, A., Fink, S. L., Goecker, E. A., Roychoudhury, P., Huang, M.-L., et al. (2021). Anti-SARS-CoV-2 antibody levels measured by the AdviseDx SARS-CoV-2 assay are concordant with previously available serologic assays but are not fully predictive of sterilizing immunity. *J. Clin. Microbiol.* 59, e0098921. doi: 10.1128/JCM.00989-21
- Castillo-Olivares, J., Wells, D. A., Ferrari, M., Chan, A. C. Y., Smith, P., Nadesalingam, A., et al. (2021). Analysis of serological biomarkers of SARS-CoV-2 infection in convalescent samples from severe, moderate and mild COVID-19 cases. *Front. Immunol.* 12, 748291. doi: 10.3389/fimmu.2021.748291
- Ciotti, M., Ciccozzi, M., Pieri, M., and Bernardini, S. (2021). The COVID-19 pandemic: Viral variants and vaccine efficacy. *Crit. Rev. Clin. Lab. Sci.* 1–10. doi: 10.1080/10408363.2021.1979462
- Cooper, G., Mao, Q., Cawt, L., Wang, Y., Dougall, T., Rigsby, P., et al. (2018). Establishment of the 1st WHO international standard for anti-EV71 serum (Human). *Biologicals*. 53, 39–50. doi: 10.1016/j.biologicals.2018.03.001
- Dimech, W., Arachchi, N., Cai, J., Sahin, T., and Wilson, K. (2013). Investigation into low-level anti-rubella virus IgG results reported by commercial immunoassays. *Clin. Vaccine Immunol.* : CVI. 20, 255–261. doi: 10.1128/CVI.00603-12
- Finney, D. J., and Schild, H. O. (1966). Parallel line assay with successive adjustment of doses. *Br. J. Pharmacol.* 28, 84–92. doi: 10.1111/j.1476-5381.1966.tb01876.x
- Frederick National Laboratory for Cancer Research. (2021). The Human SARS-CoV-2 Serology Standard. Available online at: <https://frederick.cancer.gov/initiatives/seronet/serology-standard>
- GenScript. (2021). *CPass SARS-CoV-2 Neutralization Antibody Detection Kit Instructions for Use*. Available online at: <https://www.fda.gov/media/143583/download>
- Giavarina, D., and Carta, M. (2021). Improvements and limits of anti SARS-CoV-2 antibodies assays by WHO (NIBSC 20/136) standardization. *Diagnosis (Berlin, Germany)*. doi: 10.1515/dx-2021-0126
- Holder, P. K., Maslanka, S. E., Pais, L. B., Dykes, J., Plikaytis, B. D., and Carlone, G. M. (1995). Assignment of *Neisseria meningitidis* serogroup A and C class-specific anticapsular antibody concentrations to the new standard reference serum CDC1992. *Clin. Diagn. Lab.* 2, 132–137. doi: 10.1128/cdli.2.2.132-137.1995
- Infantino, M., Pieri, M., Nuccetelli, M., Grossi, V., Lari, B., Tomassetti, F., et al. (2021). The WHO international standard for COVID-19 serological tests: towards harmonization of anti-spike assays. *Int. Immunopharmacol.* 100, 108095. doi: 10.1016/j.intimp.2021.108095
- Kempster, S. L., Almond, N., Dimech, W., Grangeot-Keros, L., Huzly, D., Icenogle, J., et al. (2020). WHO international standard for anti-rubella: Learning from its application. *Lancet. Infect. Dis.* 20, e17–e19. doi: 10.1016/S1473-3099(19)30274-9
- Knezevic, I., Mattiuzzo, G., Page, M., Minor, P., Griffiths, E., Nuebling, M., et al. (2022). WHO international standard for evaluation of the antibody response to COVID-19 vaccines: call for urgent action by the scientific community. *Lancet. Microbe*. 3:e235–e240. doi: 10.1016/S2666-5247(21)00266-4
- Kristiansen, P. A., Page, M., Bernasconi, V., Mattiuzzo, G., Dull, P., Makar, K., et al. (2021). WHO International Standard for anti-SARS-CoV-2 immunoglobulin. *Lancet (London, England)*. 397, 1347–1348. doi: 10.1016/S0140-6736(21)00527-4

- Landes, R. D., Lensing, S. Y., and Hauer-Jensen, M. (2019). Interval estimators of relative potency in toxicology and radiation countermeasure studies: Comparing methods and experimental designs. *J. Biopharmaceut. Statist.* 29, 348–358. PubMed. doi: 10.1080/10543406.2018.1535500
- Mattiuzzo, G., Bentley, E., Hassall, M., Routley, M., Richardson, S., Bernasconi, V., et al. (2020). *Establishment of the WHO international standard and reference panel for anti-SARS-CoV-2 antibody*. Geneva: World Health Organization
- Mattiuzzo, G., Knezevic, I., Hassall, M., Ashall, J., Myhill, S., Faulkner, V., et al. (2019). Harmonization of Zika neutralization assays by using the WHO International Standard for anti-Zika virus antibody. *NPJ Vaccines*. 4, 42. doi: 10.1038/s41541-019-0135-3
- McDonald, J. U., Rigsby, P., Dougall, T., and Engelhardt, O. G. (2018). Establishment of the first WHO international standard for antiserum to respiratory syncytial virus: report of an international collaborative study. *Vaccine*. 36, 7641–7649. doi: 10.1016/j.vaccine.2018.10.087
- National Institute for Biological Standards and Control. (2020). *WHO International Standard: First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (human) NIBSC code: 20/136. Instructions for use*. Available online at: <https://www.nibsc.org/documents/ifu/20-136.pdf>
- Norman, M., Gilboa, T., Ogata, A. F., Maley, A. M., Cohen, L., Busch, E. L., et al. (2020). Ultrasensitive high-resolution profiling of early seroconversion in patients with COVID-19. *Nat. Biomed. Eng.* 4, 1180–1187. doi: 10.1038/s41551-020-00611-x
- Oneworld Accuracy. (2021). COVS434 [SARS-CoV-2 Serology. Available online at: <https://results.oneworldaccuracy.com/results/catalog/browse?cm=ACCUTESTI&y=2022&l=en&f=COVS>
- Perkmann, T., Perkmann-Nagele, N., Koller, T., Mucher, P., Radakovics, A., Marculescu, R., et al. (2021). Anti-spike protein assays to determine SARS-CoV-2 antibody levels: a head-to-head comparison of five quantitative assays. *Microbiol. Spect.* 9, e0024721. doi: 10.1128/Spectrum.00247-21
- Petrone, L., Petruccioli, E., Vanini, V., Cuzzi, G., Najafi Fard, S., Alonzi, T., et al. (2021). A whole blood test to measure SARS-CoV-2-specific response in COVID-19 patients. *Clin. Microbiol. Infect.* 27, 286.e7–286.e13. doi: 10.1016/j.cmi.2020.09.051
- R Core Team. (2021). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. Available online at: <https://www.R-project.org/>
- Reed, G. F., Lynn, F., and Meade, B. D. (2002). Use of coefficient of variation in assessing variability of quantitative assays. *Clin. Vaccine Immunol.* 9, 1235–1239. doi: 10.1128/CDLI.9.6.1235-1239.2002
- Schultz, J. S., McCarthy, M. K., Rester, C., Sabourin, K. R., Annen, K., DomBourian, M., et al. (2021). Development and validation of a multiplex microsphere immunoassay using dried blood spots for SARS-CoV-2 seroprevalence: application in first responders in Colorado, USA. *J. Clin. Microbiol.* 59. doi: 10.1128/JCM.00290-21
- Solastie, A., Virta, C., Haveri, A., Ekström, N., Kantele, A., Miettinen, S., et al. (2021). A highly sensitive and specific SARS-CoV-2 spike- and nucleoprotein-based fluorescent multiplex immunoassay (FMIA) to measure IgG, IgA, and IgM class antibodies. *Microbiol. Spect.* 9, e0113121. doi: 10.1128/Spectrum.01131-21
- Tan, C. W., Chia, W. N., Qin, X., Liu, P., Chen, M. I.-C., Tiu, C., et al. (2020). A SARS-CoV-2 surrogate virus neutralization test based on antibody-mediated blockage of ACE2-spike protein-protein interaction. *Nat. Biotechnol.* 38, 1073–1078. doi: 10.1038/s41587-020-0631-z
- Taylor, S. C., Hurst, B., Charlton, C. L., Bailey, A., Kanji, J. N., McCarthy, M. K., et al. (2021). A new SARS-CoV-2 dual-purpose serology test: highly accurate infection tracing and neutralizing antibody response detection. *J. Clin. Microbiol.* 59. doi: 10.1128/JCM.02438-20
- Thermo Fisher Scientific. (2021). *MASTM SARS-CoV-2 IgG Positive Control. Catalog No.10-028-305*. Available online at: <https://www.fishersci.com/shop/products/sarscov2-igg-pos-ctl-5x3ml-pk/10028305#>
- Timiryasova, T. M., Hodge, S. A., Zheng, L., Singer, A., Vincent, D., Rahman, M., et al. (2020). Preparation and qualification of internal rabies reference standards for use in the rabies rapid fluorescent focus inhibition test. *Sci. Rep.* 10, 9893. doi: 10.1038/s41598-020-66754-8
- Windsor, W., Knight, V., Merkel, P., Lamb, M., Tucker, H., Carson, K., et al. (2021). COVID-19 serology control panel using the dried tube specimen method. *Am. Soc. Tropical Med. Hygiene*. doi: 10.1101/2021.07.07.21260101
- Wood, J. M., Major, D., Heath, A., Newman, R. W., Höschler, K., Stephenson, I., et al. (2012). Reproducibility of serology assays for pandemic influenza H1N1: Collaborative study to evaluate a candidate WHO International Standard. *Vaccine*. 30, 210–217. doi: 10.1016/j.vaccine.2011.11.019
- Zeichhardt, H., and Kammel, M. (2021). *INSTAND Summary of sample properties and target values EQAS Group No. 416 Virus Immunology – SARS-CoV-2 (Ab)*. Available online at: <https://www.instand-ev.de/instand-ringversuche/ringversuchsprogramm/test/virusimmunologie-sars-cov-2-ak/>

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Genome-Wide Analysis of the Indispensable Role of Non-structural Proteins in the Replication of SARS-CoV-2

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Understanding the process of replication and transcription of SARS-CoV-2 is essential for antiviral strategy development. The replicase polyprotein is indispensable for viral replication. However, whether all nsps derived from the replicase polyprotein of SARS-CoV-2 are indispensable is not fully understood. In this study, we utilized the SARS-CoV-2 replicon as the system to investigate the role of each nsp in viral replication. We found that except for nsp16, all the nsp deletions drastically impair the replication of the replicon, and nsp14 could recover the replication deficiency caused by its deletion in the viral replicon. Due to the unsuccessful expressions of nsp1, nsp3, and nsp16, we could not draw a conclusion about their *in trans*-rescue functions. Our study provided a new angle to understand the role of each nsp in viral replication and transcription, helping the evaluation of nsps as the target for antiviral drug development.

Keywords: SARS-CoV-2, nsp, viral replication and transcription, indispensable role, replicon

INTRODUCTION

The ongoing pandemic of COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has posed a serious threat to human health and led to heavy economic loss (V'Kovski et al., 2021). The relatively limited knowledge of this deadly virus hinders us from efficiently treating patients with COVID-19 (Feng et al., 2020). Besides the recognition and entry process (Lan et al., 2020; Yin et al., 2022), the viral replication and transcription process regulated by viral replicase proteins possesses many promising targets for antiviral strategy development (V'Kovski et al., 2021; Malone et al., 2022). Thus, the systematical analysis on the roles of proteins derived from viral replicase is an important biomedical objective.

SARS-CoV-2, belonging to the *Nidovirales* order, the *Coronaviridae* family, and the *Betacoronavirus* genus (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020), is an enveloped positive-strand RNA virus. Its genome contains at least nine open reading frames (ORFs). The 5'-terminal two-thirds of the viral genome contains two open reading frames, ORF1a and ORF1ab (Chen et al., 2020). The latter is translated by a -1 ribosomal frameshifting mechanism, in which the translational complex avoids the stop codon by altering the reading frame while encountering the "slippery" sequence at the terminus of ORF1a (Pan et al., 2008). The translational products of ORF1a and ORF1ab, polyprotein 1a (pp1a) and polyprotein

1ab (pp1ab), are responsible for the viral replication and transcription. Before going to its final roles, pp1a and pp1ab are processed up to 16 non-structural proteins (nsps) by their own proteases, papain-like protease (PLpro) located in nsp3 and 3C-like protease (3CLpro) or main protease (Mpro) of nsp5 (Pan et al., 2008; Du et al., 2021; Jiang et al., 2021).

Nsp1 (approximately 180 aa in SARS-CoV-2) is a multifunctional protein capable of altering host translation (Narayanan et al., 2008a; Kamitani et al., 2009; Lokugamage et al., 2012), triggering host mRNA cleavage (Kamitani et al., 2009; Huang et al., 2011) and decay (Kamitani et al., 2006; Narayanan et al., 2008b), inhibiting the innate immune response (Zust et al., 2007; Narayanan et al., 2008b), and inducing cytokines and chemokines (Law et al., 2007). By introducing deletions in murine hepatitis viruses (MHV) and the analysis of mutant MHV, another member of the *Betacoronavirus* genus, Brockway and Denison (2005) identified a few residues in nsp1 important for viral RNA synthesis and replication, and viral protein processing. The SARS-CoV-2 nsp1 is an evolving protein, as, besides mutations, two deletions in nsp1 were identified, including the deletion of 686–694 nt (Benedetti et al., 2020) and the deletion of 500–532 nt (Lin et al., 2021b).

Nsp2's (approximately 638 aa in SARS-CoV-2) function is not well defined, while it may be involved in regulating host intracellular signaling through the interaction with prohibitin 1 (PHB1) and PHB2 (Cornillez-Ty et al., 2009). SARS-CoV-2 with the variation of nsp2 (T85I) may lead to poor replication in Vero-CCL81 cells (Pohl et al., 2021). Furthermore, the genetic deletions of nsp2 on the reverse genetics systems of MHV and SARS-CoV lead to attenuated viral growth and RNA synthesis (Graham et al., 2005).

Nsp3 (approximately 1945 aa in SARS-CoV-2) is a large multi-domain protein. It encompasses multiple functional domains, including ubiquitin-like domain, single-stranded poly(A) binding domain, C-terminal SARS-Unique domain, PLpro domain, nucleic acid-binding domain, and two transmembrane helix motifs (Jiang et al., 2021). The PLpro activity of nsp3 is responsible for releasing nsp1, nsp2, and nsp3 *per se* from the pp1a or pp1ab. Thus, it is recognized as a promising target for antiviral drug development (Shin et al., 2020). Besides protease activity, nsp3 has deubiquitinating and interferon antagonism activities (Clementz et al., 2010) and may regulate the viral replication by interacting with viral nsps, including nsp1, nsp4, nsp6, nsp10, nsp12, nsp13, nsp14, and N (Baez-Santos et al., 2015; Jiang et al., 2021). Nsp3 could also benefit viral replication by improving the inter/intra-cellular microenvironment. SARS-CoV-2 nsp3 delays the expression of IFN- β (Lei et al., 2020).

Nsp4 (approximately 500 aa in SARS-CoV-2) also has multiple transmembrane domains. Nsp4 of SARS-CoV interacts with nsp3, contributing to the endoplasmic reticulum (ER) membrane rearrangement and the assembly of double-membrane vesicles (DMVs), which play an essential role in viral replication and transcription (Sakai et al., 2017). The essential role of nsp4 was also supported by the sequence analysis on patient samples, and the nsp4 variant (E3073A) of SARS-CoV-2 is associated with a significantly reduced fever duration (Zekri et al., 2021).

Nsp5 (approximately 306 aa in SARS-CoV-2) is one of the most widely studied proteins of SARS-CoV-2. Nsp5 plays a crucial role in the maturation of viral replicase polyprotein by cleaving pp1ab at 11 sites and subsequently releasing nsp4 to nsp16 for the assembly of replication and transcription complex (RTC) (Jin et al., 2020). Nsp5 is recognized as a promising target for antiviral drug development, and more than 86 potential inhibitors of nsp5 were selected by various studies (Yan and Gao, 2021). Its structures with or without inhibitors are resolved by a myriad of studies (Mariano et al., 2020).

Nsp6 (approximately 290 aa in SARS-CoV-2) also has transmembrane domains. Together with nsp3 and nsp4, nsp6 contributes to the formation of DMV (Angelini et al., 2013). Different from the function of nsp3-nsp4 complex in pairing membranes, nsp6 majors in membrane proliferation. Nsp6 was shown to limit autophagosome expansion (Cottam et al., 2014).

Nsp7 (approximately 83 aa in SARS-CoV-2) and nsp8 (approximately 198 aa in SARS-CoV-2) function as the cofactors for RTC (Kirchdoerfer and Ward, 2019). The involvement of nsp7 and nsp8 in RTC goes through a transition from nsp7–nsp8 hexadecameric primase complex to the nsp12–nsp7–nsp8 polymerase complex, promoting RdRP efficiency of viral RNA product synthesis (Wang et al., 2020).

Nsp9 (approximately 113 aa in SARS-CoV-2) plays a vital role in the replication of SARS-CoV-2 through its activity in ssRNA/DNA binding ability (Littler et al., 2020), which is regulated by its dimerization (de et al., 2021) and NMPylation on its conserved site (Slanina et al., 2021). SARS-CoV-2 nsp9 stimulates type I interferon response (Lei et al., 2020).

Nsp10 (approximately 139 aa in SARS-CoV-2) was identified as an interaction partner of nsp14 and nsp16 by the genome-wide screening of intraviral protein–protein interactions (Pan et al., 2008). Nsp10 interacts with nsp14 (Lin et al., 2021a) and nsp16 (Krafcikova et al., 2020) to promote their 3'–5' exonuclease and RNA ribose-2'-O-methylation activities, respectively.

Nsp11 (approximately 13 aa in SARS-CoV-2) is a cleavage product of pp1a processed by Mpro at the nsp10/11 boundary. Nsp11 shares the same first nine amino acids with nsp12 and exhibits an intrinsically disordered protein behavior (Gadhavi et al., 2021).

Nsp12 (approximately 932 aa in SARS-CoV-2), the RNA-dependent RNA polymerase (RdRP) of SARS-CoV-2, forms the RTC with nsp7 and nsp8 (Wang et al., 2020). Mutations, S759A/D760A/D761A, at the key residues in nsp12 diminished the viral replication (Jin et al., 2021). More than 95% identical to SARS-CoV counterpart, nsp12 of SARS-CoV-2 exhibits a similar sensitivity to the inhibitory effect of remdesivir, and the decreased enzymatic activity and thermostability (Peng et al., 2020). Besides RdRP activity, SARS-CoV-2 nsp12 is responsible for viral RNA capping as a GTPase, adding a GTP nucleotide to the 5' end of viral RNA via a 5'–5' triphosphate linkage (Walker et al., 2021).

Nsp13 (approximately 601 aa in SARS-CoV-2) possesses RNA helicase and the nucleoside triphosphate hydrolase (NTPase) activities, unwinding viral RNA duplex and supplying the energy for unwinding by hydrolyzing ATP, respectively, in the replication of viral RNAs (Shu et al., 2020). Thus, RNA helicase activity of nsp13 is sensitive to the concentration of ATP. The

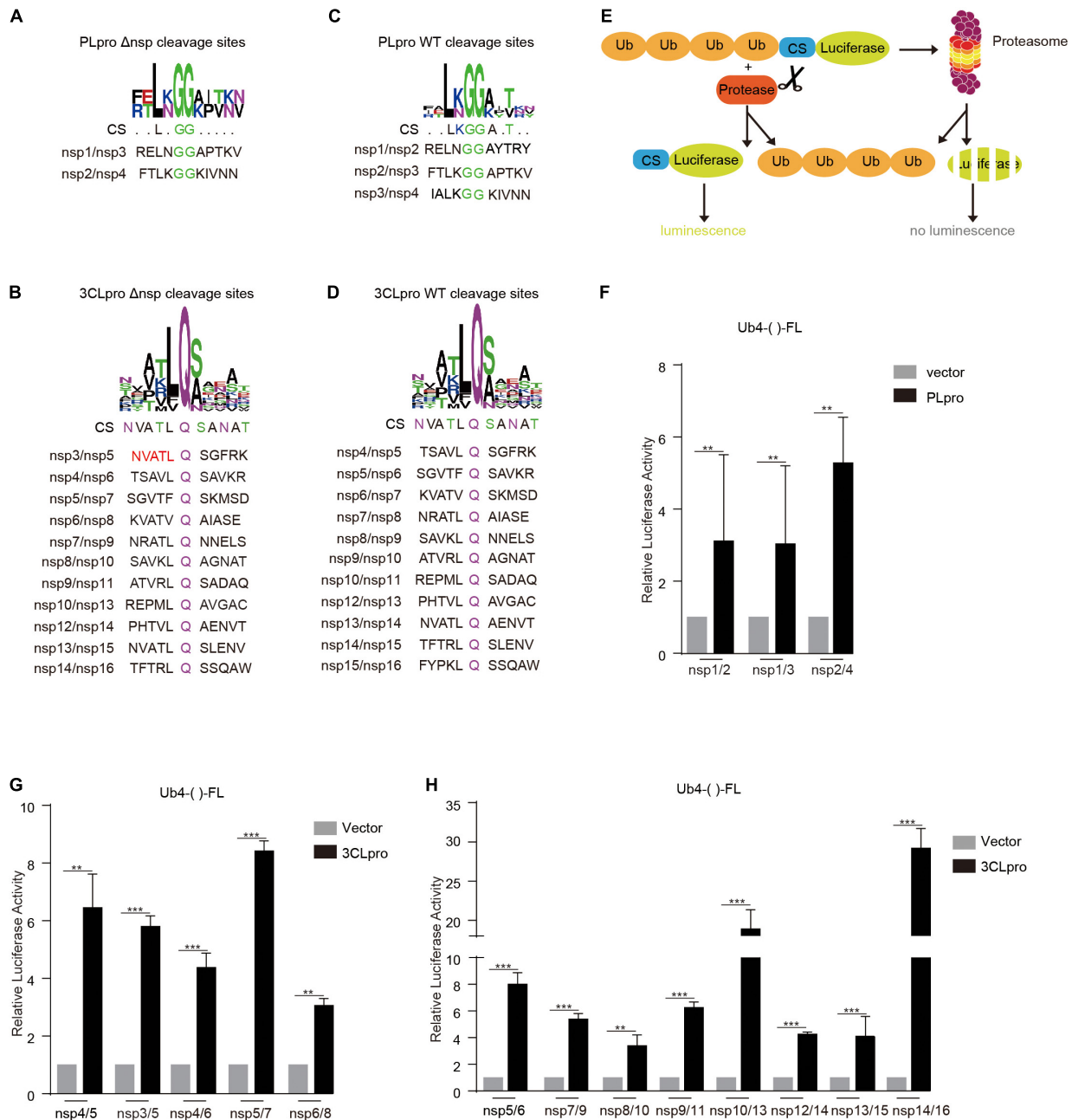


FIGURE 1 | Reconstituted cleavage sites are cleaved by PLpro and 3CLpro. The reconstituted (A,B) and WT (C,D) cleavage site sequences were analyzed using WEBLOGO. Note that the reconstituted and WT cleavage sites have similar consensus sequences (CSs). (E) Schematic illustration of PLpro and 3CLpro activity reporter system. Four tandem ubiquitins mediate the degradation of luciferase through the proteasome pathway. The cleavage site of PLpro or 3CLpro is inserted between the four tandem ubiquitins and the luciferase. PLpro or 3CLpro recognizes and cuts the cleavage site, leading to the detachment of the luciferase from the four tandem ubiquitins. The luciferase activity is measured and reflects the PLpro or 3CLpro activity. HEK293T cells were transfected with vector or PLpro (F) and 3CLpro (G,H), pRL-TK, and Ub4-FL inserted with indicated various cleavage sequences (Ub4-()-FL). 24 h post-transfection, the cells were collected, and the lysates were subjected to Dual-Luciferase Reporter (DLR™) Assay. The data represent one of three independent experiments with similar results; error bars represent the mean $\pm$ s.e.m. Two-tailed unpaired Student's *t*-test or one-way ANOVA with Bonferroni post-test correction was used to analyze the significance; ***P* < 0.01 and ****P* < 0.001; ns, not significant.

increased ATP concentrations promote the processivity of nsp13 in unwinding duplex RNA (Jang et al., 2020). Two mutations, Y541C and P504L, from variants of SARS-CoV-2, located in

the nucleotide-binding core of nsp13, weaken the transmission capacity of SARS-CoV-2, indicating the crucial role of nsp13 in viral replication (Wang et al., 2021).

Nsp14 (approximately 527 aa in SARS-CoV-2) has 3'-5' exonuclease (ExoN) and N7-guanine methyltransferase (N7-MTase) activities, which are born by N-terminal and C-terminal domains, respectively (Chen et al., 2009). The nsp14 ExoN activity is a key component of the RNA proofreading machinery, which is proposed to be essential for the stability and replication efficiency of viral genome (Becares et al., 2016). Nsp10 interacts with the N-terminal domain of nsp14, promoting ExoN activity (Ma et al., 2015). Its N7-MTase activity plays a crucial role in the synthesis of viral mRNA cap, preventing the recognition by the host cell (Becares et al., 2016). Both enzymatic activities of nsp14 are crucial for viral replication, and the mutations of D90A/E92A and D331A, impairing the ExoN and N7-MTase, respectively, drastically decreased the generation of viral genomic/subgenomic RNAs in viral replicon system (Jin et al., 2021).

Nsp15 (approximately 346 aa in SARS-CoV-2) is a uridine-specific endoribonuclease (EndoU) whose activity resides in its C-terminal domain (Frazier et al., 2021). Nsp15 plays a crucial role in viral replication, because the inactivation of EndoU by introducing mutations in nsp15 and the deletion in nsp15 drastically decreased the viral replication and recovery (Ivanov et al., 2004; Almazan et al., 2006; Kang et al., 2007; Nguyen et al., 2021).

Nsp16 (approximately 298 aa in SARS-CoV-2) mediates 2'-O-methylation of viral RNA cap structure, preventing the degradation by host nucleases (Krafcikova et al., 2020; Wilamowski et al., 2021). To perform its MTase activity, nsp16 requires nsp10 as a stimulatory factor to bind its m7GpppA-RNA substrate and S-adenosyl-L-methionine (SAM), methyl donor (Chen et al., 2011). In contrast, nsp10 also interacts with nsp14, another MTase of SARS-CoV-2, but is not required for MTase activity of nsp14 (Ma et al., 2015). SARS nsp16 plays a crucial role in the viral replication, because the elimination of the nsp16 expression by introducing the stop codon at its 5' end largely attenuated the viral replication (Almazan et al., 2006; Rohaim et al., 2021).

As summarized above, the functional characterization of nsps indicates their potential involvement in viral replication and transcription. A number of studies showed that the overexpression of some nsps could further promote the replication level of viral reverse genetics systems, indicating that these nsps play essential roles in viral replication but possibly are not sufficient in the viral replication complex, which is composed of the nsps processed from replicase polyprotein (Jin et al., 2021; Luo et al., 2021).

In this study, to clarify whether each nsp is indispensable for viral replication and transcription, we examined the replication of the viral replicon with the deletion of each nsp. To minimize the undesired impact of each nsp deletion on the viral polyprotein process, we reconstructed cleavage sites recognized by PLpro and 3CLpro and confirmed the cleavage efficiency using our PLpro/3CLpro activity reporter system. By monitoring the replication activity of viral replicons with each nsp deletion, we found that the dependencies of viral replication on each nsp varied considerably, and nsp14 can rescue the decreased replication of viral replicon caused by the loss of nsp14 *per se*.

RESULTS

The Reconstituted Cleavage Sites Can Be Efficiently Cleaved by Papain-Like Protease and 3C-Like Protease

The deletion of nsp_x (x is any number from 1 to 16) from the viral genome can lead to the failure to separate its adjacent upstream (nsp_x-1) and downstream nsps (nsp_x + 1) (Supplementary Figure 1). The functions of nsp_x-1 and nsp_x + 1 are likely altered in the fused form, nsp_x-1-nsp_x + 1. To solve this issue, we reconstituted the cleavage sites between nsp_x-1 and nsp_x + 1, adjacent to the removed nsp_x. The reconstituted cleavage sites are composed of the C-terminal amino acid sequence of nsp_x-1, double glycine (GG) or glutamine (Q), and N-terminal amino acid sequence of nsp_x + 1 (Figures 1A,B). The consensus sequence of reconstituted cleavage sites is similar to that of the wild-type cleavage sites (Figures 1C,D). For nsp3/nsp5 cleavage site, we replaced the last five amino acids, IALKG, at the C-terminal end of nsp3 with NVATL to rebuild the cleavage site recognized by 3CLpro (Figure 1B).

To verify whether the reconstituted cleavage sites could be processed by PLpro and 3CLpro, we employed the 3CLpro activity reporter system developed previously by our group (Du et al., 2021; Figure 1E). In this system, the reporter gene firefly luciferase (FL) is fused with four tandem ubiquitins (Ub4), which lead to the degradation of firefly luciferase in a ubiquitin-dependent proteasome mechanism and low activity of firefly luciferase. An in-frame amino acid sequence is inserted at the *Hind*III site between four tandem ubiquitins and firefly luciferase (Ub4-()-FL). The cleavage on this inserted sequence leads to the separation of four tandem ubiquitins and firefly luciferase, which gives a robust increase in the detected activity of firefly luciferase.

We inserted all the reconstituted cleavage site sequences at the *Hind*III site and tested the cleavage efficiency by PLpro and 3CLpro. The results showed that the cleavage efficiencies on the reconstituted cleavage sites are comparable to that of WT cleavage sites, indicating that the deletion of nsps, except nsp3 with PLpro activity and nsp5 with 3CLpro activity, should not influence the process of replicase polyprotein (Figures 1F-H).

Construction of pBAC-nCoV-Replicon-Δnsp (nCoV-Rep-Δnsp)

Next, we constructed the replicon of SARS-CoV-2 with the deletions of nsp1 to nsp16 based on pBAC-nCoV-Replicon (nCoV-Rep), which was constructed by our group previously (Jin et al., 2021). In CMV-5' UTR-ORF1ab region of nCoV-Rep, we designed eight unique restriction sites, namely, *Kas*I, *Bsi*WI, *Nhe*I, *Pac*I, *Cl*aI, *Mlu*I, *Axy*I, and *Sac*II, which separate the viral cDNA sequence into seven replaceable segments and thus are very helpful for the reconstruction operation on the replicon (Figure 2A). For the deletion of each nsp, we first selected the segment containing the target nsp with the unique restriction sites. The segments for various nsps are described in Figure 2B.

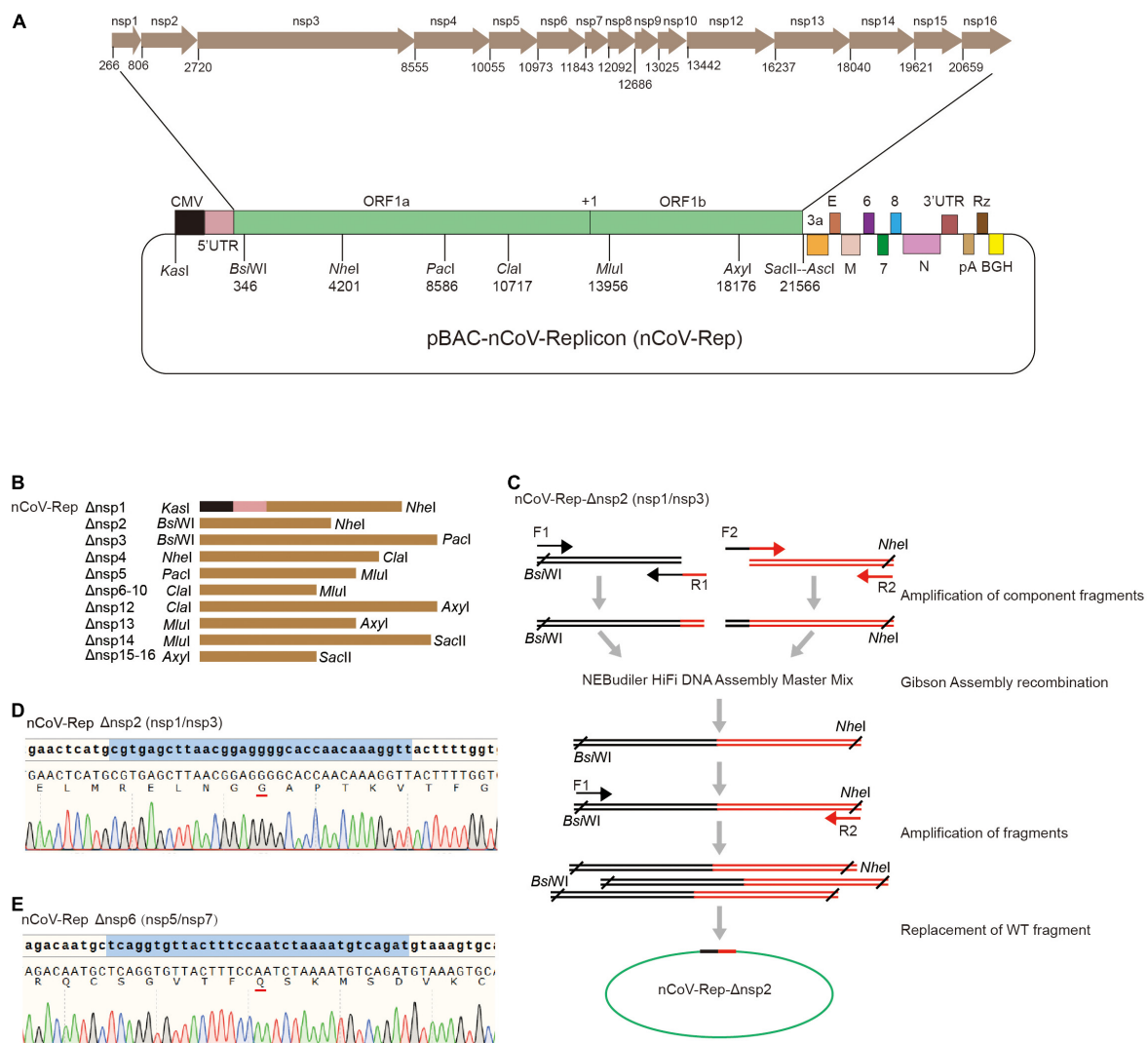


FIGURE 2 | Construction of nCoV-Rep-Δnsp. **(A)** Schematic of the SARS-CoV-2 replicon (pBAC-nCoV-Replicon) with indicated unique restriction sites and the location of each nsp. **(B)** The design of the fragments with indicated restriction sites for the deletion of each nsp. **(C)** Schematic of the construction strategy for cloning nCoV-Rep-Δnsp plasmids. The Sanger sequencing results of reconstituted sequences in nCoV-Rep-Δnsp2 **(D)** and nCoV-Rep-Δnsp6 **(E)**.

To obtain the target nsp-deleted segment, we designed middle forward (F2) and reverse (R1) primers composed of 3' terminal sequence of the upstream nsp_x-1 adjacent to the deleted nsp_x, cleavage site sequences, encoding GG or Q, and 5' terminal sequence of the downstream nsp_x + 1 adjacent to the deleted nsp_x (Figure 2C), except the primers for nsp4 deletion, which contain the mutant sequence encoding NVATL (Figure 1B). We used the primer pair of the forward primer (F1) upstream of the restriction site and the middle reverse primer (R1) and the primer pair of the reverse primer (R2) downstream of restriction site and the middle forward primer (F2) to amplify two-component fragments. We assembled two-component fragments into the segment with deletion of the target nsp using Gibson Assembly strategy and amplified the segment with the primer pair of the forward primer upstream (F1) of the restriction site and the reverse primer (R2) downstream of

the restriction site (Figure 2C). Using the unique restriction sites, we replaced WT segments with the target nsp-deleted segments and verified the segment sequence with Sanger DNA sequencing to ensure no undesired mutations (Figures 2D,E and Supplementary Figure 2).

The Deletion of Non-structural Protein 1–15 Impairs the Replicative Activity of the Replicon

To investigate the role of various nsps in viral replication and transcription, we transfected the WT replicon (nCoV-Rep) and the replicons with the deletion of various nsps (nCoV-Rep-Δnsp1 to 16) into HEK293T cells as described previously (Jin et al., 2021). We examined the subgenomic RNAs using quantitative PCR (qPCR) with the forward primer

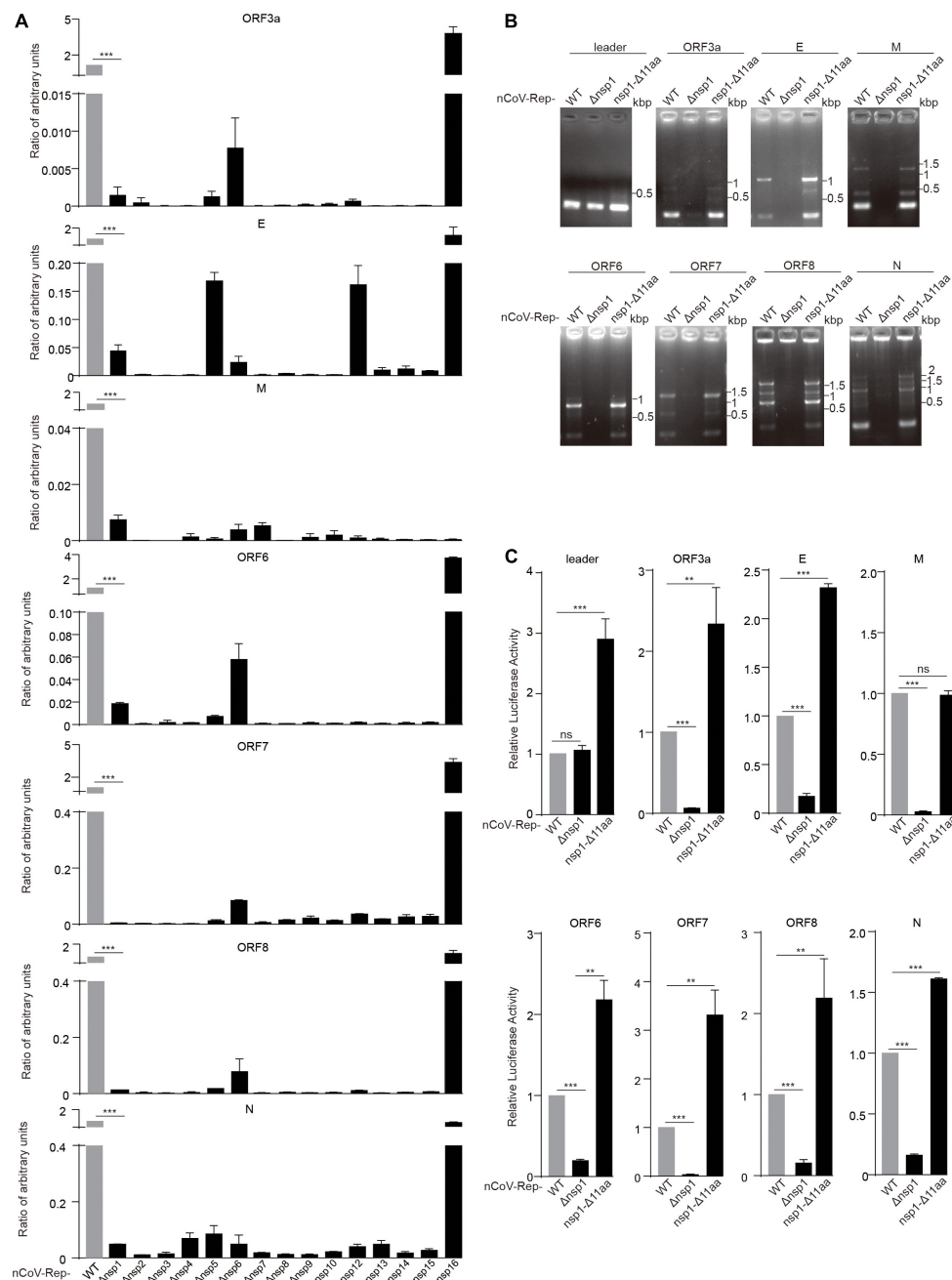


FIGURE 3 | Nsp deletion impairs the replicative activity of the replicon. HEK293T cells were transfected with nCoV-Rep WT or nCoV-Rep- Δnsp s and GFP, which was used as the control to normalize the transfection efficiency. 48 h post-transfection, the cells were harvested for qPCR assay with a 5-s extension time to quantify the subgenomic RNAs (Jin et al., 2021). The relative amounts of subgenomic RNAs for nCoV-Rep WT and $\Delta nsp1$ -16 were depicted in (A). Similarly, the relative amounts of subgenomic RNAs for nCoV-Rep WT, $\Delta nsp1$, and $nsp1-\Delta11aa$ were analyzed with RT-PCR (B) with a 1-min extension time, which is enough to amplify all the possible subgenomic RNAs and qPCR (C). Two-tailed unpaired Student's *t*-test or one-way ANOVA with Bonferroni post-test correction was used to analyze the significance; ***P* < 0.01 and ****P* < 0.001; ns, not significant.

in the leader sequence and the reverse primers in 5' UTR, ORF3a, E, M, ORF6, ORF7, ORF8, and N (Figure 3A). To only synthesize the products from the template composed of a direct fusion of leader sequence and coding regions of various ORFs, we reduced the extension time to 5 s as discussed

previously (Jin et al., 2021). The results showed that except $nsp16$ deletion, the synthesis of each subgenomic RNA is largely impaired, indicating that all the nsps except $nsp16$ could play an indispensable role in the viral replication and transcription.

Deletions in nsp1 of SARS-CoV-2 variants were reported, indicating that nsp1 may not be essential for viral replication and transcription (Lin et al., 2021b). To clarify the different impacts on viral replication between the deletion of whole nsp1 and $\Delta 500-532$ in nsp1, we constructed the replicon with the reported deletion of 11 amino acids in nsp1 (nCoV-Rep-nsp1- $\Delta 11$ aa) (Lin et al., 2021b) and examined the synthesis of subgenomic RNAs. The results showed that unlike the replicon with the deletion of the whole nsp1 (nCoV-Rep- Δ nsp1), nCoV-Rep-nsp1- $\Delta 11$ aa gave an increased synthesis of many subgenomic RNAs, in agreement with the previous report (Lin et al., 2021b; **Figures 3B,C**). This piece of data suggested that nsp1 plays an indispensable role in viral replication and transcription despite its unfavorable functions mentioned above.

Non-structural Protein14 Reconstitution Can Rescue the Impaired Replication Caused by the Non-structural Protein14 Deletion

Next, we asked whether the expression of non-replicon nsps *in trans* could rescue the impaired replication caused by the nsp deletion. We cotransfected nCoV-Rep- Δ nsp1 to 16 with nsp1 to 16 expressing plasmid into HEK293T cells. Besides quantitative PCR, we examined the N protein expression using Western blotting (WB). In agreement with the qPCR result, except nsp16, the nsp deletions largely impair N protein expression (**Figure 4** and **Supplementary Figure 3**). We found that the nsp14 could rescue the impaired viral replication caused by the nsp14 deletion, as indicated by the significantly increased expression of N protein by nsp14 expression compared with vector control. The nsp16 expression *in trans* could not increase the N expression, which is not affected by the nsp16 deletion, further supporting a dispensable role of nsp16 in viral replication and transcription (**Figure 4** and **Supplementary Figure 3**). Since we failed to detect the expression of nsp1, nsp3, and nsp16, whether these nsps could rescue the impaired viral replication by corresponding nsp deletion is still not determined.

DISCUSSION

It is well recognized that nsps derived from the replicase polyprotein of SARS-CoV-2 play crucial roles in viral replication and transcription. However, whether all these nsps are indispensable for viral replication and transcription is still not fully defined. Here, we constructed the replicon of SARS-CoV-2 with the deletion of each nsp and verified the replication activity of the mutant replicons. We found that except for nsp16, all the deletions of nsp impaired the replication of the viral replicon (**Figure 3A**). The effect caused by the deletion of nsps should not be due to the low cleavage efficiency between the two nsps adjacent to the deleted nsp, because the cleavages on the reconstructed cleavage sites are verified with our 3CLpro/PLpro activity reporter system (**Figure 1**). By restoring the nsp14 expression, the nsp14 deleted replicon regained the replication activity

and gave the expression of N gene at the 3' terminal of viral genome (**Figure 4**).

In general, our findings suggest that the viral replication relies on each nsp are varied. We found that the replicon with the deletion of nsp16 is still able to replicate itself and gives the expression of N gene, which is dependent on the discontinuous mechanism (Hussain et al., 2005). This piece of data indicating the viral 2'-O-methylation by nsp16 is likely dispensable for viral replication. Nsp16 could help the viral RNA escape from the recognition of the host innate immune system by decorating the viral RNA with 2'-O-methylation (Zust et al., 2011). We noticed an apparent increase in N gene expression, indicating that the deletion of nsp16 may promote translation efficiency. Indeed, the previous studies (Hoernes et al., 2016; Choi et al., 2018) uncovered that 2'-O-methylation decreases translation efficiency by disrupting tRNA decoding during translation elongation. Thus, we infer that besides facilitating the escape from the host immune monitoring, nsp16 could tune the expression of various subgenomic RNAs.

We found that nsp14 could rescue the replication of nsp14-deleted replicon, but the other nsps could not, indicating that nsp14 could function as a separate protein, not tightly associated with RTC as other nsps. Considering that the two reactions mediated by 3'-5' exoribonuclease (ExoN) and N7-guanine methyltransferase (N7-MTase) activities of nsp14 could perform independently, we infer that hampering the association of nsp14 with viral RTC may not be a feasible antiviral strategy.

The expression of well-known replication-associated proteins, including nsp7, nsp8, nsp9, nsp10, nsp12, nsp13, and nsp15, could not recover the impaired replication of the viral replicon with the corresponding nsp deletion, indicating that the viral RTC assembly likely prefers the *in cis* component nsps generated from the same original polypeptides. The preference is likely due to the time window and the subcellular location for the viral RTC assembly (van Hemert et al., 2008; Acheampong et al., 2022). The exogenous expression of some nsps cannot fully satisfy the requirements, so these nsps cannot restore the assembly and the proper function of viral RTC.

Three nsps containing transmembrane domains, nsp3, nsp4, and nsp6, exhibited indispensable functions in the viral replication, indicating that the membrane structure or the location of RTCs could be essential for the efficient replication of viral RNAs by providing an essential microenvironment (Cortese et al., 2020). Mutations on the cleavage sites of nsp7 to nsp10 were reported to have a different impact on the viral replication compared with the in-frame deletions of nsp7 to nsp10 coding sequences. The mutation on the nsp9-nsp10 cleavage site only resulted in an attenuated viral replication, while the deletion of the coding sequence of nsp7-nsp10 was lethal for mutant viruses, consistent with our study (Deming et al., 2007). Although no replication-associated function of nsp2 is uncovered, the deletion of nsp2 largely impaired the replication of viral replicon, consistent with the previous reports that the titers of progeny virus by SARS Δ nsp2 infection were $\sim 1\text{-log}_{10}$ reduced compared to wild-type infections (Graham et al., 2005), indicating that investigation on the nsp2's replication-associated function is a potential research direction.

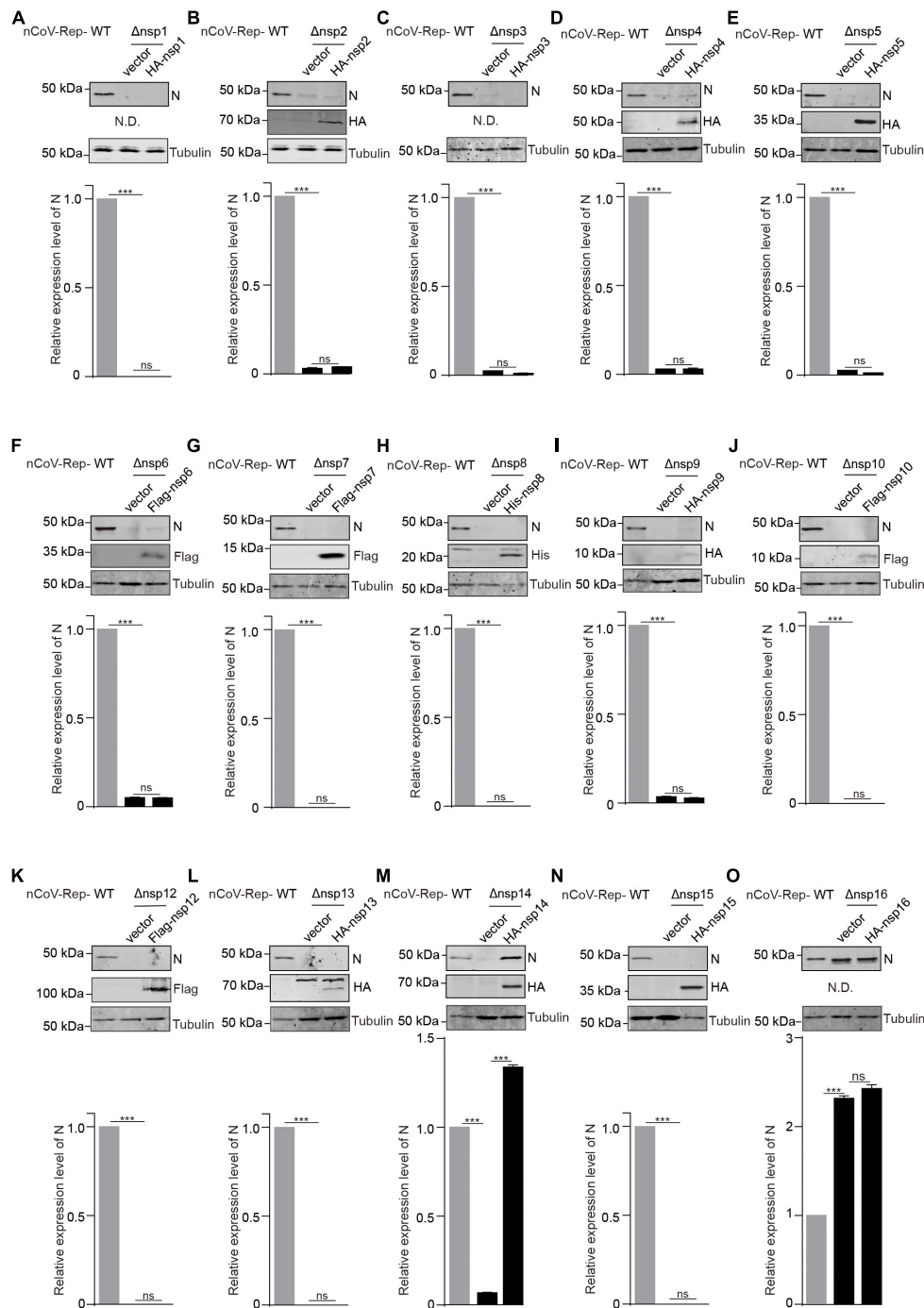


FIGURE 4 | *In trans* expression of nsp14 rescues the nsp14-deletion-induced impaired replication. (A–O) HEK293T cells were transfected with nCoV-Rep WT or nCoV-Rep- Δ nsp with vector or corresponding nsp expressing plasmid. 48 h post-transfection, the cells were collected and subjected to WB, blotted with indicated antibodies. N is the nucleocapsid protein antibody. N.D. is not detected. The densitometry of immunoblot bands was determined using Image Studio™ Lite Software (LI-COR Biosciences). The amounts of N protein were normalized with tubulin. Two-tailed unpaired Student's *t*-test was used to analyze the significance; ****P* < 0.001; ns, not significant.

We acknowledge that our findings only briefly described the role of each nsp on viral replication and transcription. The conclusion drawn here based on the replicon system awaits further studies in detail using different systems, such as

recombinant live viruses. Our findings here may provide a new angle to look at the role of various nsps in viral replication, suggesting more essential nsps and associations between nsps for antiviral strategy development.

TABLE 1 | Primers for cloning cleavage sites into Ub4-FL vector.

Primer name	Forward primer (5'–3')	Reverse primer (5'–3')
HindIII-Δnsp2	AGCTTCGTGAGCTTAACGGAGGGGACCAACAAAGGTTA	AGCTTAACCTTTGTTGGTGCCCTCCGTTAAGCTCACGA
HindIII-Δnsp3	AGCTTTTCACACTCAAAGGCGGTAAATTTGTTAATAATA	AGCTTATTATTAACAATTTTACGGCCTTTGAGTGTGAAA
HindIII-Δnsp4	AGCTTAATGTGGCAACTTTACAAAGTGGTTTTAGAAAAA	AGCTTTTTTCTAAACCACTTTGTAAAGTTGCCACATTA
HindIII-Δnsp5	AGCTTACCTCAGCTGTTTTGCAGAGTGCAAGTGAAAAAGAA	AGCTTTCTTTTCACTGCACCTCTGCAAAACAGCTGAGGTA
HindIII-Δnsp6	AGCTTTCAGGTGTTACTTTCCAATCTAAAATGTCAGATA	AGCTTATCTGACATTTTAGATTGGAAGTAACACCTGAA
HindIII-Δnsp7	AGCTTAAAGTAGCCACTGTACAGGCTATAGCCTCAGAGA	AGCTTCTCTGAGGCTATAGCCTGTACAGTGGCTACTTTA
HindIII-Δnsp8	AGCTTAACAGGGCAACCTTACAAAATAATGAGCTTAGTA	AGCTTACTAAGCTCATTATTTTGAAGTTGCCCTGTTA
HindIII-Δnsp9	AGCTTTCTGCTGTCAAATTACAGGCTGGTAATGCAACAA	AGCTTTGTTGCATTACAGCCTGTAATTTGACAGCAGAA
HindIII-Δnsp10	AGCTTGCCACAGTACGCTACAATCAGCTGATGCACAAA	AGCTTTTGTGCATCAGCTGATTGTAGACGTACTGTGGCA
HindIII-Δnsp12	AGCTTCGCGAAGCCATGCTTCAGGCTGTTGGGGCTTGTA	AGCTTACAAGCCCCAACAGCCTGAAGCATGGGTTTCGCGA
HindIII-Δnsp13	AGCTTCGCGATACAGTCTTACAGGCTGAAAATGTAACAA	AGCTTTGTACATTTTCAGCCTGTAAAGCTGTATGCGGA
HindIII-Δnsp14	AGCTTAATGTGGCAACTTTACAAAGTTTAGAAAATGTGA	AGCTTCACATTTTCTAACTTTGTAAAGTTGCCACATTA
HindIII-Δnsp15	AGCTTACTTTTACAAGACTTCAGCTAGTCAAGCGTGGA	AGCTTCCACGCTTGACTAGACTGAAGTCTTGTAAAAGTA

MATERIALS AND METHODS

Cell Culture and Transfection

HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, C11960500), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, A3160801), 100 unit/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific, 15140-122) at 37°C with 5% CO₂.

HEK293T cells were transfected at approximately 60% confluency with various nCoV-Rep vectors using Hieff TransTM Liposomal Transfection Reagent (Yeasen Biotech, Cat#40802ES03) following the manufacturer's instructions. 48 h post-transfection, the cells were subjected to various assays.

Plasmid Construction

The construction of Ub4-nsp1/3-FL to Ub4-nsp14/16-FL is described as follows: First, the oligos of the positive and negative strands (1 µL for each at the concentration of 10 µM, listed in **Table 1**) were phosphorylated with T4 polynucleotide kinase (NEB M0201S) in T4 ligase buffer (NEB M0202S) at 37°C for 30 min. Second, the oligos were denatured for 10 min at 95°C and cooled down slowly (approximately 30 min) to room temperature. Third, the Ub4-FL (Du et al., 2021) was linearized with *HindIII* and purified. Lastly, the annealed oligos were ligated with linearized Ub4-FL at the ratio of 1:10, and the ligation products were transformed into the DH5α component cells. The clones were verified with Sanger DNA sequencing (Tsingke Biotechnology Co., Ltd., Beijing).

The nCoV-Rep was constructed in our previous work (Jin et al., 2021). Considering the vectors' capacity in cloning and ability to stably maintain the foreign DNA fragments, we employed the bacterial artificial chromosome (BAC) vector to clone the full-length cDNA of SARS-CoV-2. So far, six strategies have been successfully applied to construct the reverse genetics systems of coronaviruses: the RNA recombination-based, the vaccinia virus vector-based, the yeast-based recombination system-based, the circular polymerase extension reaction-based,

BAC vector-based, and the *in vitro* ligation-based strategies. Among these strategies, BAC-based and the *in vitro* ligation-based strategies are the most widely used to construct reverse genetics systems of coronaviruses (Wang et al., 2022). Compared with the *in vitro* ligation-based strategy, the BAC-based strategy is more component in constructing the biosafe replicon and performing no live virus-involved quantitative studies on the replication and transcription of viral RNAs. To achieve the expression of viral genomic cDNA sequence in cells, we fused type II promoter CMV with the N-terminus of viral genomic cDNA and installed a transcriptional terminator BGH downstream of the C-terminus of viral genomic cDNA. To obtain the authentic 3' terminus of viral genomic RNA, we inserted the HDV ribozyme between 3' terminus of viral RNA and the BGH terminator. After the viral RNA is transcribed by the CMV promoter, the RNA sequence derived from BGH terminator can be removed through HDV ribozyme-mediated splicing mechanism, and the complete viral RNA is generated.

The constructions of nCoV-Rep-Δnsp1 to nCoV-Rep-Δnsp15 are described as follows. First, two unique restriction sites upstream and downstream of the nsp to be removed in nCoV-Rep (Jin et al., 2021) were selected. The middle forward/reverse primers (**Table 2**) include 3' terminal sequence of the nsp prior to the nsp to be removed, 5' terminal sequence of the nsp after the nsp to be removed, and the sequence generating the cleavage sites for *PLpro* or *3CLpro*. The fragments upstream and downstream of the nsp to be moved were amplified with the primer combinations of the primer upstream of the restriction site and middle reverse primer and that of the primer downstream of the restriction site and middle forward primer, respectively. The two fragments were assembled seamlessly with NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621). The assembled new fragments were amplified with the primers upstream and downstream of the restriction sites and inserted into nCoV-Rep vector to replace WT fragment between the selected two restriction sites. The DH10B component cells were employed for transformation, and the clones were verified with Sanger DNA sequencing (Tsingke Biotechnology Co., Ltd., Beijing).

TABLE 2 | Primers for constructing the fragments with the deletion of each nsp.

Primer name	Forward primer (5'–3')	Reverse primer (5'–3')
Δnsp1	GGTAAGATGGCATACACTCGCTATGTCGATAACAACCTTC	CATAGCGAGTGTATGCCATCTTACCTTTTCGGTCACACCCG
Δnsp2	GCTTAACGGAGGGGACCAACAAAGGTTACTTTTGGTG	GTTGGTGCCCTCCGTTAAGCTCACGC
Δnsp3	CGGTAAATTTGTTAATAATTGTTGAAGCAGTTAATTAAGTTAC	CCAATTATTAACAATTTTACCGCTTTGAGTGTGAAGG
Δnsp4	CAAAGAATGTGGCAACTTTACAAAGTGGTTTTAGAAAAATGGCATTCCC	GTAAAGTTGCCACATTCTTTGTTGTACACATTAAACAATTGTCTAGTAG
Δnsp5	CAGCTGTTTTGCAGAGTGCAGTGAAAGAACAATCAAGGG	CACTGCACTCTGCAAAACAGCTGAGGTGATAGAG
Δnsp6	GTTACTTTCCAATCTAAATGTCAGATGTAAGTGCACATCAGTAG	CTGACATTTTAGATTGGAAAGTAACACCTGAGCATTGTCTAAC
Δnsp7	GCCACTGTACAGGCTATAGCCTCAGAGTTTATGTTCCCTTC	CTGAGGCTATAGCCTGTACAGTGGCTACTTTGATACAAGG
Δnsp8	GGGCAACCTTACAAAATAATGAGCTTAGTCTGTTGCACTAC	GCTCATTATTTTGAAGGTTGCCCTGTTGTCCAG
Δnsp9	GTCAAATTACAGGCTGGTAATGCAACAGAAGTGCC	CATTACCAGCCTGTAATTTGACAGCAGAATTGGCCC
Δnsp10	GTACGTCTACAATCAGCTGATGCACAATCGTTTTTAAACG	GTGCATCAGCTGATTGTAGACGTACTGTGGCAGCTAAAC
Δnsp12	CCATGCTTCAGGCTGTTGGGGCTTGTGTTCTTTG	CAAGCCCCAACAGCCTGAAGCATGGGTTTCGCG
Δnsp13	CAGTCTTACAGGCTGAAAATGTAACAGGACTCTTTAAAGATTGTAG	GTTACATTTTCAGCCTGTAAGACTGTATGCGGTGTGTACATAG
Δnsp14	CTTTACAAAGTTTAGAAAATGTGGCTTTTAATGTTGTAAATAAGG	CATTTTCTAAACTTTGTAAGTTGCCACATTCTACGTG
Δnsp15	CAAGACTTCAGTCTAGTCAAGCGTGGCAACCG	CGCTTGACTAGACTGAAGTCTGTAAAAGTGTTCAGAGG
Δnsp16	CCCAAATTACATAAACAACATCCGCGGGGGC	GATTGTTGCTTTATTGTAATTTGGGTAAATGTTTCTACATGGCC
nsp1-Δ11aa	GTTTCGGATGCTCGAACTGCAGAACTCGAAGGCATTTCAGTACGG	GCCTTCGAGTTCTGCAGTTCGAGCATCCGAAC

The strategy to construct nCoV-Rep-nsp1-Δ11aa was described previously for introducing mutations in nsp12 and nsp14 (Jin et al., 2021). In brief, the segment between *Bsi*WI and *Nhe*I was chosen for mutagenesis. The forward and reverse primers (Table 2) for generating the deletion of 500–532 nt (Δ11aa) were used to amplify the fragments upstream or downstream of the mutant site. The two fragments were assembled seamlessly with NEBuilder HiFi DNA Assembly Master Mix. The assembled products were amplified by PCR and used to replace the wild-type *Bsi*WI-*Nhe*I segment.

The construction of nCoV-Rep-Δnsp16 is relatively straightforward compared with other nsp deletion mutants. The *Axy*I-*Sac*II fragment without nsp16 was inserted into nCoV-Rep vector to replace WT fragment between *Axy*I and *Sac*II sites.

The construction of LPC-nsp1 to LPC-nsp16 is referred to as in our previous work (Jiang et al., 2021).

Dual-Luciferase Reporter Assay

One day prior to transfection, HEK293T cells (1×10^5) were plated in 24-well plates. Various Ub4-CS-FL (0.5 μg), RL-TK (0.1 μg), and LPC-3CLpro-HA or LPC-PLpro-HA were transfected into the cells using Hieff TransTM Liposomal Transfection Reagent. Forty eight hour post-transfection, the cells were lysed in 50 μL 1x Passive Lysis Buffer (PLB, Promega, E1941). The activities of firefly and renilla luciferase were measured using Dual-Glo[®] Luciferase Assay System to determine the relative luciferase activities.

RNA Extraction, Real-Time Quantitative PCR, and RT-PCR

Total RNA was isolated from the HEK293T cells using TRIzol reagent (Thermo Fisher Scientific, Cat#15596026) and treated with RNase-free DNase (Takara, Dalian, China, EN0521)

according to the manufacturer's instructions. The quality of RNA was examined using the electrophoresis on 1% agarose gel, and the purity of RNA was verified on the basis of the ratio of OD260/280 on NanoDrop One (Thermo Scientific). Two μg of total RNA was used as a template to synthesize cDNA using the cDNA synthesis kit (Thermo Fisher Scientific, Cat#K1622). The cDNAs were subjected to the real-time quantitative PCR (qPCR), which were performed on a 96TM Real Time PCR Detection System (Applied BiosystemsTM 7500), in 10 μL reaction mixtures containing 5 μL SYBR[®] TB Green[®] Premix Ex TaqTM (Tli RNaseH Plus) (Takara, Dalian, China, Cat#RR420A). The thermal profile consists of 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 5 s at 60°C. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression values. The details for the design of primers and qPCR conditions were described as previously (Jin et al., 2021). The RT-PCR was performed with 2 × Hieff[®] PCR Master Mix (Yeasen, Shanghai, China, 10102ES08), extension time of PCR program is 1 min, and PCR products were examined in the 1.5% agarose gel after DNA electrophoresis.

Western Blot

Cell samples were collected and lysed in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA, and 50 mM Tris-HCl pH7.4, supplemented with cCompleteTM Protease Inhibitor Cocktail). All samples' concentration was quantified with BCA assay (Thermo Fisher Scientific, 23227) following the manufacturer's instructions. The lysates were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated in PBS containing 0.05% Tween 20 and 5% non-fat milk (blocking buffer) for 1 h at room temperature and then left in the blocking buffer containing primary antibody of HA (Proteintech, 10011878), His (Proteintech, 10004365), Flag (Proteintech, 00098867), Tubulin (Proteintech, 66031-1-Ig), or

Nucleocapsid (Sino Biological, 40143-R019) at 4°C overnight. The following day, after being washed with PBS containing 0.05% Tween 20, the membranes were incubated with appropriate secondary antibody (Goat anti-Rabbit, LI-COR, D10121-05; Goat anti-Mouse, LI-COR, D10217-05, at 1:10,000). The final blots were developed on Odyssey CLx Imaging System (LI-COR Biosciences).

Statistics

Except for specially stated, all the experiments were performed at least three times. The data analyses were finished using Student's *t*-test of SPSS (version 21.0; SPSS Inc., Chicago, IL). When *P*-value was less than 0.05, the results were considered significant.

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

XP and J-AP conceived the ideas, designed the experiments, and wrote the manuscript. All authors performed experiments or data analysis, read, and approved the final manuscript.

REFERENCES

- Acheampong, K. K., Schaff, D. L., Emert, B. L., Lake, J., Reffsin, S., Shea, E. K., et al. (2022). Subcellular detection of SARS-CoV-2 RNA in human tissue reveals distinct localization in alveolar Type 2 pneumocytes and alveolar macrophages. *mBio* 13:e0375121. doi: 10.1128/mbio.03751-21
- Almazan, F., Dediego, M. L., Galan, C., Escors, D., Alvarez, E., Ortego, J., et al. (2006). Construction of a severe acute respiratory syndrome coronavirus infectious cDNA clone and a replicon to study coronavirus RNA synthesis. *J. Virol.* 80, 10900–10906. doi: 10.1128/JVI.00385-06
- Angelini, M. M., Akhlaghpour, M., Neuman, B. W., and Buchmeier, M. J. (2013). Severe acute respiratory syndrome coronavirus nonstructural proteins 3, 4, and 6 induce double-membrane vesicles. *mBio* 4, e524–e513. doi: 10.1128/mBio.00524-13
- Baez-Santos, Y. M., St John, S. E., and Mesecar, A. D. (2015). The SARS-coronavirus papain-like protease: structure, function and inhibition by designed antiviral compounds. *Antiviral Res.* 115, 21–38. doi: 10.1016/j.antiviral.2014.12.015
- Becares, M., Pascual-Iglesias, A., Nogales, A., Sola, I., Enjuanes, L., and Zuniga, S. (2016). Mutagenesis of coronavirus nsp14 reveals its potential role in modulation of the innate immune response. *J. Virol.* 90, 5399–5414. doi: 10.1128/JVI.03259-15
- Benedetti, F., Snyder, G. A., Giovanetti, M., Angeletti, S., Gallo, R. C., Ciccozzi, M., et al. (2020). Emerging of a SARS-CoV-2 viral strain with a deletion in nsp1. *J. Transl. Med.* 18:329. doi: 10.1186/s12967-020-02507-5
- Brockway, S. M., and Denison, M. R. (2005). Mutagenesis of the murine hepatitis virus nsp1-coding region identifies residues important for protein processing, viral RNA synthesis, and viral replication. *Virology* 340, 209–223. doi: 10.1016/j.virology.2005.06.035
- Chen, Y., Cai, H., Pan, J., Xiang, N., Tien, P., Ahola, T., et al. (2009). Functional screen reveals SARS coronavirus nonstructural protein nsp14 as a novel cap N7 methyltransferase. *Proc. Natl. Acad. Sci. U.S.A.* 106, 3484–3489. doi: 10.1073/pnas.0808790106

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.907422/full#supplementary-material>

Supplementary Figure 1 | Design of each nsp deletion. Each nsp was deleted by fusing its adjacent upstream and adjacent downstream nsps, except nsp1 and nsp16. The new cleavage site sequences were depicted. The deletion of nsp1 and nsp16 left a new start codon before nsp2 and a stop codon after nsp15, respectively.

Supplementary Figure 2 | Sanger sequencing results for nCoV-Rep-Δnsps. The Sanger sequencing results covering the newly formed cleavage site regions in various nCoV-Rep-Δnsps were depicted.

Supplementary Figure 3 | Investigation of *in trans*-rescue function of various nsps in the viral replication by quantifying the amount the N (related to Figure 4). (A–O) The RNA samples described in Figures 4A–O were subjected to real-time quantitative PCR assay. The amounts of N subgenomic RNA (sgRNA) from various samples were normalized with internal control GAPDH.

- Chen, Y., Liu, Q., and Guo, D. (2020). Emerging coronaviruses: genome structure, replication, and pathogenesis. *J. Med. Virol.* 92, 418–423.
- Chen, Y., Su, C., Ke, M., Jin, X., Xu, L., Zhang, Z., et al. (2011). Biochemical and structural insights into the mechanisms of SARS coronavirus RNA ribose 2'-O-methylation by nsp16/nsp10 protein complex. *PLoS Pathog.* 7:e1002294. doi: 10.1371/journal.ppat.1002294
- Choi, J., Indrisunaite, G., DeMirci, H., Jeong, K. W., Wang, J., Petrov, A., et al. (2018). 2'-O-methylation in mRNA disrupts tRNA decoding during translation elongation. *Nat. Struct. Mol. Biol.* 25, 208–216. doi: 10.1038/s41594-018-0030-z
- Clementz, M. A., Chen, Z., Banach, B. S., Wang, Y., Sun, L., Ratia, K., et al. (2010). Deubiquitinating and interferon antagonism activities of coronavirus papain-like proteases. *J. Virol.* 84, 4619–4629. doi: 10.1128/JVI.02406-09
- Cornillez-Ty, C. T., Liao, L., Yates, J. R. III, Kuhn, P., and Buchmeier, M. J. (2009). Severe acute respiratory syndrome coronavirus nonstructural protein 2 interacts with a host protein complex involved in mitochondrial biogenesis and intracellular signaling. *J. Virol.* 83, 10314–10318. doi: 10.1128/JVI.00842-09
- Coronaviridae Study Group of the International Committee on Taxonomy of Viruses (2020). The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat. Microbiol.* 5, 536–544. doi: 10.1038/s41564-020-0695-z
- Cortese, M., Lee, J. Y., Cerikan, B., Neufeldt, C. J., Oorschot, V. M. J., Kohrer, S., et al. (2020). Integrative imaging reveals SARS-CoV-2-induced reshaping of subcellular morphologies. *Cell Host Microbe* 28, 853–866e5. doi: 10.1016/j.chom.2020.11.003
- Cottam, E. M., Whelband, M. C., and Wileman, T. (2014). Coronavirus NSP6 restricts autophagosome expansion. *Autophagy* 10, 1426–1441. doi: 10.4161/auto.29309
- de, O. A. J., Pinheiro, S., Zamora, W. J., Alves, C. N., Lameira, J., and Lima, A. H. (2021). Structural, energetic and lipophilic analysis of SARS-CoV-2 non-structural protein 9 (NSP9). *Sci. Rep.* 11:23003. doi: 10.1038/s41598-021-02366-0
- Deming, D. J., Graham, R. L., Denison, M. R., and Baric, R. S. (2007). Processing of open reading frame 1a replicase proteins nsp7 to nsp10 in murine hepatitis virus strain A59 replication. *J. Virol.* 81, 10280–10291. doi: 10.1128/JVI.00017-07

- Du, L., Xie, Y., Zheng, K., Wang, N., Gao, M., Yu, T., et al. (2021). Oxidative stress transforms 3CLpro into an insoluble and more active form to promote SARS-CoV-2 replication. *Redox Biol.* 48:102199. doi: 10.1016/j.redox.2021.102199
- Feng, W., Zong, W., Wang, F., and Ju, S. (2020). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2): a review. *Mol. Cancer* 19:100.
- Frazier, M. N., Dillard, L. B., Krahn, J. M., Perera, L., Williams, J. G., Wilson, I. M., et al. (2021). Characterization of SARS2 Nsp15 nuclease activity reveals it's mad about U. *Nucleic Acids Res.* 49, 10136–10149. doi: 10.1093/nar/gkab719
- Gadhav, K., Kumar, P., Kumar, A., Bhardwaj, T., Garg, N., and Giri, R. (2021). Conformational dynamics of 13 amino acids long NSP11 of SARS-CoV-2 under membrane mimetics and different solvent conditions. *Microb. Pathog.* 158:105041. doi: 10.1016/j.micpath.2021.105041
- Graham, R. L., Sims, A. C., Brockway, S. M., Baric, R. S., and Denison, M. R. (2005). The nsp2 replicase proteins of murine hepatitis virus and severe acute respiratory syndrome coronavirus are dispensable for viral replication. *J. Virol.* 79, 13399–13411. doi: 10.1128/JVI.79.21.13399-13411.2005
- Hoernes, T. P., Clementi, N., Faserl, K., Glasner, H., Breuker, K., Lindner, H., et al. (2016). Nucleotide modifications within bacterial messenger RNAs regulate their translation and are able to rewire the genetic code. *Nucleic Acids Res.* 44, 852–862. doi: 10.1093/nar/gkv1182
- Huang, C., Lokugamage, K. G., Rozovics, J. M., Narayanan, K., Semler, B. L., and Makino, S. (2011). SARS coronavirus nsp1 protein induces template-dependent endonucleolytic cleavage of mRNAs: viral mRNAs are resistant to nsp1-induced RNA cleavage. *PLoS Pathog.* 7:e1002433. doi: 10.1371/journal.ppat.1002433
- Hussain, S., Pan, J., Chen, Y., Yang, Y., Xu, J., Peng, Y., et al. (2005). Identification of novel subgenomic RNAs and noncanonical transcription initiation signals of severe acute respiratory syndrome coronavirus. *J. Virol.* 79, 5288–5295. doi: 10.1128/JVI.79.9.5288-5295.2005
- Ivanov, K. A., Hertzog, T., Rozanov, M., Bayer, S., Thiel, V., Gorbalenya, A. E., et al. (2004). Major genetic marker of nidoviruses encodes a replicative endoribonuclease. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12694–12699. doi: 10.1073/pnas.0403127101
- Jang, K. J., Jeong, S., Kang, D. Y., Sp, N., Yang, Y. M., and Kim, D. E. (2020). A high ATP concentration enhances the cooperative translocation of the SARS coronavirus helicase nsp13 in the unwinding of duplex RNA. *Sci. Rep.* 10:4481. doi: 10.1038/s41598-020-61432-1
- Jiang, Y., Tong, K., Yao, R., Zhou, Y., Lin, H., Du, L., et al. (2021). Genome-wide analysis of protein-protein interactions and involvement of viral proteins in SARS-CoV-2 replication. *Cell Biosci.* 11:140. doi: 10.1186/s13578-021-00644-y
- Jin, Y. Y., Lin, H., Cao, L., Wu, W. C., Ji, Y., Du, L., et al. (2021). A convenient and biosafe replicon with accessory genes of SARS-CoV-2 and its potential application in antiviral drug discovery. *Virol. Sin.* 36, 913–923. doi: 10.1007/s12250-021-00385-9
- Jin, Z., Du, X., Xu, Y., Deng, Y., Liu, M., Zhao, Y., et al. (2020). Structure of M(pro) from SARS-CoV-2 and discovery of its inhibitors. *Nature* 582, 289–293.
- Kamitani, W., Huang, C., Narayanan, K., Lokugamage, K. G., and Makino, S. (2009). A two-pronged strategy to suppress host protein synthesis by SARS coronavirus Nsp1 protein. *Nat. Struct. Mol. Biol.* 16, 1134–1140. doi: 10.1038/nsmb.1680
- Kamitani, W., Narayanan, K., Huang, C., Lokugamage, K., Ikegami, T., Ito, N., et al. (2006). Severe acute respiratory syndrome coronavirus nsp1 protein suppresses host gene expression by promoting host mRNA degradation. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12885–12890. doi: 10.1073/pnas.0603144103
- Kang, H., Bhardwaj, K., Li, Y., Palaninathan, S., Sacchetti, J., Guarino, L., et al. (2007). Biochemical and genetic analyses of murine hepatitis virus Nsp15 endoribonuclease. *J. Virol.* 81, 13587–13597. doi: 10.1128/JVI.00547-07
- Kirchdoerfer, R. N., and Ward, A. B. (2019). Structure of the SARS-CoV nsp12 polymerase bound to nsp7 and nsp8 co-factors. *Nat. Commun.* 10:2342. doi: 10.1038/s41467-019-10280-3
- Krafickova, P., Silhan, J., Nencka, R., and Boura, E. (2020). Structural analysis of the SARS-CoV-2 methyltransferase complex involved in RNA cap creation bound to sinefungin. *Nat. Commun.* 11:3717. doi: 10.1038/s41467-020-17495-9
- Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., et al. (2020). Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature* 581, 215–220. doi: 10.1038/s41586-020-2180-5
- Law, A. H., Lee, D. C., Cheung, B. K., Yim, H. C., and Lau, A. S. (2007). Role for nonstructural protein 1 of severe acute respiratory syndrome coronavirus in chemokine dysregulation. *J. Virol.* 81, 416–422. doi: 10.1128/JVI.02336-05
- Lei, X., Dong, X., Ma, R., Wang, W., Xiao, X., Tian, Z., et al. (2020). Activation and evasion of type I interferon responses by SARS-CoV-2. *Nat. Commun.* 11:3810. doi: 10.1038/s41467-020-17665-9
- Lin, J. W., Tang, C., Wei, H. C., Du, B., Chen, C., Wang, M., et al. (2021b). Genomic monitoring of SARS-CoV-2 uncovers an Nsp1 deletion variant that modulates type I interferon response. *Cell Host Microbe.* 29, 489–502e8. doi: 10.1016/j.chom.2021.01.015
- Lin, S., Chen, H., Chen, Z., Yang, F., Ye, F., Zheng, Y., et al. (2021a). Crystal structure of SARS-CoV-2 nsp10 bound to nsp14-ExoN domain reveals an exonuclease with both structural and functional integrity. *Nucleic Acids Res.* 49, 5382–5392. doi: 10.1093/nar/gkab320
- Littler, D. R., Gully, B. S., Colson, R. N., and Rossjohn, J. (2020). Crystal structure of the SARS-CoV-2 non-structural protein 9, Nsp9. *iScience* 23:101258. doi: 10.1016/j.isci.2020.101258
- Lokugamage, K. G., Narayanan, K., Huang, C., and Makino, S. (2012). Severe acute respiratory syndrome coronavirus protein nsp1 is a novel eukaryotic translation inhibitor that represses multiple steps of translation initiation. *J. Virol.* 86, 13598–13608. doi: 10.1128/JVI.01958-12
- Luo, Y., Yu, F., Zhou, M., Liu, Y., Xia, B., Zhang, X., et al. (2021). Engineering a reliable and convenient SARS-CoV-2 replicon system for analysis of viral RNA synthesis and screening of antiviral inhibitors. *mBio* 12, e2754–e2720. doi: 10.1128/mBio.02754-20
- Ma, Y., Wu, L., Shaw, N., Gao, Y., Wang, J., Sun, Y., et al. (2015). Structural basis and functional analysis of the SARS coronavirus nsp14-nsp10 complex. *Proc. Natl. Acad. Sci. U.S.A.* 112, 9436–9441. doi: 10.1073/pnas.1508686112
- Malone, B., Urakova, N., Snijder, E. J., and Campbell, E. A. (2022). Structures and functions of coronavirus replication-transcription complexes and their relevance for SARS-CoV-2 drug design. *Nat. Rev. Mol. Cell Biol.* 23, 21–39. doi: 10.1038/s41580-021-00432-z
- Mariano, G., Farthing, R. J., Lale-Farjat, S. L. M., and Bergeron, J. R. C. (2020). Structural characterization of SARS-CoV-2: where we are, and where we need to be. *Front. Mol. Biosci.* 7:605236. doi: 10.3389/fmolb.2020.605236
- Narayanan, K., Huang, C., and Makino, S. (2008b). SARS coronavirus accessory proteins. *Virus Res.* 133, 113–121.
- Narayanan, K., Huang, C., Lokugamage, K., Kamitani, W., Ikegami, T., Tseng, C. T., et al. (2008a). Severe acute respiratory syndrome coronavirus nsp1 suppresses host gene expression, including that of type I interferon, in infected cells. *J. Virol.* 82, 4471–4479. doi: 10.1128/JVI.02472-07
- Nguyen, H. T., Falzarano, D., Gerdt, V., and Liu, Q. (2021). Construction of a noninfectious SARS-CoV-2 replicon for antiviral-drug testing and gene function studies. *J. Virol.* 95:e0068721. doi: 10.1128/JVI.00687-21
- Pan, J., Peng, X., Gao, Y., Li, Z., Lu, X., Chen, Y., et al. (2008). Genome-wide analysis of protein-protein interactions and involvement of viral proteins in SARS-CoV replication. *PLoS One* 3:e3299. doi: 10.1371/journal.pone.0003299
- Peng, Q., Peng, R., Yuan, B., Zhao, J., Wang, M., Wang, X., et al. (2020). Structural and biochemical characterization of the nsp12-nsp7-nsp8 core polymerase complex from SARS-CoV-2. *Cell Rep.* 31:107774. doi: 10.1016/j.celrep.2020.107774
- Pohl, M. O., Busnadiego, I., Kufner, V., Glas, I., Karakus, U., Schmutz, S., et al. (2021). SARS-CoV-2 variants reveal features critical for replication in primary human cells. *PLoS Biol.* 19:e3001006. doi: 10.1371/journal.pbio.3001006
- Rohaim, M. A., El Nagggar, R. F., Clayton, E., and Munir, M. (2021). Structural and functional insights into non-structural proteins of coronaviruses. *Microb. Pathog.* 150:104641. doi: 10.1016/j.micpath.2020.104641
- Sakai, Y., Kawachi, K., Terada, Y., Omori, H., Matsuura, Y., and Kamitani, W. (2017). Two-amino acids change in the nsp4 of SARS coronavirus abolishes viral replication. *Virology* 510, 165–174. doi: 10.1016/j.virol.2017.07.019
- Shin, D., Mukherjee, R., Grewe, D., Bojkova, D., Baek, K., Bhattacharya, A., et al. (2020). Papain-like protease regulates SARS-CoV-2 viral spread and innate immunity. *Nature* 587, 657–662. doi: 10.1038/s41586-020-2601-5
- Shu, T., Huang, M., Wu, D., Ren, Y., Zhang, X., Han, Y., et al. (2020). SARS-coronavirus-2 Nsp13 possesses NTPase and RNA helicase activities that can be inhibited by bismuth salts. *Virol. Sin.* 35, 321–329. doi: 10.1007/s12250-020-00242-1

- Slanina, H., Madhugiri, R., Bylapudi, G., Schultheiss, K., Karl, N., Gulyaeva, A., et al. (2021). Coronavirus replication-transcription complex: vital and selective NMPylation of a conserved site in nsp9 by the NiRAN-RdRp subunit. *Proc. Natl. Acad. Sci. U.S.A.* 118:e2022310118. doi: 10.1073/pnas.2022310118
- van Hemert, M. J., van den Worm, S. H., Knoops, K., Mommaas, A. M., Gorbalenya, A. E., and Snijder, E. J. (2008). SARS-coronavirus replication/transcription complexes are membrane-protected and need a host factor for activity in vitro. *PLoS Pathog.* 4:e1000054. doi: 10.1371/journal.ppat.1000054
- V'Kovski, P., Kratzel, A., Steiner, S., Stalder, H., and Thiel, V. (2021). Coronavirus biology and replication: implications for SARS-CoV-2. *Nat. Rev. Microbiol.* 19, 155–170. doi: 10.1038/s41579-020-00468-6
- Walker, A. P., Fan, H., Keown, J. R., Knight, M. L., Grimes, J. M., and Fodor, E. (2021). The SARS-CoV-2 RNA polymerase is a viral RNA capping enzyme. *Nucleic Acids Res.* 49, 13019–13030. doi: 10.1093/nar/gkab1160
- Wang, Q., Wu, J., Wang, H., Gao, Y., Liu, Q., Mu, A., et al. (2020). Structural basis for RNA replication by the SARS-CoV-2 polymerase. *Cell* 182, 417–428e13. doi: 10.1016/j.cell.2020.05.034
- Wang, R., Chen, J., Gao, K., Hozumi, Y., Yin, C., and Wei, G. W. (2021). Author correction: analysis of SARS-CoV-2 mutations in the United States suggests presence of four substrains and novel variants. *Commun. Biol.* 4:311. doi: 10.1038/s42003-021-01867-y
- Wang, W., Peng, X., Jin, Y., Pan, J. A., and Guo, D. (2022). Reverse genetics systems for SARS-CoV-2. *J. Med. Virol.* 94. doi: 10.1002/jmv.27738
- Wilamowski, M., Sherrell, D. A., Minasov, G., Kim, Y., Shuvalova, L., Lavens, A., et al. (2021). 2'-O methylation of RNA cap in SARS-CoV-2 captured by serial crystallography. *Proc. Natl. Acad. Sci. U.S.A.* 118:e2100170118. doi: 10.1073/pnas.2100170118
- Yan, F., and Gao, F. (2021). An overview of potential inhibitors targeting non-structural proteins 3 (PL(pro) and Mac1) and 5 (3CL(pro)/M(pro)) of SARS-CoV-2. *Comput. Struct. Biotechnol. J.* 19, 4868–4883. doi: 10.1016/j.csbj.2021.08.036
- Yin, W., Xu, Y., Xu, P., Cao, X., Wu, C., Gu, C., et al. (2022). Structures of the omicron spike trimer with ACE2 and an anti-omicron antibody. *Science* 375, 1048–1053. doi: 10.1126/science.abn8863
- Zekri, A. N., Mohanad, M., Hafez, M. M., Soliman, H. K., Hassan, Z. K., Abouelhoda, M., et al. (2021). Genome sequencing of SARS-CoV-2 in a cohort of Egyptian patients revealed mutation hotspots that are related to clinical outcomes. *Biochim. Biophys. Acta Mol. Basis Dis.* 1867:166154. doi: 10.1016/j.bbdis.2021.166154
- Zust, R., Cervantes-Barragan, L., Habjan, M., Maier, R., Neuman, B. W., Ziebuhr, J., et al. (2011). Ribose 2'-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. *Nat. Immunol.* 12, 137–143.
- Zust, R., Cervantes-Barragan, L., Kuri, T., Blakqori, G., Weber, F., Ludewig, B., et al. (2007). Coronavirus non-structural protein 1 is a major pathogenicity factor: implications for the rational design of coronavirus vaccines. *PLoS Pathog.* 3:e109. doi: 10.1371/journal.ppat.0030109

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SARS-CoV-2 RNA in Wastewater Was Highly Correlated With the Number of COVID-19 Cases During the Fourth and Fifth Pandemic Wave in Kobe City, Japan

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of the current coronavirus disease 2019 (COVID-19) pandemic and associated respiratory infections, has been detected in the feces of patients. Therefore, determining SARS-CoV-2 RNA levels in sewage may help to predict the number of infected people within the area. In this study, we quantified SARS-CoV-2 RNA copy number using reverse transcription quantitative real-time PCR with primers and probes targeting the N gene, which allows the detection of both wild-type and variant strain of SARS-CoV-2 in sewage samples from two wastewater treatment plants (WWTPs) in Kobe City, Japan, during the fourth and fifth pandemic waves of COVID-19 between February 2021 and October 2021. The wastewater samples were concentrated *via* centrifugation, yielding a pelleted solid fraction and a supernatant, which was subjected to polyethylene glycol (PEG) precipitation. The SARS-CoV-2 RNA was significantly and frequently detected in the solid fraction than in the PEG-precipitated fraction. In addition, the copy number in the solid fraction was highly correlated with the number of COVID-19 cases in the WWTP basin (WWTP-A: $r=0.8205$, $p<0.001$; WWTP-B: $r=0.8482$, $p<0.001$). The limit of capturing COVID-19 cases per 100,000 people was 0.75 cases in WWTP-A and 1.20 cases in WWTP-B, respectively. Quantitative studies of RNA in sewage can be useful for administrative purposes related to public health, including issuing warnings and implementing preventive measures within sewage basins.

Keywords: COVID-19, SARS-CoV-2, sewage, wastewater, environmental surveillance

INTRODUCTION

The coronavirus disease 2019 (COVID-19) outbreak, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), started in December 2019 and then spread worldwide in 2020 (Huang et al., 2020). In Japan, the COVID-19 pandemic can be divided into five major waves as of December 2021 (Worldometer, 2020). COVID-19 infections occurred mainly

due to alpha and delta variants in the fourth (March to June 2021) and fifth (August to October 2021) waves, respectively (Hodcroft, 2021). SARS-CoV-2 mainly causes symptoms of upper respiratory tract infections, but it may also cause severe pneumonia including acute respiratory distress syndrome (ARDS; Wu et al., 2020). Because SARS-CoV-2 can also infect the digestive organs, high levels of the virus can be detected in the feces of infected individuals (Lescure et al., 2020). Stool samples from 48.1% of patients tested positive for SARS-CoV-2 RNA and 70.3% of these patients had stool viral RNA that remained positive even when respiratory specimens were negative, as shown in a meta-analysis study (Cheung et al., 2020). Thus, SARS-CoV-2 is not only a case of respiratory distress, but is also one of the most important causative agents of human gastroenteritis.

Since municipal wastewater contains microorganisms derived from human feces, the concentration of pathogens in sewage is affected by infectious disease epidemics, mainly gastroenteritis, in the watershed population. For example, a previous study showed that the RNA copy number of human gastroenteric norovirus in sewage was significantly related to the number of gastroenteritis cases in the wastewater treatment plant (WWTP) basin (Kazama et al., 2017). In addition, hepatitis E virus was detectable in raw sewage when 1%–4% of residents in a WWTP basin were infected (Miura et al., 2016). Monitoring the prevalence of SARS-CoV-2 in wastewater is also considered to be an effective approach for predicting the COVID-19 epidemic following the detection of SARS-CoV-2 RNA in sewage in several countries (Ahmed et al., 2020a; La Rosa et al., 2020b; Wang et al., 2020; Wurtzer et al., 2020, 2022; Albastaki et al., 2021; Wehrendt et al., 2021; Kevill et al., 2022). In some of these studies, the RNA copy number of SARS-CoV-2 in the sewage was correlated with the number of COVID-19 clinical cases (Medema et al., 2020a; Carrillo-Reyes et al., 2021; Nagarkar et al., 2021; Street et al., 2021; Wurtz et al., 2021; Monteiro et al., 2022). In Japan, SARS-CoV-2 RNA was first detected in secondary treated wastewater before chlorination at a WWTP in Yamanashi Prefecture in April 2020 (Haramoto et al., 2020). Although experiments to detect SARS-CoV-2 RNA have been carried out on wastewater in other regions in Japan for 1–3 months (Hata et al., 2021; Kitamura et al., 2021; Nagashima et al., 2021; Torii et al., 2021), few studies have reported the relationship between the number of COVID-19 cases and the amount of RNA detected with long-term monitoring.

Owing to the low concentration of pathogenic viruses in wastewater, a method for concentrating and detecting these viruses is necessary (Haramoto et al., 2018). Various methods aimed at concentrating RNA in environmental samples to improve detection have been evaluated (Ahmed et al., 2020b; Weidhaas et al., 2021). Among them, electronegative membrane adsorption, polyethylene glycol (PEG) precipitation, and ultrafiltration are frequently used to detect non-enveloped viruses such as poliovirus and norovirus (World Health Organization, 2003; Kazama et al., 2017). Several studies have employed these methods to enrich SARS-CoV-2 RNA (Foladori et al., 2020; La Rosa et al., 2020a; Sangkham, 2021); however,

since SARS-CoV-2 is an enveloped virus, the concentration efficiency of the virus differs from that of non-enveloped viruses. Recently, comparative studies of extraction methods showed that SARS-CoV-2 RNA was more abundantly detected in the solid fraction of sewage samples, i.e., the pellet obtained by centrifugation of sewage samples (Kitamura et al., 2021; Westhaus et al., 2021). On the other hand, another study reported that approximately 90% of the SARS-CoV-2 RNA was present in the liquid phase of the influent wastewater (Weidhaas et al., 2021). The assessment of RNA concentration from sewage solids is important, even though solid residues are largely removed in studies on wastewater treatment.

Evaluating the efficiency of the process after wastewater concentration requires the use of a control virus. Pepper mild mottle virus (PMMoV) is the most abundant virus in human feces (Zhang et al., 2006), owing to which it can be easily quantified without spiking in a wastewater sample. High concentrations of PMMoV have been detected in water environment (Rosario et al., 2009; Haramoto et al., 2013; Hughes et al., 2017), and this virus has been used as an internal control for virus detection in wastewater in several studies (D'Aoust et al., 2021; Gerrity et al., 2021; Rosiles-González et al., 2021).

In the present study, we examined the pelleted solid fraction and the product of PEG precipitation of the supernatant fraction of wastewater samples for SARS-CoV-2 RNA. Wastewater was collected once a week from two WWTPs in Kobe, Japan, during the fourth and fifth pandemic waves of COVID-19, and the relationship between the SARS-CoV-2 RNA copy number in the two sample types was correlated with the reported number of COVID-19 cases in the corresponding sewage basin.

MATERIALS AND METHODS

Sample Collection

Influent wastewater samples were collected once a week from 24 February to 27 October 2021 at WWTP-A ($n=36$) and WWTP-B ($n=36$) in Kobe City, Japan. The samples were grabbed from the influent, which comprised wastewater before treatment at the WWTPs. All sampling was performed at a fixed time every Wednesday, except on May 6 (WWTP-A and WWTP-B) and August 12 (WWTP-B), in which samples were collected on a Thursday. The samples were collected in sterile plastic bottles and kept frozen at -20°C until analysis. As of December 2021, the city had 1,515,907 inhabitants, of which 98.7% were covered by six WWTPs. WWTP-A and WWTP-B covered 51.5% of the population, received 51.3% of the total wastewater, and treated a total flow of $364,100\text{ m}^3$ per day. The amounts of rainfall (mm/day) and influent flow (m^3/day) were measured as routine work at each WWTP.

RNA Extraction

Viral RNA was extracted from each sewage sample after centrifugation, to produce a solid fraction, and after PEG precipitation of the supernatant, to produce a PEG-precipitated fraction, following the procedures of previous studies with minor modifications (Jones and Johns, 2009; Kitamura et al., 2021).

Specifically, 160 ml of each sample was divided equally into four aliquots (40 ml each) held in 50 ml tubes and centrifuged at $10,000 \times g$ for 30 min. RNA was extracted from the resulting pellet (solid fraction sample) using the NucleoBond RNA Soil kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Meanwhile, the entire supernatant was precipitated using PEG 8000 (final concentration 10%; Promega, Madison, WI, United States) and NaCl (final concentration 1 M; Wako, Tokyo, Japan) by incubating at 4°C overnight with gentle rotation. After centrifugation at $10,000 \times g$ for 60 min, the precipitate was resuspended in 500 μl of phosphate buffer solution (pH 7.0, 0.067 mol/L; Nacalai Tesque, Kyoto, Japan). RNA was extracted from 140 μl of the PEG-precipitated suspension using a QIAamp Viral RNA Kit (Qiagen, Hilden, Germany). RNA was also extracted from 140 μl of raw unconcentrated sewage samples using a QIAamp Viral RNA Kit (Qiagen).

Reverse Transcription-Quantitative PCR

To quantify viral RNA in the samples, reverse transcription-quantitative PCR (RT-qPCR) was performed using the Thermal Cycler Dice Real Time System III (Takara Bio, Shiga, Japan). SARS-CoV-2 RNA was quantified in the solid fraction, the PEG-precipitated sample, and unconcentrated sewage samples using the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, Foster City, CA, United States) with combination of CDC 2019-nCoV_N1 and CDC 2019-nCoV_N2 primers and probes, which can be used to detect both the wild type and variant strains. The primer sequences used are described in **Supplementary Table S1**. Thermal cycling conditions included an initial incubation at 50°C for 5 min and initial denaturation at 95°C for 20 s, followed by 45 cycles of denaturation at 95°C for 3 s and annealing and extension at 60°C for 30 s, as per the manufacturer's instructions. PMMoV RNA was also quantified in the same samples using the One Step PrimeScript III RT-qPCR Mix (Takara Bio). Thermal cycling conditions for PMMoV included an initial incubation at 52°C for 5 min and initial denaturation at 95°C for 10 s, followed by 45 cycles of denaturation at 95°C for 5 s and annealing and extension at 60°C for 30 s, as per the manufacturer's instructions. All RT-qPCR analyses included both positive (standard DNA/RNA) and negative (water) controls. The analysis of SARS-CoV-2 samples was performed in duplicate; samples in which only one of the reactions showed a positive amplification were considered as negative overall. To obtain a standard curve for each assay, 10-fold dilution series of a standard plasmid DNA (PMMoV; 5×10^3 – 5×10^6 ; Haramoto et al., 2013) or RNA (2.5×10^0 and 5×10^0 – 5×10^4 ; SARS-CoV-2 RNA positive control; Takara Bio) solutions were prepared for each assay. RNA copy numbers were calculated from the Ct values using the standard curves. The limit of quantification for SARS-CoV-2 was set at 2.5 copies/reaction (**Supplementary Figure S1**).

Calculation of Viral RNA Copy Number and Recovery Rate

The copy number of the viral RNA calculated using RT-qPCR was corrected to copy/L as previously described (Qiu et al., 2022), as follows:

RNA copy number (copy / L)

$$= \left(\begin{array}{l} \text{RNA copy number (copy / reaction)} \\ \times \frac{V_{\text{extracted RNA}}}{V_{\text{RNA in each PCR reaction}}} \\ \times \frac{V_{\text{wastewater concentrate}}}{V_{\text{wastewater concentrate for RNA extraction}}} \times \frac{1000}{V_{\text{initial wastewater}}} \end{array} \right)$$

where $V_{\text{extracted RNA}}$ is the total volume of the extracted RNA, $V_{\text{RNA in each PCR reaction}}$ is the volume of RNA assayed in a RT-PCR reaction, $V_{\text{wastewater concentrate}}$ is the sample volume after concentration, $V_{\text{wastewater concentrate for RNA extractions}}$ is the volume of wastewater concentrate used for RNA extraction, and $V_{\text{initial wastewater}}$ is the volume of initial wastewater sample processed.

Meanwhile, the recovery rate (%) was calculated using PMMoV quantitative value as follows:

Recovery rate (%)

$$= \frac{\text{PMMoV RNA copy number of concentrated sample (copy / L)}}{\text{PMMoV RNA copy number of unconcentrated sample (copy / L)}} \times 100$$

Statistical Analysis

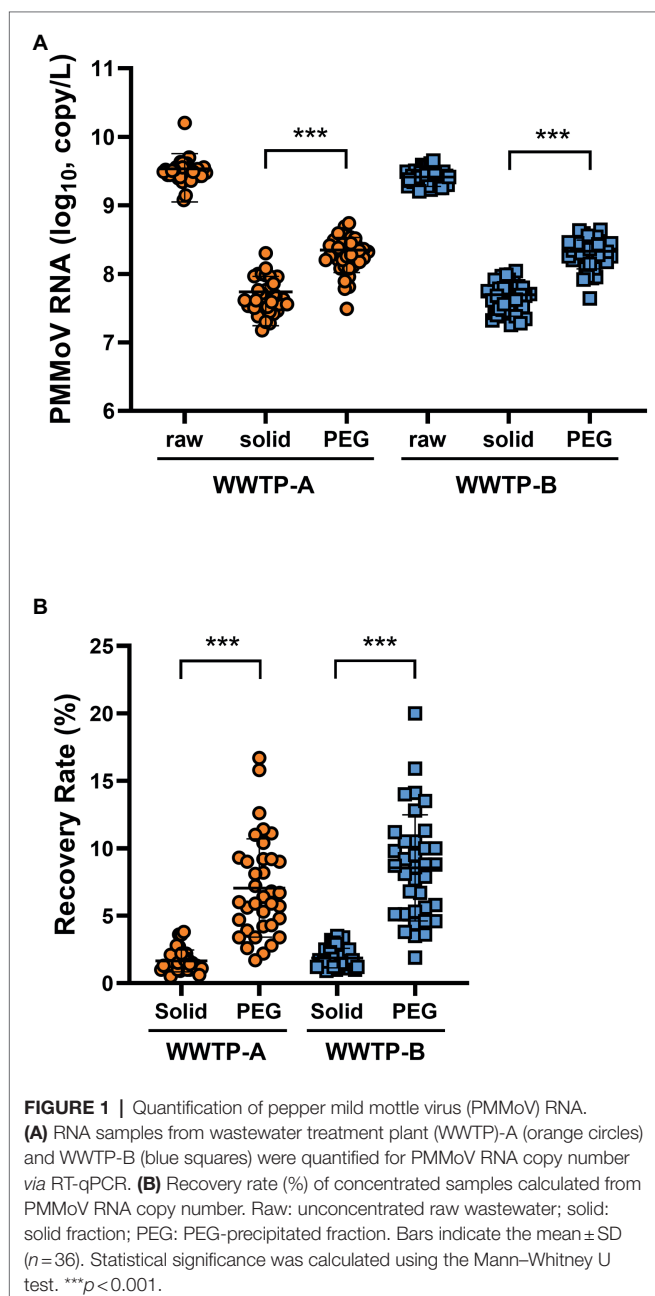
The daily newly reported number of COVID-19 cases was obtained from the Coronavirus Infection Status Report, which is a public database from Kobe City (2021). This database includes the symptomatic cases reported by hospitals and private COVID-19 test centers, as well as asymptomatic cases tested for contact tracing conducted by Public Health Management Center, Kobe City. The number of COVID-19 cases in each investigated basin of the WWTPs were provided by the Public Health Division, Public Health Management Center, Kobe City. Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Prism Software, San Diego, CA, United States). The slope, intercept, and coefficient of determination (R^2) values between standard RNA and Ct value, and case numbers and RNA copy numbers were calculated using linear regression; in the latter case, regression through the origin was used. The detection frequency of SARS-CoV-2 between the solid and liquid fractions was assessed using Fisher's exact test. The Mann-Whitney U test was used to compare RNA copy numbers of the different sewage treatment samples. To compare case numbers and RNA copy numbers, and amount of rainfall/influent flow and RNA copy numbers, the correlation coefficient (r) was calculated using Spearman's correlation coefficient.

RESULTS

Comparison of the Solid Fraction and PEG-Precipitated Sewage Samples

The PMMoV was used as a control for the RNA extraction process. The PMMoV RNA copy numbers extracted from the

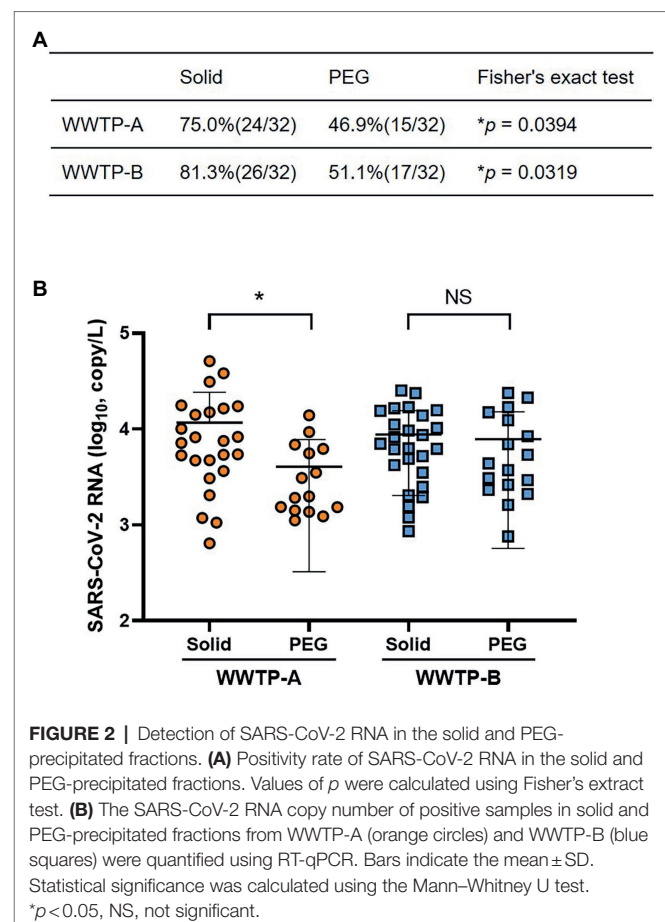
non-enriched (raw), solid, and PEG-precipitated samples were in the range 1.2×10^9 – 1.6×10^{10} , 1.5×10^7 – 2.0×10^8 , and 3.1×10^7 – 5.5×10^8 copies/L, respectively (Figure 1A). The recovery rates calculated from the PMMoV RNA copy numbers of solid and PEG-precipitated samples were in the range 0.5%–3.8% and 1.7%–20% (Figure 1B). The RNA copy number and recovery rate of PMMoV RNA in PEG-precipitated fraction were significantly higher than in solid fraction. The detection frequency of SARS-CoV-2 RNA in solid samples was significantly higher than that in PEG-precipitated samples in both WWTP-A and WWTP-B (Figure 2A). SARS-CoV-2 RNA copy numbers in the solid fraction and PEG-precipitated fraction were in the range 3.1×10^2 – 3.8×10^4 and 7.6×10^2 – 2.4×10^4 , respectively



(Figure 2B). While SARS-CoV-2 RNA copy numbers in the solid fraction samples were significantly higher than in the PEG-precipitated fraction from WWTP-A, no significant difference was observed between both the fraction from WWTP-B.

Association Between RNA Copy Number and Infected Case Number

The SARS-CoV-2 RNA copy number in the solid fraction was highly correlated with the number of COVID-19 cases reported between 24 February 2021 and 27 October 2021 (WWTP-A: $r=0.8205$, $p < 0.001$; WWTP-B: $r=0.8482$, $p < 0.001$; Figure 3). While SARS-CoV-2 RNA in the PEG-precipitated fraction and unconcentrated raw samples was significantly correlated with the number of COVID-19 cases between 24 February 2021 and 27 October 2021 (PEG: WWTP-A: $r=0.6237$, $p < 0.001$; WWTP-B: $r=0.7803$, $p < 0.001$, and Raw: WWTP-A: $r=0.6285$, $p < 0.001$; WWTP-B: $r=0.4517$, $p=0.0057$; Supplementary Figure S2), the correlation between RNA copy number and COVID-19 cases was lower than that in the solid fraction. The relationships between SARS-CoV-2 RNA and COVID-19 cases in WWTPs basin were evaluated using linear regression analysis (Figure 4). In this study, detection limit of SARS-CoV-2 RNA by RT-PCR was 2.5 copy/reaction, which is calculated to be 625 copy/L in the solid fraction. The limit of capturing COVID-19 cases per 100,000 people



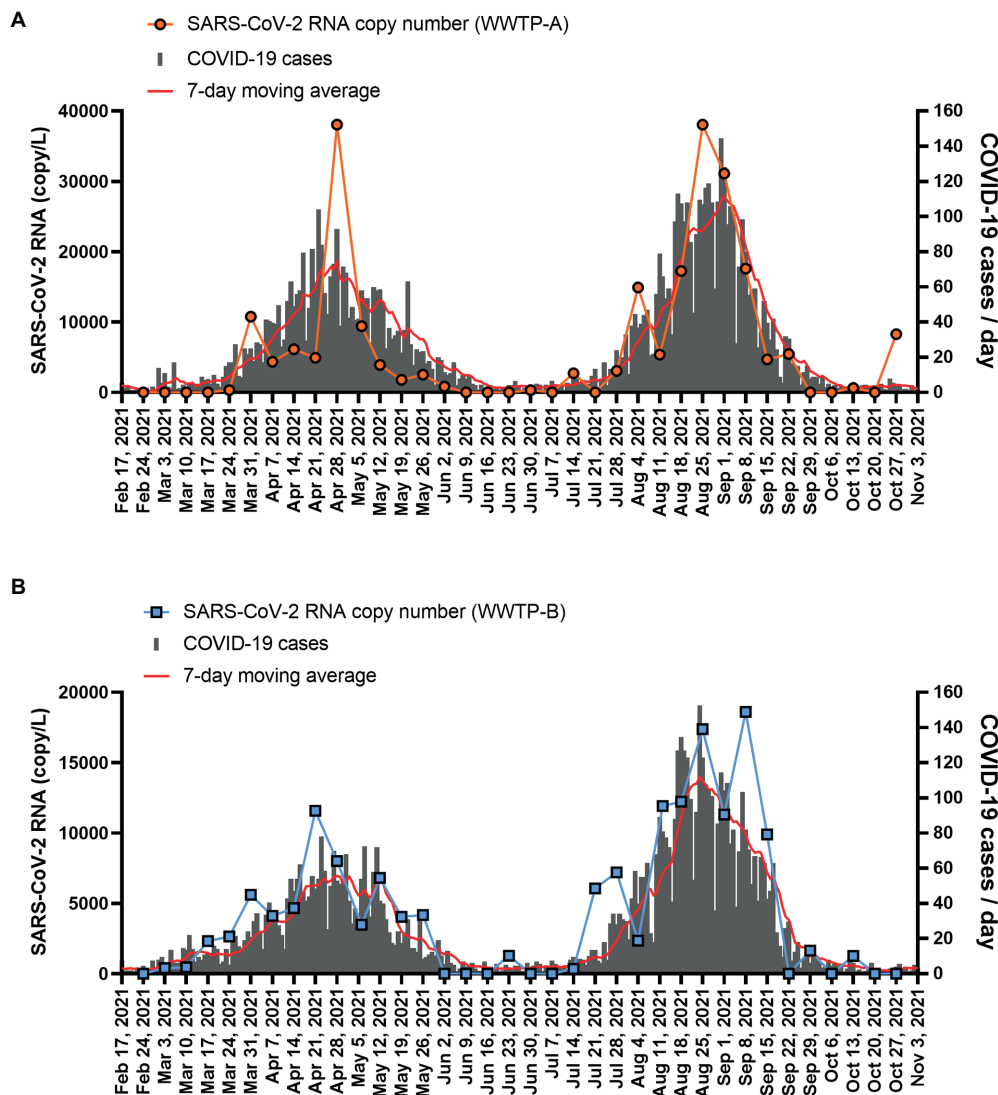


FIGURE 3 | SARS-CoV-2 RNA copy number in the solid fraction and COVID-19 case numbers. The SARS-CoV-2 copy numbers in the solid fraction from (A) WWTP-A (orange circle) and (B) WWTP-B (blue squares) are plotted. The number of new COVID-19 cases per day in WWTP basin is indicated by the gray bars, and the seven-day moving average is indicated by the red line.

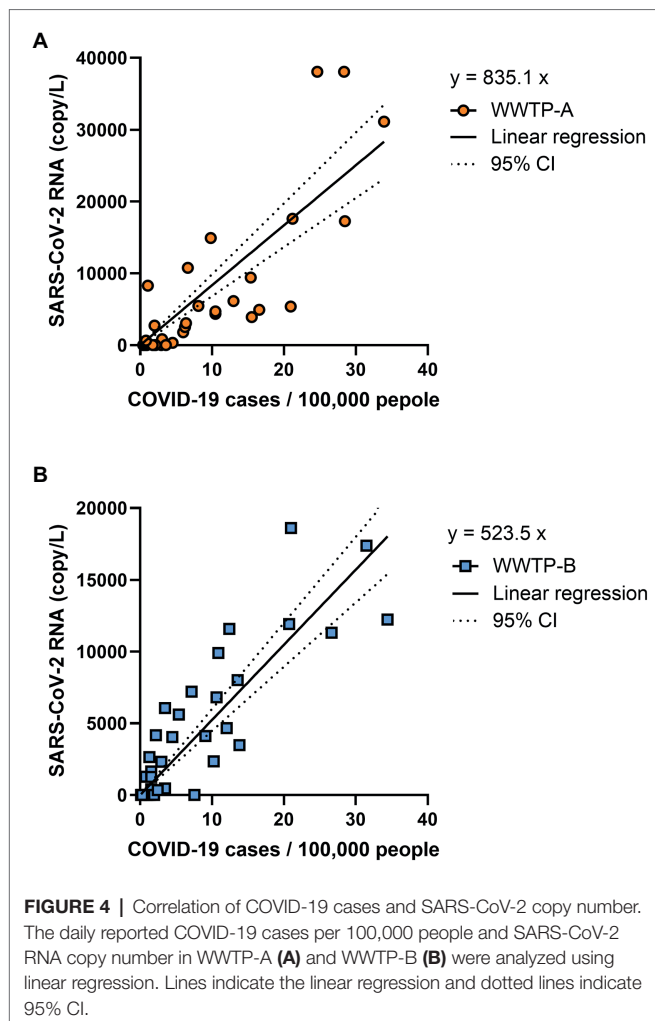
calculated using slope were 0.75 cases in WWTP-A and 1.20 cases in WWTP-B, respectively. When the effect of rain on viral RNA concentrations was evaluated, no inverse correlation was found between the amount of rainfall and SARS-CoV-2 RNA in solid fraction (WWTP-A: $r=0.1973$, $p=0.2047$; WWTP-B: $r=0.1539$, $p=0.3701$; **Supplementary Figures S3A,B**). Likewise, no inverse correlation between the amount of influent flow and RNA concentration was observed (WWTP-A: $r=0.4088$, $p=0.0133$; WWTP-B: $r=0.1652$, $p=0.3356$; **Supplementary Figures S3C,D**).

DISCUSSION

In this study, SARS-CoV-2 RNA was detected at higher levels in the solid fractions than in the PEG-precipitated fractions,

and the RNA copy numbers reflected the infection trend numbers during the fourth and fifth pandemic waves in Kobe, Japan.

The fraction in which higher RNA copy numbers were measured differed between non-enveloped PMMoV and enveloped SARS-CoV-2; PMMoV RNA was higher in the PEG-precipitated liquid fraction, whereas SARS-CoV-2 RNA was higher in the solid fraction. This difference may be related to the structure of the viruses. In one study, up to 26% of enveloped viruses, such as mouse hepatitis virus and bacteriophage $\phi 6$, were bound to the solid fraction, whereas only 6% of non-enveloped viruses, such as bacteriophages MS2 and T3, were similarly bound in wastewater samples (Ye et al., 2016). The results of our study are consistent with those of a previous study that reported the detection efficiency of



PMMoV in the PEG-precipitated liquid fraction significantly higher than in the solid fraction (Graham et al., 2021). Similar to our findings, RNA extraction from the solid fraction was better than that from liquid fraction for detecting SARS-CoV-2 in previous reports comparing enrichment methods, including pelleted solid fraction, PEG precipitation, electronegative membrane adsorption, and ultrafiltration (Kitamura et al., 2021; Westhaus et al., 2021). In contrast, another study reported that approximately 90% of the SARS-CoV-2 RNA was present in the liquid phase of the influent wastewater compared to the RNA sorbed on the influent solids (Weidhaas et al., 2021). Tomasino et al. (2021) reported that no significant differences were observed in Ct values of SARS-CoV-2 RNA between the liquid and solid phases. These differences are thought to be due to the centrifuge conditions for solid collection. In this study, since centrifugal condition described in previous studies (Kitamura et al., 2021; Westhaus et al., 2021) did not completely precipitate the solid, we set a strong centrifugal condition for ease of work and efficient solid recovery. The different solid removal strategies may result in a high or low representation of the virus in the solid fraction. To minimize errors in RT-PCR detection and quantification, it is recommended that

SARS-CoV-2 RNA should be concentrated from both liquid and solid phases of wastewater (Ahmed et al., 2022). Furthermore, in this study, SARS-CoV-2 RNA could be detected using a non-enrichment method as a result of the large number of COVID-19 cases. A previous study reported that the RNA copy number of unenriched wastewater correlated with the number of cases in Marseille, France (Wurtz et al., 2021). These results suggest that the wastewater enrichment methods are not always necessary in areas with high number of COVID-19 cases.

Recovery rate using the PMMoV copy number have been conducted for concentration efficiency of wastewater. Previous studies that compared the liquid and solid fraction showed that the detection efficiency of PMMoV ranged 8.0%–30% in the PEG-precipitated liquid fraction and 6.0%–17% in the solid fraction (Graham et al., 2021), and 12%–102% from liquid fractions and 9.4%–62% from solid fractions (Alamin et al., 2022). Our reported recovery values tended to be lower than the range reported in previous studies. The copy numbers of PMMoV RNA were in the range 8.2×10^6 – 3.1×10^8 copy/L in the liquid fraction and 1.6×10^2 – 1.0×10^7 copy/L in the solid fraction (Kitamura et al., 2021). Hasing et al. (2021) reported that the copy numbers of PMMoV were median values of 8.98×10^6 (interquartile range, 6.38×10^6 – 1.20×10^7) copies per 100 ml in the liquid fraction, and 1.71×10^6 (interquartile range, 1.52×10^6 – 2.58×10^6) copies per 100 ml in the solids. PMMoV copy numbers in our study did not deviate significantly from the ranges in previous studies, suggesting that the process of wastewater concentration had been performed properly.

In our study, collection of wastewater samples was performed by spot grab sampling, which can affect the SARS-CoV-2 RNA concentration because wastewater flow in a WWTP is increased by the rain. Rainfall was expected to have little effect on RNA concentrations as no inverse correlation between amount of rainfall/influent flow and SARS-CoV-2 RNA in solid was observed. Previous studies showed that composite samples, which were collected by flow-weighting for 24 h, were more detectable than grab samples for SARS-CoV-2 RNA in wastewater (Gerrity et al., 2021; Monteiro et al., 2022). However, grab sampling had been performed for SARS-CoV-2 RNA detection (Randazzo et al., 2020; Carrillo-Reyes et al., 2021; Kitamura et al., 2021; Street et al., 2021; Wehrendt et al., 2021), and correlated with COVID-19 cases (Kitamura et al., 2021; Street et al., 2021). While grab samples have the limitation of low sensitivity, they could be a useful sampling method because they have the advantage of being collected quickly, do not require automated equipment, and were able to reflect COVID-19 cases in our study.

Our data showed that SARS-CoV-2 RNA was detected at higher concentrations as the number of COVID-19 cases increased. This result is consistent with that of a previous study in Tokyo, Japan, which compared SARS-CoV-2 RNA levels in the solid fraction of wastewater with the number of COVID-19 cases from June 2020 to August 2020 (Kitamura et al., 2021). The number of SARS-CoV-2 RNA in primary settled solids collected from primary clarifier was correlated

with COVID-19 cases in a study conducted in California, United States (Graham et al., 2021; Wolfe et al., 2021). The number of positive COVID-19 cases has also been correlated with SARS-CoV-2 RNA in wastewater in other countries (Medema et al., 2020a; Carrillo-Reyes et al., 2021; Wurtz et al., 2021); however, the results differed on whether the detection of SARS-CoV-2 RNA increased before or coincident with the number of COVID-19 cases. The viral load in wastewater preceded clinical data by 4 days to 2 weeks in some studies (Medema et al., 2020a; Randazzo et al., 2020; Trottier et al., 2020; Claro et al., 2021; Wu et al., 2022), whereas no time difference was reported in other studies (Peccia et al., 2020; Kitamura et al., 2021). Our data also showed no time difference when correlating the number of COVID-19 cases and SARS-CoV-2 RNA levels. In addition, a study performed daily composite collection of wastewater and reported that although the trend in SARS-CoV-2 RNA levels preceded the number of cases during the first infection wave in France, both measures followed a similar curve in the second infection wave (Wurtz et al., 2021). The difference in the trends of the two waves was probably due to differences in the duration of recognition of the number of cases, that is, in the early stages of a pandemic it is difficult to determine the number of cases as reporting is relatively late, which suggests that the detection of viral RNA precedes case load. In Kobe City, because of active investigation of close contacts and efforts to ascertain the number of infected people, the time difference in reporting the number of cases may be reduced; thus, the viral RNA level and the number of cases correlate without an apparent time difference. In addition, the detection of SARS-CoV-2 RNA from WWTP-B occurred earlier than in WWTP-A. In fact, the number of COVID-19 cases in the WWTP-B basin tended to peak earlier than in WWTP-A.

Linear regression analysis between SARS-CoV-2 RNA copy number and COVID-19 cases showed that the limit of capturing COVID-19 cases per 100,000 people was 0.75 cases in WWTP-A and 1.20 cases in WWTP-B, respectively. SARS-CoV-2 RNA in wastewater was quantifiable in some WWTP basins with daily positive test rates of less than 1 per 10,000 people (Wilder et al., 2021). To detect of SARS-CoV-2, approximately 0.12% and 0.09% of the total population in the WWTP basin area were required to be assessed (Chavarria-Miró et al., 2021). SARS-CoV-2 in wastewater samples collected from five WWTPs in Japan was more likely to be detected when there were more than 10 confirmed cases of COVID-19 per 100,000 people in the basin area, but it was detectable in wastewater even before the number of cases reached 1 per 100,000 people (Hata et al., 2021). The current study found that the capturing COVID-19 cases was equal to or higher than in previous studies. In this study, RNA concentration was detectable in the range 7.6×10^2 – 2.4×10^4 copy/L when COVID-19 cases per 100,000 people were in the range 0.77–34.4. Previous studies showed that SARS-CoV-2 RNA was detected in the following ranges at the following COVID-19 case rates (per 100,000 people); 1.7×10^3 – 3.8×10^5 copy/L at 4.8–57.3 cases (D'Aoust et al., 2021), 1.2×10^1 – 2.2×10^3 copy/L at 0.1–100

cases (Medema et al., 2020a), and 3.0×10^3 – 2.0×10^4 copy/L at 30–174 cases (Westhaus et al., 2021). Medema et al. (2020b) reported a simulation model of the number of COVID-19 infected people in the population and concentration of SARS-CoV-2 RNA in sewage, and estimated that RNA copy number was approximately 10^2 – 10^5 copy/L at 10–100 cases per 100,000 people. Our results are consistent with these previous studies, suggesting that RNA concentration reflects COVID-19 case numbers.

In Japan, the fourth and fifth waves of SARS-CoV-2 RNA were useful predictions of manifesting COVID-19 cases. The present data indicate that the detection of SARS-CoV-2 RNA in sewage can be used to monitor and predict trends in SARS-CoV-2 infections. This monitoring may provide valuable data even when the number of patients diagnosed with COVID-19 at clinical sites becomes low owing to mass vaccination. In the mass vaccination era, the number of asymptomatic cases is expected to increase, making it more difficult to determine the actual number of cases in the community. The usefulness of wastewater-based epidemiology, which can determine the number of both infected and asymptomatic persons in a community, will increase in the future. These results show the potential of using sewage monitoring, such as RNA levels, in public health, including responding to and the issuing of health warnings within sewerage basins.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, and further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

YT, TM, and TI: conceptualization. YT and EI: methodology and writing—original draft preparation. NO and TM: resources. YT, EI, SM, AM, RN, and NN: investigation. YT: formal analysis and funding acquisition. RN and TI: supervision. YT, EI, SM, AM, RN, NN, NO, TM, and TI: writing—review and editing. 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/fmicb.2022.892447/full#supplementary-material>

REFERENCES

- Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J. W., et al. (2020a). First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. *Sci. Total Environ.* 728:138764. doi: 10.1016/j.scitotenv.2020.138764
- Ahmed, W., Bivins, A., Bertsch, P. M., Bibby, K., Choi, P. M., Farkas, K., et al. (2020b). Surveillance of SARS-CoV-2 RNA in wastewater: methods optimisation and quality control are crucial for generating reliable public health information. *Curr. Opin. Environ. Sci. Health.* doi: 10.1016/j.coesh.2020.09.003 [Epub ahead of print].
- Ahmed, W., Simpson, S. L., Bertsch, P. M., Bibby, K., Bivins, A., Blackall, L. L., et al. (2022). Minimizing errors in RT-PCR detection and quantification of SARS-CoV-2 RNA for wastewater surveillance. *Sci. Total Environ.* 805:149877. doi: 10.1016/j.scitotenv.2021.149877
- Alamin, M., Tsuji, S., Hata, A., Hara-Yamamura, H., and Honda, R. (2022). Selection of surrogate viruses for process control in detection of SARS-CoV-2 in wastewater. *Sci. Total Environ.* 823:153737. doi: 10.1016/j.scitotenv.2022.153737
- Albastaki, A., Naji, M., Lootah, R., Almeheiri, R., Almulla, H., Almarri, I., et al. (2021). First confirmed detection of SARS-CoV-2 in untreated municipal and aircraft wastewater in Dubai, UAE: The use of wastewater based epidemiology as an early warning tool to monitor the prevalence of COVID-19. *Sci. Total Environ.* 760:143350. doi: 10.1016/j.scitotenv.2020.143350
- Carrillo-Reyes, J., Barragán-Trinidad, M., and Buitrón, G. (2021). Surveillance of SARS-CoV-2 in sewage and wastewater treatment plants in Mexico. *J. Water Process Eng.* 40:101815. doi: 10.1016/j.jwpe.2020.101815
- Chavarria-Miró, G., Anfruns-Estrada, E., Martínez-Velázquez, A., Vázquez-Portero, M., Guix, S., Paraira, M., et al. (2021). Time evolution of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in wastewater during the first pandemic wave of COVID-19 in the metropolitan area of Barcelona, Spain. *Appl. Environ. Microbiol.* 87, e02750–e02720. doi: 10.1128/AEM.02750-20
- Cheung, K. S., Hung, I. F. N., Chan, P. P. Y., Lung, K. C., Tso, E., Liu, R., et al. (2020). Gastrointestinal manifestations of SARS-CoV-2 infection and virus load in fecal samples from a Hong Kong cohort: systematic review and meta-analysis. *Gastroenterology* 159, 81–95. doi: 10.1053/j.gastro.2020.03.065
- Claro, I. C. M., Cabral, A. D., Augusto, M. R., Duran, A. F. A., Graciosa, M. C. P., Fonseca, F. L. A., et al. (2021). Long-term monitoring of SARS-CoV-2 RNA in wastewater in Brazil: a more responsive and economical approach. *Water Res.* 203:117534. doi: 10.1016/j.watres.2021.117534
- D'Aoust, P. M., Mercier, E., Montpetit, D., Jia, J. J., Alexandrov, I., Neault, N., et al. (2021). Quantitative analysis of SARS-CoV-2 RNA from wastewater solids in communities with low COVID-19 incidence and prevalence. *Water Res.* 188:116560. doi: 10.1016/j.watres.2020.116560
- Supplementary Figure S1** | Detection limit of SARS-CoV-2 RNA using RT-qPCR. Each copy of the standard SARS-CoV-2 RNA (Ct value) was confirmed using RT-qPCR measurement. The slope, intercept, and coefficient of determination (R^2) values were calculated using linear regression.
- Supplementary Figure S2** | SARS-CoV-2 RNA copy number in the PEG-precipitated or unconcentrated sample and COVID-19 case numbers. The SARS-CoV-2 copy numbers in the liquid fraction concentrated using PEG precipitation (A,B) or unconcentrated raw sample (C,D) from WWTP-A (A,C; orange circles) and WWTP-B (B,D; blue squares) are plotted. The number of new COVID-19 cases per day in WWTP basin is indicated by the gray bars, and the seven-day moving average is indicated by the red line.
- Supplementary Figure S3** | The amount of rainfall and SARS-CoV-2 RNA copy number in the solid fraction. The SARS-CoV-2 RNA copy number in the solid fraction from WWTP-A (A,C; orange circles) and WWTP-B (B,D; blue squares) are plotted. The amount of rainfall in each WWTPs is indicated by the purple bars (A,B) and the amount of influent flow is indicated by the black line (C,D).
- Foladori, P., Cutrupi, F., Segata, N., Manara, S., Pinto, F., Malpei, F., et al. (2020). SARS-CoV-2 from faeces to wastewater treatment: what do we know? A review. *Sci. Total Environ.* 743:140444. doi: 10.1016/j.scitotenv.2020.140444
- Gerrity, D., Papp, K., Stoker, M., Sims, A., and Frehner, W. (2021). Early-pandemic wastewater surveillance of SARS-CoV-2 in southern Nevada: methodology, occurrence, and incidence/prevalence considerations. *Water Res. X.* 10:100086. doi: 10.1016/j.wroa.2020.100086
- Graham, K. E., Loeb, S. K., Wolfe, M. K., Catoe, D., Sinnott-Armstrong, N., Kim, S., et al. (2021). SARS-CoV-2 RNA in wastewater settled solids is associated with COVID-19 cases in a large urban sewer shed. *Environ. Sci. Technol.* 55, 488–498. doi: 10.1021/acs.est.0c06191
- Haramoto, E., Kitajima, M., Hata, A., Torrey, J. R., Masago, Y., Sano, D., et al. (2018). A review on recent progress in the detection methods and prevalence of human enteric viruses in water. *Water Res.* 135, 168–186. doi: 10.1016/j.watres.2018.02.004
- Haramoto, E., Kitajima, M., Kishida, N., Konno, Y., Katayama, H., Asami, M., et al. (2013). Occurrence of pepper mild mottle virus in drinking water sources in Japan. *Appl. Environ. Microbiol.* 79, 7413–7418. doi: 10.1128/AEM.02354-13
- Haramoto, E., Malla, B., Thakali, O., and Kitajima, M. (2020). First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. *Sci. Total Environ.* 737:140405. doi: 10.1016/j.scitotenv.2020.140405
- Hasing, M., Yu, J., Qiu, Y., Maal-Bared, R., Bhavanam, S., Lee, B., et al. (2021). Comparison of detecting and quantitating SARS-CoV-2 in wastewater using moderate-speed centrifuged solids versus an ultrafiltration method. *Water* 13:2166. doi: 10.3390/w13162166
- Hata, A., Hara-Yamamura, H., Meuchi, Y., Imai, S., and Honda, R. (2021). Detection of SARS-CoV-2 in wastewater in Japan during a COVID-19 outbreak. *Sci. Total Environ.* 758:143578. doi: 10.1016/j.scitotenv.2020.143578
- Hodcroft, E. (2021). CoVariants. Available at: <https://covariants.org/> (Accessed 17 December 2021).
- Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 395, 497–506. doi: 10.1016/S0140-6736(20)30183-5
- Hughes, B., Beale, D. J., Dennis, P. G., Cook, S., and Ahmed, W. (2017). Cross-comparison of human wastewater-associated molecular markers in relation to fecal indicator bacteria and enteric viruses in recreational beach waters. *Appl. Environ. Microbiol.* 83, e00028–e000217. doi: 10.1128/AEM.00028-17
- Jones, T. H., and Johns, M. W. (2009). Improved detection of F-specific RNA coliphages in fecal material by extraction and polyethylene glycol precipitation. *Appl. Environ. Microbiol.* 75, 6142–6146. doi: 10.1128/AEM.00436-09
- Kazama, S., Miura, T., Masago, Y., Konta, Y., Tohma, K., Manaka, T., et al. (2017). Environmental surveillance of Norovirus genogroups I and II for

- sensitive detection of epidemic variants. *Appl. Environ. Microbiol.* 83, e03406–e034016. doi: 10.1128/AEM.03406-16
- Kevill, J. L., Pellett, C., Farkas, K., Brown, M. R., Bassano, I., Denise, H., et al. (2022). A comparison of precipitation and filtration-based SARS-CoV-2 recovery methods and the influence of temperature, turbidity, and surfactant load in urban wastewater. *Sci. Total Environ.* 808:151916. doi: 10.1016/J.SCITOTENV.2021.151916
- Kitamura, K., Sadamasu, K., Muramatsu, M., and Yoshida, H. (2021). Efficient detection of SARS-CoV-2 RNA in the solid fraction of wastewater. *Sci. Total Environ.* 763:144587. doi: 10.1016/j.scitotenv.2020.144587
- Kobe City (2021). Coronavirus Infection Status Report (in Japanese). Available at: https://www.city.kobe.lg.jp/a73576/kenko/health/infection/protection/covid_19.html (Accessed December 10, 2021).
- La Rosa, G., Bonadonna, L., Lucentini, L., Kenmoe, S., and Suffredini, E. (2020a). Coronavirus in water environments: occurrence, persistence and concentration methods—a scoping review. *Water Res.* 179:115899. doi: 10.1016/J.WATRES.2020.115899
- La Rosa, G., Iaconelli, M., Mancini, P., Bonanno Ferraro, G., Veneri, C., Bonadonna, L., et al. (2020b). First detection of SARS-CoV-2 in untreated wastewaters in Italy. *Sci. Total Environ.* 736:139652. doi: 10.1016/J.SCITOTENV.2020.139652
- Lescure, F. X., Bouadma, L., Nguyen, D., Parisey, M., Wicky, P. H., Behillil, S., et al. (2020). Clinical and virological data of the first cases of COVID-19 in Europe: a case series. *Lancet Infect. Dis.* 20, 697–706. doi: 10.1016/S1473-3099(20)30200-0
- Medema, G., Been, F., Heijnen, L., and Pettersson, S. (2020b). Implementation of environmental surveillance for SARS-CoV-2 virus to support public health decisions: opportunities and challenges. *Curr. Opin. Environ. Sci. Health.* 17, 49–71. doi: 10.1016/j.coesh.2020.09.006
- Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., and Brouwer, A. (2020a). Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. *Environ. Sci. Technol. Lett.* 7, 511–516. doi: 10.1021/acs.estlett.0c00357
- Miura, T., Lhomme, S., Le Saux, J. C., Le Mehaute, P., Guillois, Y., Couturier, E., et al. (2016). Detection of hepatitis E virus in sewage after an outbreak on a French Island. *Food Environ. Virol.* 8, 194–199. doi: 10.1007/s12560-016-9241-9
- Monteiro, S., Rente, D., Cunha, M. V., Gomes, M. C., Marques, T. A., Lourenço, A. B., et al. (2022). A wastewater-based epidemiology tool for COVID-19 surveillance in Portugal. *Sci. Total Environ.* 804:150264. doi: 10.1016/J.SCITOTENV.2021.150264
- Nagarkar, M., Keely, S. P., Jahne, M., Wheaton, E., Hart, C., Smith, B., et al. (2021). SARS-CoV-2 monitoring at three sewersheds of different scales and complexity demonstrates distinctive relationships between wastewater measurements and COVID-19 case data. *Sci. Total Environ.* 816:151534. doi: 10.1016/J.SCITOTENV.2021.151534
- Nagashima, M., Kawakami, M., Hayashi, M., Kumagai, R., Kasuya, F., Yoshida, I., et al. (2021). RNA Detection by RT-qPCR and non-isolation of SARS-CoV-2 in concentrated wastewater (June–August 2020, Tokyo). *Jpn. J. Infect. Dis.* doi: 10.7883/yoken.JJID.2021.055 [Epub ahead of print].
- Peccia, J., Zulli, A., Brackney, D. E., Grubaugh, N. D., Kaplan, E. H., Casanovas-Massana, A., et al. (2020). Measurement of SARS-CoV-2 RNA in wastewater tracks community infection dynamics. *Nat. Biotechnol.* 38, 1164–1167. doi: 10.1038/S41587-020-0684-Z
- Qiu, Y., Yu, J., Pabbaraju, K., Lee, B. E., Gao, T., Ashbolt, N. J., et al. (2022). Validating and optimizing the method for molecular detection and quantification of SARS-CoV-2 in wastewater. *Sci. Total Environ.* 812:151434. doi: 10.1016/j.scitotenv.2021.151434
- Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simón, P., Allende, A., and Sánchez, G. (2020). SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. *Water Res.* 181:115942. doi: 10.1016/J.WATRES.2020.115942
- Rosario, K., Symonds, E. M., Sinigalliano, C., Stewart, J., and Breitbart, M. (2009). Pepper mild mottle virus as an indicator of fecal pollution. *Appl. Environ. Microbiol.* 75, 7261–7267. doi: 10.1128/AEM.00410-09
- Rosiles-González, G., Carrillo-Jovel, V. H., Alzate-Gaviria, L., Betancourt, W. Q., Gerba, C. P., Moreno-Valenzuela, O. A., et al. (2021). Environmental surveillance of SARS-CoV-2 RNA in wastewater and groundwater in Quintana Roo, Mexico. *Food Environ. Virol.* 13, 457–469. doi: 10.1007/s12560-021-09492-y
- Sangkham, S. (2021). A review on detection of SARS-CoV-2 RNA in wastewater in light of the current knowledge of treatment process for removal of viral fragments. *J. Environ. Manag.* 299:113563. doi: 10.1016/J.JENVMAN.2021.113563
- Street, R., Mathee, A., Mangwana, N., Dias, S., Sharma, J. R., Ramharack, P., et al. (2021). Spatial and temporal trends of SARS-CoV-2 RNA from wastewater treatment plants over 6 weeks in Cape Town, South Africa. *Int. J. Environ. Res. Public Health* 18:12085. doi: 10.3390/IJERPH182212085
- Tomasino, M. P., Semedo, M., Vieira, E., Moreira, P., Ferraz, E., Rocha, A., et al. (2021). SARS-CoV-2 RNA detected in urban wastewater from Porto, Portugal: method optimization and continuous 25-week monitoring. *Sci. Total Environ.* 792:148467. doi: 10.1016/j.scitotenv.2021.148467
- Torii, S., Furumai, H., and Katayama, H. (2021). Applicability of polyethylene glycol precipitation followed by acid guanidinium thiocyanate-phenol-chloroform extraction for the detection of SARS-CoV-2 RNA from municipal wastewater. *Sci. Total Environ.* 756:143067. doi: 10.1016/J.SCITOTENV.2020.143067
- Trottier, J., Darques, R., Ait Mouheb, N., Partiot, E., Bakhache, W., Deffieu, M. S., et al. (2020). Post-lockdown detection of SARS-CoV-2 RNA in the wastewater of Montpellier, France. *One Heal* 10:100157. doi: 10.1016/J.ONEHLT.2020.100157
- Wang, S., Green, H. C., Wilder, M. L., Du, Q., Kmush, B. L., Collins, M. B., et al. (2020). High-throughput wastewater analysis for substance use assessment in Central New York during the COVID-19 pandemic. *Environ. Sci. Process Impacts* 22, 2147–2161. doi: 10.1039/D0EM00377H
- Wehrendt, D. P., Massó, M. G., Gonzales Machuca, A., Vargas, C. V., Barrios, M. E., Campos, J., et al. (2021). A rapid and simple protocol for concentration of SARS-CoV-2 from sewage. *J. Virol. Methods* 297:114272. doi: 10.1016/J.JVIROMET.2021.114272
- Weidhaas, J., Aanderud, Z. T., Roper, D. K., VanDerslice, J., Gaddis, E. B., Ostermiller, J., et al. (2021). Correlation of SARS-CoV-2 RNA in wastewater with COVID-19 disease burden in sewersheds. *Sci. Total Environ.* 775:145790. doi: 10.1016/j.scitotenv.2021.145790
- Westhaus, S., Weber, F. A., Schiwy, S., Linnemann, V., Brinkmann, M., Wiedera, M., et al. (2021). Detection of SARS-CoV-2 in raw and treated wastewater in Germany – suitability for COVID-19 surveillance and potential transmission risks. *Sci. Total Environ.* 751:141750. doi: 10.1016/J.SCITOTENV.2020.141750
- Wilder, M. L., Middleton, F., Larsen, D. A., Du, O., Fenty, A., Zeng, T., et al. (2021). Co-quantification of crAssphage increases confidence in wastewater-based epidemiology for SARS-CoV-2 in low prevalence areas. *Water Res.* X. 11:100100. doi: 10.1016/j.wroa.2021.100100
- Wolfe, M. K., Topol, A., Knudson, A., Simpson, A., White, B., Vugia, D. J., et al. (2021). High-frequency, high-throughput quantification of SARS-CoV-2 RNA in wastewater settled solids at eight publicly owned treatment works in Northern California shows strong association with COVID-19 incidence. *mSystems* 6:e0082921. doi: 10.1128/mSystems.00829-21
- World Health Organization (2003). Guidelines for environmental surveillance of poliovirus circulation. Available at: <https://apps.who.int/iris/handle/10665/67854> (Accessed December 21, 2021).
- Worldometer (2020). COVID-19 coronavirus pandemic. Available at: <https://www.worldometers.info/coronavirus/japan> (Accessed December 20, 2021).
- Wu, C., Chen, X., Cai, Y., Xia, J., Zhou, X., Xu, S., et al. (2020). Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Intern. Med.* 180, 934–943. doi: 10.1001/jamainternmed.2020.0994
- Wu, F., Xiao, A., Zhang, J., Moniz, K., Endo, N., Armas, F., et al. (2022). SARS-CoV-2 RNA concentrations in wastewater foreshadow dynamics and clinical presentation of new COVID-19 cases. *Sci. Total Environ.* 805:150121. doi: 10.1016/J.SCITOTENV.2021.150121
- Wurtz, N., Lacoste, A., Jardot, P., Delache, A., Fontaine, X., Verlande, M., et al. (2021). Viral RNA in city wastewater as a key indicator of COVID-19 recrudescence and containment measures effectiveness. *Front. Microbiol.* 12:664477. doi: 10.3389/FMICB.2021.664477
- Wurtzer, S., Marechal, V., Mouchel, J. M., Maday, Y., Teyssou, R., Richard, E., et al. (2020). Evaluation of lockdown effect on SARS-CoV-2 dynamics through viral genome quantification in waste water, greater Paris, France, 5 March to 23 April 2020. *Euro Surveill.* 25:2000776. doi: 10.2807/1560-7917.ES.2020.25.50.2000776
- Wurtzer, S., Waldman, P., Levert, M., Cluzel, N., Almayrac, J. L., Charpentier, C., et al. (2022). SARS-CoV-2 genome quantification in wastewaters at regional

- and city scale allows precise monitoring of the whole outbreaks dynamics and variants spreading in the population. *Sci. Total Environ.* 810:152213. doi: 10.1016/J.SCIOTENV.2021.152213
- Ye, Y., Ellenberg, R. M., Graham, K. E., and Wigginton, K. R. (2016). Survivability, partitioning, and recovery of enveloped viruses in untreated municipal wastewater. *Environ. Sci. Technol.* 50, 5077–5085. doi: 10.1021/acs.est.6b00876
- Zhang, T., Breitbart, M., Lee, W. H., Run, J. Q., Wei, C. L., Soh, S. W., et al. (2006). RNA viral community in human feces: prevalence of plant pathogenic viruses. *PLoS Biol.* 4:e3. doi: 10.1371/journal.pbio.0040003

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SARS-CoV-2 Whole-Genome Sequencing Using Oxford Nanopore Technology for Variant Monitoring in Wastewaters

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Since the beginning of the Coronavirus Disease-19 (COVID-19) pandemic, multiple Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) mutations have been reported and led to the emergence of variants of concern (VOC) with increased transmissibility, virulence or immune escape. In parallel, the observation of viral fecal shedding led to the quantification of SARS-CoV-2 genomes in wastewater, providing information about the dynamics of SARS-CoV-2 infections within a population including symptomatic and asymptomatic individuals. Here, we aimed to adapt a sequencing technique initially designed for clinical samples to apply it to the challenging and mixed wastewater matrix, and hence identify the circulation of VOC at the community level. Composite raw sewage sampled over 24 h in two wastewater-treatment plants (WWTPs) from a city in western France were collected weekly and SARS-CoV-2 quantified by RT-PCR. Samples collected between October 2020 and May 2021 were submitted to whole-genome sequencing (WGS) using the primers and protocol published by the ARTIC Network and a MinION Mk1C sequencer (Oxford Nanopore Technologies, Oxford, United Kingdom). The protocol was adapted to allow near-full genome coverage from sewage samples, starting from ~5% to reach ~90% at depth 30. This enabled us to detect multiple single-nucleotide variant (SNV) and assess the circulation of the SARS-CoV-2 VOC Alpha, Beta, Gamma, and Delta. Retrospective analysis of sewage samples shed light on the emergence of the Alpha VOC with detection of first co-occurring signature mutations in mid-November 2020 to reach predominance of this variant in early February 2021. In parallel, a mutation-specific qRT-PCR assay confirmed the spread of the Alpha VOC but detected it later than WGS. Altogether, these data show that SARS-CoV-2 sequencing in sewage can be used for early detection of an emerging VOC in a population and confirm its ability to track shifts in variant predominance.

Keywords: SARS-CoV-2, variant of concern, wastewater-based epidemiology, next-generation sequencing, Oxford Nanopore Technology, sewage, ARTIC

INTRODUCTION

Shedding of SARS-CoV-2 *via* human feces results in the presence of viral genetic material in human sewage, thus allowing wastewater-based epidemiology (WBE). WBE relies on the fact that anytime a stable molecule or micro-organism is excreted by humans and later drained into wastewater, the original concentration excreted by the serviced population can be inferred from sewage sample analysis (Madoux-Humery et al., 2016; Mao et al., 2020; Polo et al., 2020). Prior to the COVID-19 pandemic, other members of the *Coronaviridae* family had already been identified in wastewater (Wang et al., 2005; Bibby and Peccia, 2013) but not for epidemiological purpose. This approach is particularly interesting as it provides additional information about the dynamics of SARS-CoV-2 infections at the community level. Indeed, it includes symptomatic but also asymptomatic individuals which can represent between 10.1 and 23.0% of the infected population for SARS-CoV-2 and largely contribute to the silent spread of the disease (He et al., 2021). WBE has been used since the beginning of the COVID-19 pandemic in several countries and numerous studies demonstrated temporal correlations between SARS-CoV-2 RNA titers in sewage and the number of human cases in the corresponding population (Ahmed et al., 2020; Medema et al., 2020; Wu et al., 2020, 2022; Wurtzer et al., 2020; Amerreh et al., 2021). These results indicate that monitoring of wastewater can serve as an early warning tool to inform public health authorities (Farkas et al., 2020). This approach, previously used for human enteric viruses (Miura et al., 2016), is innovative concerning a respiratory, enveloped virus.

Most of these WBE studies used quantitative reverse transcription-PCR to detect and estimate the viral concentration. This technique is sensitive and specific but it gives little information on the genomic sequence. With the increase of SARS-CoV-2 genetic diversity and hence emergence of new lineages, genome analysis is essential to monitor evolution, transmission, and spread of variants of the virus. It also implies that additional techniques such as sequencing and/or mutation-specific SARS-CoV-2 RT-PCR tests should be considered for WBE. Since Chinese health authorities first shared the SARS-CoV-2 complete genome sequence, >8,800,000 genomes have been sequenced as of March 2022, mostly from clinical samples. This worldwide effort in SARS-CoV-2 whole genome sequencing (WGS) was made possible, among many factors, by the design of multiplex PCR panels such as those shared by the ARTIC Network (Tyson et al., 2020). Because of the virus genetic diversity, these data allowed to describe groups and associate them with geographic and temporal pattern of virus spread. This diversity is described by the Nextstrain project¹ which divides SARS-CoV-2 into 25 major clades (19A-B, 20A-20J, and 21A-M) based on high prevalence, signature mutations and geographic spread (Hadfield et al., 2018).

The wastewater matrix poses several challenges for sequencing of SARS-CoV-2: (1) the viral load is low compared to most clinical samples, (2) the genetic diversity represents a mix of strains infecting many different people, (3) a high proportion of

the viral genomes is unprotected and likely fragmented (Wurtzer et al., 2021b), precluding the amplification and sequencing of whole genomes from single RNA molecules, (4) the matrix itself contains a high diversity of other genetic materials and chemicals, some known as PCR inhibitors. To circumvent these issues, SARS-CoV-2 whole-genome sequencing (WGS) protocols need to be adapted at all steps—RNA extraction, cDNA synthesis, genome amplification, library preparation, and bioinformatics analysis. To date, several studies have demonstrated that SARS-CoV-2 RNA sequencing from wastewater could help to understand the city- or country-scale circulation of SARS-CoV-2 variants (Nemudryi et al., 2020; Agrawal et al., 2021; Bar-Or et al., 2021; Crits-Christoph et al., 2021; Fontenele et al., 2021; Izquierdo-Lara et al., 2021; Jahn et al., 2021; Prado et al., 2021; Rouchka et al., 2021; Rubio-Acero et al., 2021; Wilton et al., 2021). SARS-CoV-2 WGS in sewage was conducted using multiplex PCR panels combined mostly with Illumina sequencing (Ai et al., 2021; Bar-Or et al., 2021; Fontenele et al., 2021; Hillary et al., 2021; Izquierdo-Lara et al., 2021; Jahn et al., 2021; Mondal et al., 2021; Prado et al., 2021; Rouchka et al., 2021; Rubio-Acero et al., 2021; Wurtz et al., 2021), and more rarely with Oxford Nanopore Technology (Nemudryi et al., 2020; Izquierdo-Lara et al., 2021; Rios et al., 2021). Here, we aimed to adapt a sequencing technique using the widely used and frequently updated ARTIC-400 panel of primers (Tyson et al., 2020) and Oxford Nanopore Technology (ONT), initially designed for clinical samples, to apply it to the challenging wastewater matrix. This technique enabled to observe single nucleotide variants specific of the Alpha and Beta variants of concern (VOC) and to detect the Alpha VOC at the community level in a French city, earlier than using a variant-specific quantitative RT-PCR assay.

MATERIALS AND METHODS

Virus Stocks and Cell Lines

Mengovirus (MgV) strain pMC0 (kindly provided by A. Bosch, University of Barcelona, Spain) was propagated in HeLa cells as previously described (Martin et al., 1996).

Sample Collection and Extraction

Untreated wastewater (raw sewage) samples were collected on a weekly basis at two wastewater treatment plants (WWTPs), WWTP1 and WWTP2, serving a total of 644,000 inhabitants (446,000 and 198,000, respectively) in the same city, between October 2020 and May 2021. For this study, 38 samples from WWTP1 and 38 from WWTP2 were used for sequencing. In addition, four samples used for method adaptation were collected in WWTP3, serving 22,000 inhabitants in a smaller city, between March and April 2021. All samples are listed in **Supplementary Table 1**. The 24-h flow-dependent composite samples (1–2 L) were collected in the morning, transported on ice to the laboratory and stored at 4°C for 0–2 days before the first analysis consisting in SARS-CoV-2 RNA quantification. For the retrospective part of our study, wastewater samples were analyzed after storage at –20°C for up to >1 year and thawed by overnight incubation at 4°C. All samples were homogenized and a

¹<https://nextstrain.org>

subsample of 11 mL was ultracentrifugated for 1 h at $100,000 \times g$ as described in Wurtzer et al. (2020). Pellet was resuspended in 500 μ L of Phosphate-Buffered Saline (PBS). Nucleic acids (NAs) were subsequently extracted by using the NucliSens kit and the NucliSens miniMAG purification system (bioMérieux, Marcy L'Etoile, France) following the manufacturer's instructions, with 2 mL lysis buffer, 50 μ L magnetic silica and eluted in 100 μ L elution buffer. Extracted NAs were further cleaned up using the OneStep PCR inhibitor removal kit (Zymo Research, Irvine, CA, United States), following the manufacturer's instructions.

Process Control

Mengovirus, a murine picornavirus, was used as a process control for nucleic acid (NA) extraction. MgV or other non-enveloped viruses were used as process control for SARS-CoV-2 WBE by other teams previously (Barril et al., 2021; Alamin et al., 2022; Brnić et al., 2022). Here it was considered adequate as our concentration step relies on ultracentrifugation, which is efficient on both enveloped and non-enveloped virions, and early tests showed similar efficiencies using a porcine coronavirus (data not shown). Briefly, 100 μ L of MgV solution (10^6 cRNA) were added to each 11 mL wastewater subsample prior to ultracentrifugation and each series of NA extractions included an extraction control in the form of 100 μ L of pure MgV solution. MgV concentration in NAs extracted from sewage samples were compared to that of the extraction control to calculate the extraction efficiency of each sample (Supplementary Table 1).

Quantitative One-Step Reverse Transcription and PCR and Genome Copy Quantification

The Ultrasens kit (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) was used for all quantitative one-step reverse transcription and PCR (qRT-PCR) assays, following the manufacturer's instructions, using an Aria Mx or MxP3000 real-time PCR system (Agilent Technologies, Santa Clara, United States) (Desdouits et al., 2021). The MgV qRT-PCR assay was carried out as previously described (Le Guyader et al., 2009) on 5 μ L of pure NA extract and of a 10-fold dilution, to assess the presence of PCR inhibitors. After verification of extraction efficiency using MgV, 5 μ L of pure NA extract in triplicate were screened for SARS-CoV-2 using two sets of primers and probes: IP4, targeting the polymerase gene, used to quantify SARS-CoV-2, and Δ 69/70, targeting the 69/70 HV deletion on the spike gene, designed to assess and quantify the Alpha VOC (Supplementary Table 2; Wurtzer et al., 2021a). Thermal profile was adapted to comply with the one-step qRT-PCR kit requirements: reverse-transcription for 15 min at 55°C, first denaturation and *Taq* polymerase activation for 5 min at 95°C, and 45 cycles of denaturation (94°C, 15 s), annealing (58°C, 30 s) and extension (65°C, 30 s) followed by fluorescence acquisition. For quantification, 5-point standard curves in duplicate were made by serial dilution of a SARS-CoV-2 RNA transcript (CNR des virus respiratoires, Pasteur Institute, Paris, France) for the IP4 PCR, and of a NA extracted from B.1.1.7 strain for the Δ 69/70 PCR (Centre de Recherche en Transplantation et Immunologie,

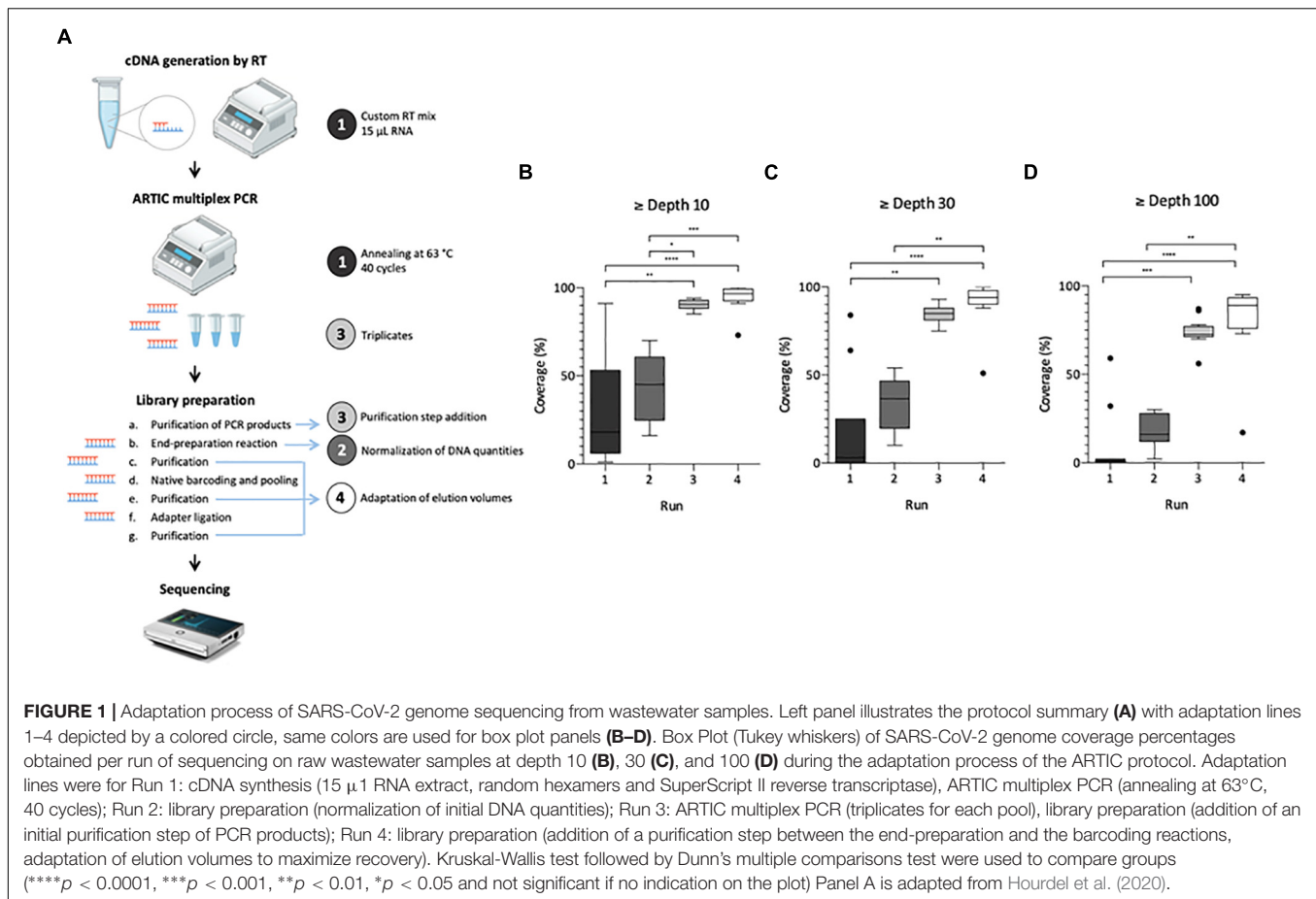
UMR1064, ITUN, Nantes, France). Good laboratory practices were observed throughout the analysis process, with dedicated separate rooms for wastewater processing, NA extraction, preparation of PCR mixtures, template addition, positive controls addition, and amplification. No-template controls were included in all qRT-PCR assays and proved always negative.

cDNA Generation

Reverse transcription was performed with 15 μ L of NAs extracted from SARS-CoV-2 positive wastewater samples using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) following a modified protocol (Strubbia et al., 2019). Briefly, 15 μ L of RNA, either freshly extracted and stored at 4°C for up to 2 weeks, or stored frozen at -20°C for up to 10 months and thawed at room temperature, were mixed with 4.6 μ L random hexamers (Thermo Fisher Scientific) in presence of 3 μ L 10 $\times$ ligase buffer (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) and 2.4 μ L 100 mM MgCl_2 . The reaction was incubated at room temperature for 2 min and the following components were added to the mix: 2 μ L 10 $\times$ ligase buffer, 1 μ L SuperScript II Reverse Transcriptase, 1 μ L dNTPs at 25 mM each, 1 μ L DTT and 15 μ L nuclease-free water. Then, the reaction was incubated for 90 min at 37°C and for 20 min at 70°C.

Library Preparation and Sequencing

Generated cDNA were used as a template for SARS-CoV-2-specific multiplex PCR. The ARTIC v3 Panel (designed by Josh Quick, University of Birmingham and marketed by Integrated DNA Technologies, United States) consists of 98 amplicons of approximately 400 bp in length, spanning the entire genome (Tyson et al., 2020). These primers were used in two PCR pools according to the ARTIC network's instructions (ncov-2019-sequencing-protocol-v3-locost). PCR were performed in triplicate for each pool using 8.5 μ L cDNA as template, under the following conditions: heat activation for 30 s at 98°C and 40 cycles of denaturation (95°C, 15 s), annealing and extension (63°C, 5 min). Amplicons for the same sample were pooled and used as a template for library synthesis following the ARTIC nCoV-2019 sequencing protocol v3 (ncov-2019-sequencing-protocol-v3-locost). A few modifications were performed as described below (Figure 1A). Pooled amplicons were purified with 0.8 $\times$ SPRIselect beads (Beckman Coulter, Fullerton, CA, United States) following the manufacturer's instructions and Fullerton, CA eluted in 10 μ L nuclease-free water. Concentrations were measured by fluorescence in a Qubit 3 (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) following the manufacturer's instructions. Purified amplicons were diluted with nuclease-free water in 8.3 μ L total, using the sample having the lowest concentration to define the quantity added for each sample (150–400 ng). A purification step was added following the end-preparation reaction using 1 $\times$ SPRIselect beads and resuspending in 5 μ L nuclease-free water. Then, 3.75 μ L of the purified end-preparation reaction mixture were mixed with barcodes accordingly using the Oxford Nanopore native barcoding kit (NBD-104, Oxford Nanopore Technologies,



Oxford, United Kingdom). The last purified product was eluted in 13 μ L of elution buffer. Finally, the library was loaded on a R9.4.1 flow cell placed onto a MinION Mk1C sequencer for a 14–18 h run. Any difference between the described method and the ARTIC nCoV-2019 sequencing protocol v3 is part of the adaptation process (Figure 1A).

SARS-CoV-2 Sequence Analysis

After the sequencing runs, fast5 data files were base-called using Guppy (version 4.3.4, Oxford Nanopore Technologies, Oxford, United Kingdom) to generate fastq files (available at <https://data-dataref.ifremer.fr/bioinfo/ifremer/obepine/lsem/data/dna-sequence-raw/>). Successfully base-called reads were further analyzed following the ARTIC nCoV-2019 pipeline version 1.2.1,² which included demultiplexing, read filtering, primers and barcode trimming. The resulting alignment file was used for single nucleotide variants (SNVs) calling using LoFreq version 2.1.5 with minimum base quality of 20 and 20 $\times$ coverage, relative to Wuhan-Hu-1/2019 reference genome (GenBank: MN908947.3). Short indels calling was also performed using Lofreq after a preprocessing step to insert indel qualities. Samtools was used to read alignment files and an Awk-based script enabled to extract genome coverage percentages at depth 10, 30, and 100. Samtools also allowed the extraction of mean

genome coverage across the distinct amplicons trimmed for primer and overlapping sequences, for each sample. For VOC analysis, we excluded samples with depth 30 coverage <70%. On the basis of previous studies (Martin et al., 2020; Izquierdo-Lara et al., 2021), single nucleotide variants (SNVs) and indels with coverage < 30, average quality < 30, frequency < 5%, and homopolymer run > 4 (for indels only) were excluded. The detected SNVs were filtered by position and compared with the signature mutations for alpha, beta, gamma and delta VOC described in <https://nextstrain.org> and listed in **Supplementary Table 3**. Additional details on sequencing runs are available in the **Table 1**.

Statistical Analysis

GraphPad Prism v 9.0.0 (GraphPad Software, San Diego, CA, United States) was used for data representation and statistical analysis. Comparisons for evaluation of the impact of each adaptation line during the adaptation process and freezing on the adapted sequencing protocol were performed using Kruskal-Wallis test followed by Dunn's multiple comparisons test. Correlations between SARS-CoV-2 genomic concentrations and genome coverage for all samples and each group individually (i.e., fresh or frozen RNA and frozen wastewater) were assessed using the Spearman non-parametric test. Differences were considered statistically significant when $p < 0.05$.

²<https://artic.network/ncov-2019>

TABLE 1 | Additional details on sequencing runs.

#	Objective*	Total samples	Library quantity (ng)	Total mapped reads	Median depth 30 coverage (%)
1	Adaptation	12	50	32,293	3
2	Adaptation	8	38	39,530	36
3	Adaptation	12	122	295,224	85
4	Adaptation and prospective	10	346	288,608	94
5	Retrospective	12	317	285,529	90
6	Retrospective	12	222	315,498	88
7	Retrospective	12	106	144,754	68
8	Retrospective	9	194	112,553	55

*Objective of the sequencing run: protocol adaptation for WW samples (runs 1–4), prospective sequencing of fresh WW samples (run 4), retrospective sequencing of stored WW samples (runs 5 to 8).

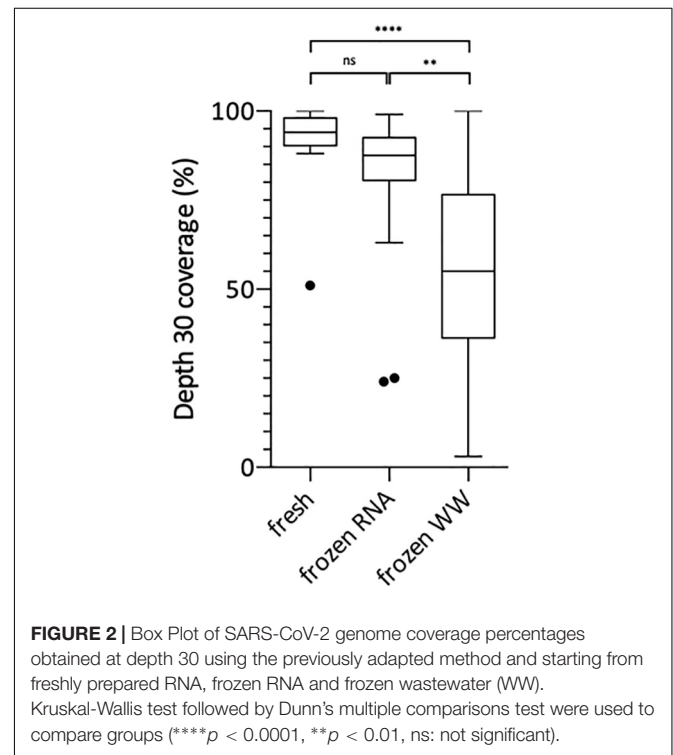
RESULTS

Sequencing Protocol Adaptation for Wastewater Samples

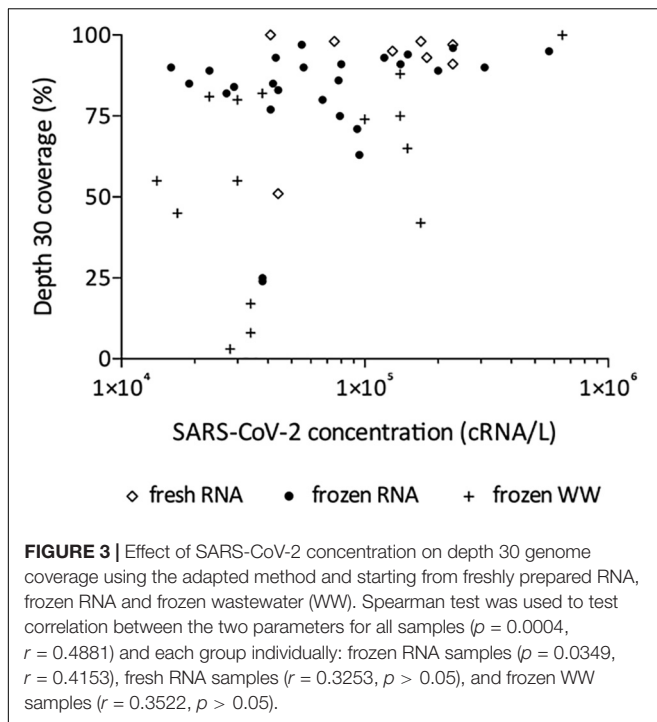
The first aim of this study was to adapt the ARTIC V3 Lo-cost protocol, initially designed for clinical samples, in order to use it for SARS-CoV-2 WGS in raw wastewater samples. Four sequencing runs were needed to achieve this objective as illustrated on **Figure 1**. For each run, modifications made to the original protocol are indicated on **Figure 1A**. Results obtained for the first run were heterogeneous and median coverages were low (18, 3 and 0% at depth 10, 30, and 100, respectively) (**Figures 1B–D**), confirming the need to adapt the initial protocol to sewage samples. Normalizing the DNA quantity for each sample enabled to reduce genome coverage disparity in run 2 and increase median coverage (45, 37, and 16%, respectively) albeit not significant (**Figures 1B–D**). For the third run, pooling triplicate ARTIC PCR and purifying the PCR products allowed to significantly improve these results with medians of 91, 85, and 73%, at depth 10, 30, and 100, respectively (**Figures 1B–D**). Finally, the adaptation of elution volumes enabled further improvement of the process in run 4 with medians of 97, 94, and 89% at depth 10, 30, and 100, respectively (**Figures 1B–D**). Altogether, these modifications enabled the implementation of an adapted protocol suitable for SARS-CoV-2 genome sequencing in wastewater samples.

Impact of Freezing and SARS-CoV-2 Concentration

Following this technical adaptation, a retrospective analysis was conducted using samples stored as frozen NA extracts or raw wastewater. This allowed us to compare the sequencing depth and coverage reached with fresh and frozen material (**Figure 2** and **Supplementary Table 1**). Best coverage percentages were obtained starting from freshly prepared RNA extracts with a median of 94% ranging from 51 to 100% at depth 30 (**Figure 2**). When using frozen RNA extracts as initial matrix for cDNA synthesis, genome coverage percentages were reduced to 88% and distribution seemed more heterogeneous ranging from 24 to 99%, but this difference was not statistically significant (**Figure 2**). The use of frozen wastewater samples had a significant impact causing a strong coverage decrease and an increase in distribution



heterogeneity ($p < 0.0001$ and $p < 0.01$ when compared to fresh and frozen RNA respectively), with a median of 55% ranging from 3 to 100% (**Figure 2**). Then, we studied the impact of SARS-CoV-2 concentration, measured by qRT-PCR, on the depth 30 genome coverage (**Figure 3** and **Supplementary Table 1**). A weak correlation was observed between the two parameters when considering all samples ($p = 0.0004$, $r = 0.4881$) or the frozen RNA samples only ($p = 0.0349$, $r = 0.4153$) but not the fresh or frozen WW samples ($r = 0.3253$ and $r = 0.3522$, respectively, $p > 0.05$ for both). There was also no correlation between the extraction efficiencies and the coverage at depth 30 ($p = 0.5639$, $r = 0.08448$). Overall, these results suggest that, using our protocol, the genome coverage is mildly affected by the SARS-CoV-2 concentration in the range covered here (from 1×10^4 to 1×10^6 cRNA/L), and highlight the adverse impact of RNA extract or wastewater sample freezing on the quality of sequencing data.



ARTIC Multiplex PCR Efficiency

The ARTIC multiplex PCR V3 creates 98 overlapping amplicons enabling amplification of the full SARS-CoV-2 genome, but with potential heterogeneous yields (Tyson et al., 2020). Here, using our adapted protocol, we observed that some of these amplicons were systematically very poorly covered despite good global genome coverage (Figure 4A). These dropouts (median of sequencing depth < 30) are amplicons #9, #23, #45, #64, #66, #67, #74, #86, and #91 and span regions summarized in Figure 4B and Supplementary Table 4. Potential mutations occurring in these regions could be missed following our protocol. Two already known mutations: A2692T (synonymous) carried by the Beta VOC and T6954C (I2230T) carried by the Alpha VOC, are covered by such amplicons (#9 and #23, respectively) (Supplementary Table 4). In our study of SARS-CoV-2 VOC circulation (Figure 5), the A2692T mutation was never detected but we managed to identify high frequency SNVs for the T6954C mutation in samples exhibiting the highest sequencing depths for the #23 amplicon (Figure 4A). Eventually, the vast majority of amplicons (91%) were sequenced at a median depth > 30, enabling SARS-CoV-2 genome sequencing and the detection of most VOC signature mutations in the challenging wastewater matrix.

Circulation of SARS-CoV-2 VOC in the City

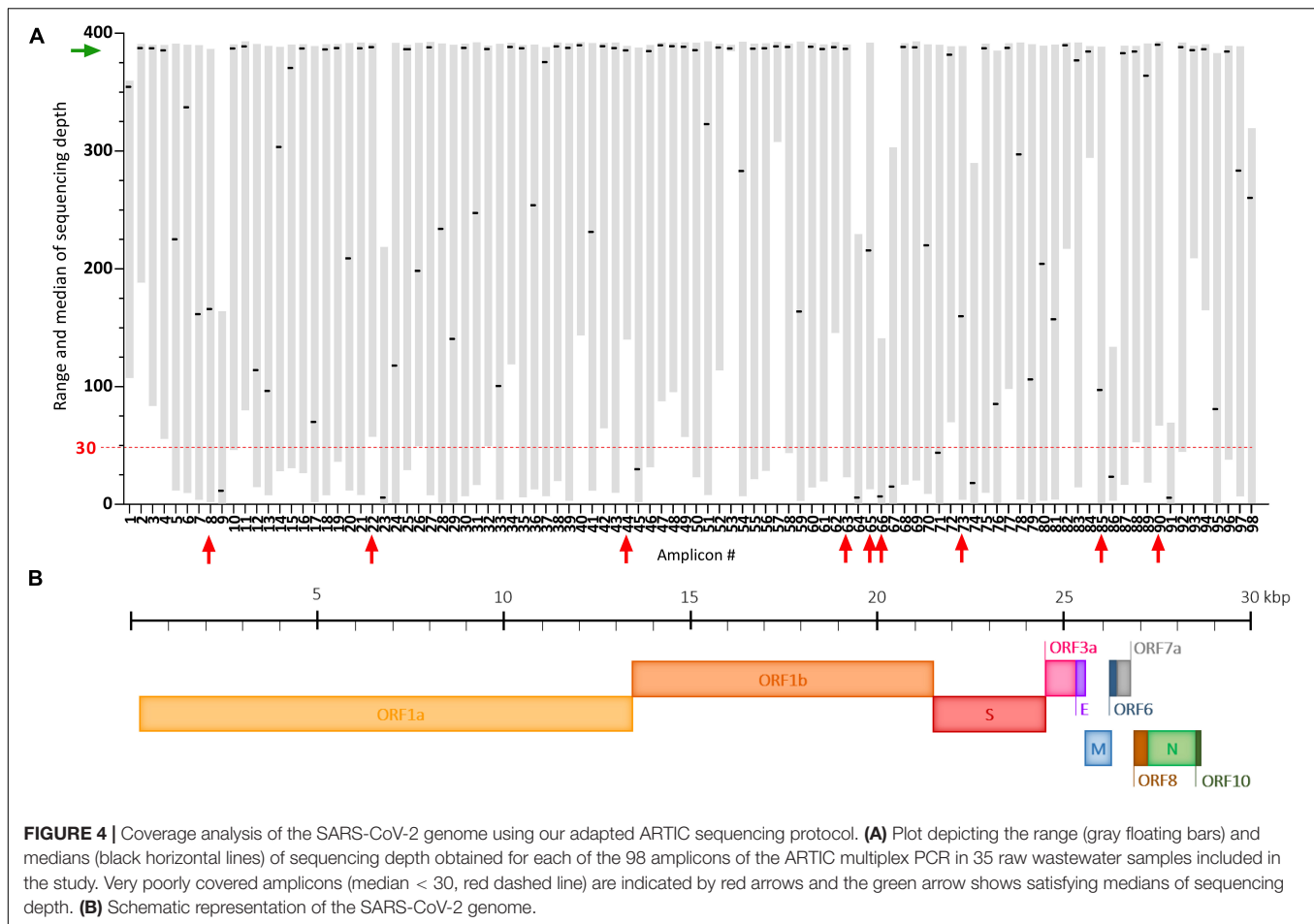
Having adapted the sequencing protocol to fit to wastewater matrix, we carried on with the analysis of SARS-CoV-2 variants in samples collected between October 2020 and May 2021 from two WWTPs (WWTP1 and WWTP2) from the same city in western France. Of 45 WW samples submitted to the

adapted sequencing protocol, we retained 35 with depth 30 coverage > 70% (ranging from 74 to 100% with a median of 88%) for analysis of VOC circulation (Supplementary Table 1). Among those, 19 (54%) came from WWTP2 and 16 (46%) from WWTP1. Detected SNVs were analyzed and compared to signature mutations of the four VOC: Alpha, Beta, Gamma, and Delta (Supplementary Table 3). Their frequencies for each sample are plotted in Figure 5. Early SARS-CoV-2 mutations such as P323L and D614G were detected throughout the period analyzed and enabled method validation. We observed multiple signature mutations of the Alpha VOC accumulating over the analyzed period (Figure 5, blue). Importantly, oldest samples (October 2020 to early November 2020) exhibit none or only one mutation of the Alpha VOC, whereas more than 25 Alpha VOC specific mutations were detected for the most recent samples (April–May 2021). Most signature mutations were detected in combination as soon as early January 2021. Altogether, these data indicate that the Alpha VOC was introduced in the city during the analyzed period to finally become predominant, probably in early February 2021. Some signature mutations of the Beta VOC were detected sporadically, sometimes as combinations of 2–3 signature mutations for the same sample, but this was erratic over time and mutation frequencies remained low. These data are compatible with a weak circulation of the Beta VOC in the studied city during this period. Finally, we found no significant occurrence of Gamma and Delta variants signature mutations over the analyzed period.

Tracking the Emergence of the Alpha VOC in the City

To better define the date of the Alpha VOC introduction in the city, we plotted the number and frequency of detected Alpha VOC signature mutations throughout the analyzed period (Figures 6A,B). From October 2020 to early November 2020, no Alpha VOC signature mutation can be detected except one (20-Oct-20 in WWTP1), for which the frequency is just above our threshold of 5%. First co-occurrences of signature mutations appear in mid-November 2020 for WWTP1 and mid-December for WWTP2, with respectively, 8 and 5 Alpha VOC specific mutations at a median frequency < 20%. From mid-November to the end of January, the number of Alpha VOC signature mutations tended to increase while the median frequencies remained around 20%. On two instances, Alpha VOC signature mutations were not detected. Finally, from February onward, the number of detected Alpha VOC signature mutations plateaued to its maximum of 20–25, while the median frequency increased to reach a maximum of 50% in April–May 2020. Individual mutation frequencies remained highly heterogeneous, varying from 10 to 85% in most samples.

Interestingly, some of these mutations being covered by the same amplicon, their presence in the same read was studied in samples corresponding to the introduction of the Alpha VOC. The three mutations responsible for the D3L substitution (28280–28282 GAT-CTA), highly specific of the Alpha VOC (see text footnote 1), were always found together on the same read for the following samples: 17-nov-20 WWTP1, 18-dec-20 WWTP2,



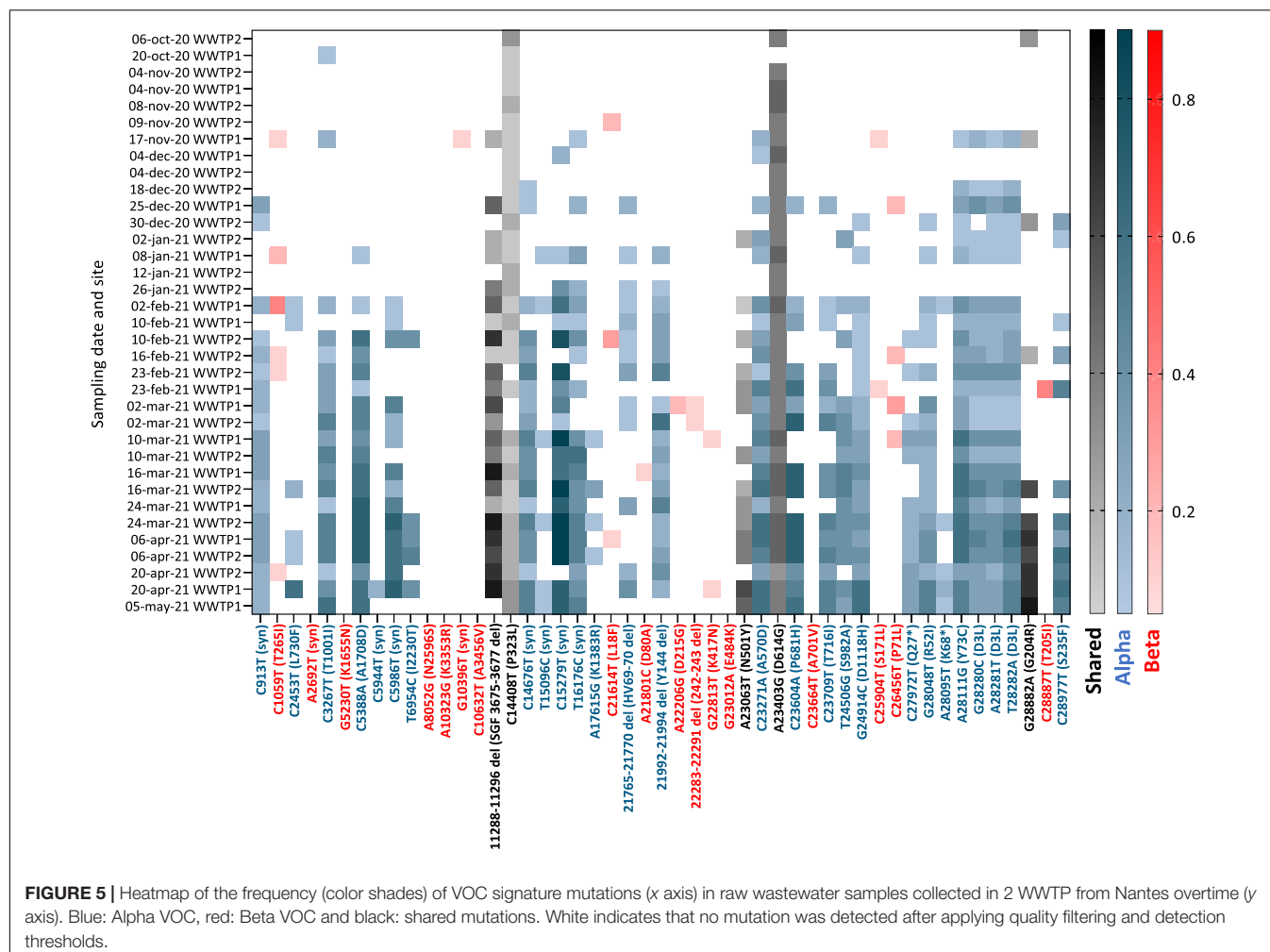
25-dec-20 WWTP1, and 30-dec-20 WWTP2 (**Supplementary Figure 1**). For the 25-dec-20 WWTP1 sample, the C23604A (P681H) and C23709T (T716I) mutations were found together in 35 out of 100 reads. Finally, for the 30-dec-20 WWTP2 sample, the G28048T (R52I) and A28111G (Y73C) mutations were identified together in 6 out of 307 reads and the G28882A (G204R) and C28977T (S235F) mutations in 21 out of 57 reads. These data show that viral strains with multiple signature mutations specific of the Alpha VOC circulated in the studied city as early as mid-November 2020.

To further validate our observations, we compared these sequencing results to quantitative data generated by two qRT-PCR assays, one SARS-CoV-2 generic qRT-PCR (IP4, see “Materials and Methods”) run on fresh samples and one specifically targeting the Δ 69/70, performed retrospectively to quantify the Alpha VOC (**Figure 7**). From December 2020 to May 2021, we detected SARS-CoV-2 genomes in wastewaters at around 10^4 – 10^5 cRNA/L. The first Δ 69/70 qRT-PCR positive results occurred on 12 January 2021 in WWTP1 (A) and 10 February 2021 in WWTP2 (B), with concentrations very close to the limit of detection (LOD). The Δ 69/70 was again detected on February 23 for both WWTPs at high levels ($>10^5$ cRNA/L). For both WWTPs, we can see a decrease at the end of March

2021, followed by a progressive increase to reach a peak at the end of April 2021. The Δ 69/70 qRT-PCR results showed more fluctuations, and detected the Alpha VOC later, than the sequencing approach, but both techniques agree on the detection of the Alpha VOC by January 2021 in the studied city, first as a minority strain, and show a gradual replacement of the initial SARS-CoV-2 strain with the Alpha VOC.

DISCUSSION

Given the increasing prevalence of new SARS-CoV-2 variants, identifying VOC and monitoring their spread in the population is crucial. SARS-CoV-2 WGS has proven to be a substantial tool facilitating the understanding of COVID-19 outbreak transmission dynamics and the surveillance of viral genetic diversity (World Health Organisation [Who], 2020). To be efficient, clinical surveillance should rely on rapid and widespread PCR testing, along with a thorough SARS-CoV-2 WGS program. In most locations equipped with a sewage collection system, the use of Environmental Surveillance (ES), through wastewater sequencing of SARS-CoV-2 genomes, could contribute to achieve this goal in a timely and cost-effective manner compared to the individual-centered testing. Sewage samples also hold



many advantages over clinical sampling considering collection is relatively easy, ethical issues and sampling bias (i.e., favoring severe cases) are limited, and only a few samples are needed to have a global picture of viral diversity in a community, including asymptomatic infections (Farkas et al., 2020; Michael-Kordatou et al., 2020). This has already been shown and used with other viruses (Lodder et al., 2012; Manor et al., 2014). Yet, they also represent a difficult matrix with a low viral concentration, hence requiring the adaptation of dedicated methods for efficient SARS-CoV-2 WGS.

Here we successfully adapted the SARS-CoV-2 sequencing technique described by the ARTIC Network (see text footnote 2) for clinical samples, to sewage samples. The ARTIC-400 multiplex PCR was shown to offer good performance with degraded or high Ct samples (Tyson et al., 2020), and thus appeared well suited for the complex wastewater matrix, considering that longer amplicons might be difficult to obtain from partially fragmented genomes (Wurtzer et al., 2021b). Since our study was conducted, others have shown that this primer scheme is indeed more efficient than others on raw influent wastewater (Lin et al., 2021). Here, compared to the published ARTIC protocol, changes were introduced at the RT, PCR and

library preparation steps to increase the initially low genome coverage breadth and depth (Figure 1). To our knowledge, performing each pool of the ARTIC multiplex PCR in triplicates and pooling them was not reported in other studies and had a major impact here, with about a 2-fold increase of coverage breadth for a given depth. This confirms that the success of WGS protocols highly depends on the availability of enough high-quality genetic material to maximize sequencing yield and the soundness of sequence data. These changes are not specific to the wastewater matrix and may also be useful for sequencing SARS-CoV-2 from difficult matrices, when the viral load is low and/or the genome fragmented.

This adapted protocol allowed us to sequence SARS-CoV-2 genomes, with high coverage depth and breadth (>70% at >30×) despite low viral concentrations as measured by qRT-PCR. We observed a weak correlation between genome coverage and SARS-CoV-2 viral load, as previously reported by similar studies using ONT sequencing (Izquierdo-Lara et al., 2021; Lin et al., 2021). Of note, the application of the adapted method on stored samples to perform retrospective studies shed light on the adverse impact of freezing wastewater samples on the quality of sequencing data. Indeed, enveloped viruses like

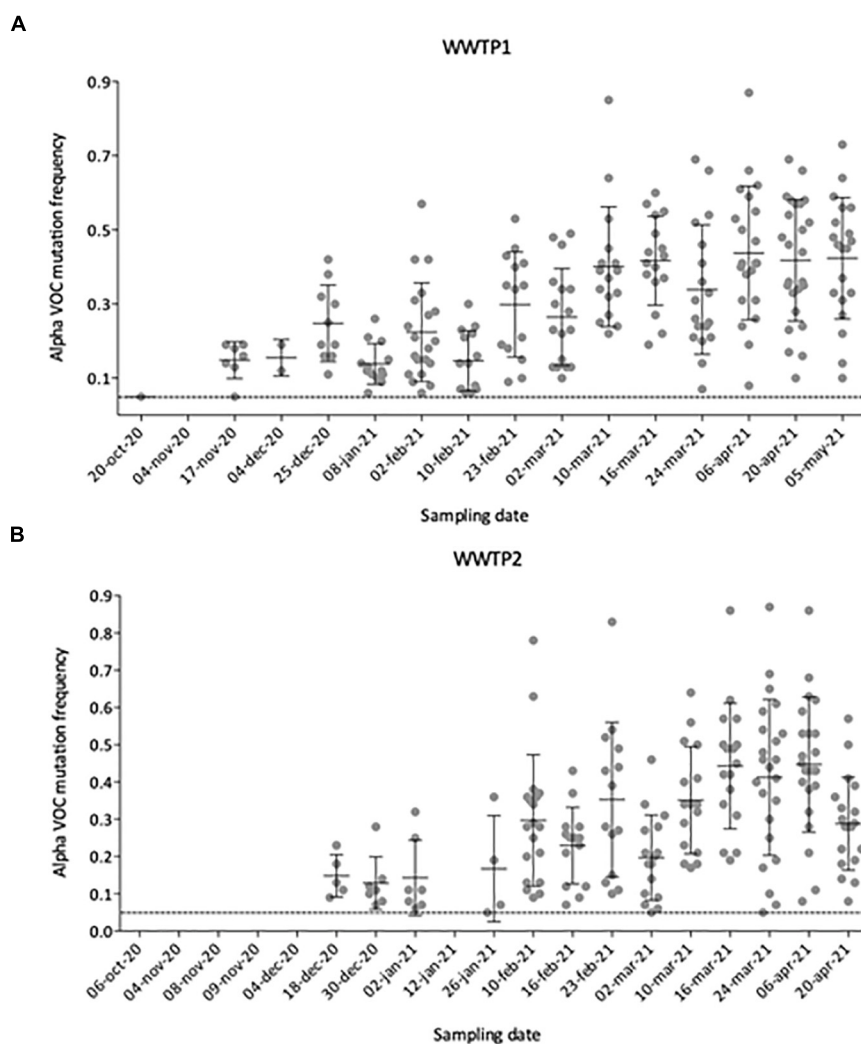


FIGURE 6 | Number and individual frequencies (dots) of SARS-CoV-2 Alpha VOC over time in 35 raw wastewater samples from WWTP1 (A) and WWTP2 (B), with median frequency (horizontal lines) and standard deviation (error bars). Only mutation with frequencies above 5% (dotted line) were considered.

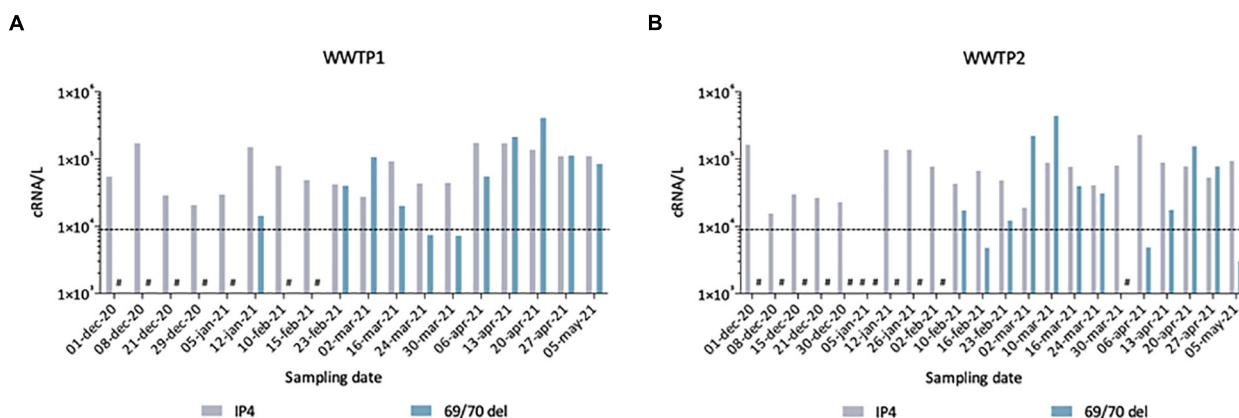


FIGURE 7 | Quantification of total SARS-CoV-2 (IP4) and Alpha VOC (69/70 del) estimated by qRT-PCR in raw wastewater samples from WWTP1 (A) and WWTP2 (B). Some samples gave signals below the theoretical limit of detection (LOD) of 9×10^3 cRNA/L (dotted line), others gave no signal/no Ct (#).

SARS-CoV-2 are commonly considered to be sensitive to freeze-thaw cycles. Besides, raw wastewater can contain detergent and other chemical products, which might contribute to disrupt the viral particles. Consequently, we recommend the use of RNA extracted from fresh wastewater samples to perform SARS-CoV-2 quantification and sequencing according to the methods described in this study.

To efficiently monitor SARS-CoV-2 variants, methods should remain fast and affordable. Thus, we favored ONT sequencing, which is known for its lower entry and per base sequencing cost (compared to second generation sequencing technologies) and its ability to generate real-time data (Chang et al., 2020). Indeed, the method we describe here can provide information within 3 days of sewage collection, including the time for sample preparation, PCR, sequencing, data export and SNV analysis, for a cost of 55 € per sample (from RNA to sequence, using flow cells twice). This is higher than the previously reported 10€ (around 12 €) per clinical sample for ARTIC V3 (Tyson et al., 2020) but is still cost-efficient for epidemiological monitoring since sequencing SARS-CoV-2 genome from a WW sample gives information at a population level compared to an individual level for a clinical sample. Both time and price could be further reduced by bulk ordering of flow cells and reagents, additional adaptations of the library preparation, higher multiplexing and automated data analysis. Furthermore, the MinION sequencer is a portable device allowing on-field sequencing in WWTP on small series of samples, which may also contribute to reduce the time-to-result in some settings. However, one important limitation of ONT is its higher error rate when compared to second generation sequencing technologies (Chang et al., 2020). To ensure reliable identification of VOC, we applied stringent thresholds combining the per-base and per-read sequencing quality, breadth and depth of coverage ($\geq 70\%$ of genome at > 30 sequencing depth), SNV frequency ($> 5\%$) as well as, for indels, homopolymer length (< 4). The absence of VOC signature mutations detection in oldest samples (October to early November 2020) confirms the validity of these thresholds. Of note, use of new flow cells with reduced error rate could allow reaching a deeper and broader coverage while reducing the thresholds to detect rare variants (R10.4, Oxford Nanopore Technologies, Oxford, United Kingdom).

Despite a satisfying production yield for a vast majority of the ARTIC amplicons, some regions were systematically absent or very poorly covered in our hands (Figure 4A). These amplicon dropouts are not news to the ARTIC Network, which already produced some work in order to fix this issue giving rise to the V3 primer scheme (Tyson et al., 2020). However, a study still reported #74 amplicon dropout with the V3 primer scheme, as in our study, and fixed the problem by adjusting concentration of its primer set (Pater et al., 2021). Other panels generating longer amplicons, designed for devices compatible with long-read sequencing such as the MinION, could also be considered. Yet, it is important to keep in mind that most studies testing these approaches were performed on clinical samples and may not reflect what occurs with wastewater samples in which targeted genomes can be fragmented and potentially not equally preserved. Indeed, we observed here dropouts for

several amplicons aside #74 that may represent WW-specific problematic regions of the viral genome, because of lower stability or higher sensitivity to PCR inhibitors.

Frequent updates of the primer panel are also necessary to adapt to new variants and avoid under-detection of certain mutations, as achieved by recently by optimizing the ARTIC V4 version for sequencing the Delta VOC (Davis et al., 2021). Importantly, the V3 primer panel was recently used to sequence the Omicron VOC in an aircraft wastewater sample, suggesting that our method can still be applied in the frame of Omicron dominance (Ahmed et al., 2022). Future updates of the ARTIC-400 primer panels could be considered to further adapt our protocol to the current circulating variants.

Major consensus genotypes detected in wastewaters were previously found to be identical to clinical genomes from the same area and can identify the predominant virus strain circulating in a population (Crits-Christoph et al., 2021). However, this approach is not suitable to identify alternative genotypes in the population being studied, which constitutes the strength of wastewater-based sequencing. In addition, it results in artificial chimeric consensus genotypes that do not depict an actual virus (Izquierdo-Lara et al., 2021). Here, we made the choice to not generate consensus genomes with sequencing data obtained from sewage samples, and rather focused on the identification of SARS-CoV-2 VOC.

We show occurrences of Alpha VOC signature mutations at high frequencies and some Beta VOC signature mutations at low frequencies, while the Gamma and Delta VOC were not observed in WW collected in the studied city. This is consistent with the known circulation of these variants in France (Santé Publique France, 2021), where the Alpha VOC became predominant during the study period, while Beta and Gamma VOC remained rare. We observed three phases of the Alpha VOC spread (Figures 6A,B). In the first phase, characterized by a unique Alpha VOC signature mutation occurring at a very low frequency, we consider that the Alpha VOC was not detected. The third phase, starting in February 2021, can be confidently interpreted as the spread of the Alpha VOC, given the high number and frequencies of signature mutations and the documented circulation of this VOC in France at the time (Gaymard et al., 2021). The second phase, between mid-November and the end of January, combines fewer signature mutations with erratic detection. Indeed, a small number of Alpha VOC-specific mutations (Figure 6), not always the same (Figure 5), were detected at low frequencies. In WWTP2 especially, some mutations detected in late December 2020 or early January 2021 were not observed with samples from January 12th and 26th. This could be the early sign of the Alpha VOC clusters appearing and disappearing in the served population. Since it might also result from the co-circulation of multiple minority strains with independent mutations, we took advantage of ARTIC amplicons spanning several of these mutations, and confirmed that multiple reads bore couples or triads of signature mutations representing true haplotypes rather than independent, randomly co-occurring SNV. Together, our results strongly suggest that the Alpha VOC or closely related strains were introduced in the studied city during November

2020. This is in agreement with previous studies where SARS-CoV-2 was sequenced in WW samples. In the United Kingdom, Wilton and his co-workers were able to detect the Alpha VOC in WWs from London as early as November 2020 by nested-PCR amplification and sequencing of two regions of the Spike gene (Wilton et al., 2021). The Alpha VOC was also detected through WGS by mid-December in WW from Switzerland (Jahn et al., 2021), in December 2020 in Israel (Bar-Or et al., 2021) and in January 2021 in Nice, France (Rios et al., 2021).

In previous studies, the frequency of the Alpha VOC in SARS-CoV-2 strains infecting the population was estimated from those of signature mutations in WW data (Jahn et al., 2021; Rios et al., 2021; Wilton et al., 2021). Here, the frequencies of the different Alpha VOC signature mutations were highly heterogeneous, comprised between 5% (our threshold) and 85% with the median plateauing at 50%. This could be due in part to differences in amplification efficiencies, since mutations covered by the same amplicon often display similar frequencies (Figure 5). This also likely arose from our choice to consider all mutations known to be specific for the Alpha VOC lineage (Supplementary Table 3), even when they emerged later or only occurred in a fraction of these viruses, such as A28095T. Yet, some Alpha VOC signature mutations, known to occur in the whole lineage, were also less frequently detected than others, such as the 21765-70 and 21992-4 deletions, which was already shown in another study combining ARTIC-400 and ONT sequencing (Rios et al., 2021) and might be due to the sequencing approach. These biases, and possibly others, result in an underestimation of the actual magnitude of Alpha VOC frequency in the population when considering median frequencies of its mutations in WW.

An alternative to WGS, mutation-specific RT-PCR, was used in parallel to detect and quantify the Alpha VOC in our samples. Its design allows targeting the SΔ69-70 mutation, highly specific of the Alpha VOC at the time of the study, with a PCR efficiency and a limit of detection in the range of classical, pan-SARS-CoV-2 qRT-PCR sets (Wurtzer et al., 2021a). Here, SΔ69-70 results were more fluctuant and belated than sequencing data, with a first detection in January followed by weeks of absence of detection before the rapid increase in Alpha VOC concentration mid-February. Since confidence in high Ct values decreases, it is known that the error estimates increase at low virus concentrations (Polo et al., 2018) such as those observed for SARS-CoV-2 in wastewaters, especially for the Alpha VOC at the beginning of its spreading the population. Our data suggest that WGS of SARS-CoV-2 is more sensitive than mutation-specific qRT-PCR assays for detecting an emerging VOC, probably because it combines the detection of multiple signature mutations. It is also necessary to confirm the co-occurrence of several mutations as haplotypes, and conclusively identify a VOC. Yet, since VOC-specific qRT-PCR can provide faster and quantitative results (Wurtzer et al., 2021a) both approaches are complementary, each addressing specific needs and phases of VOC circulation (identification vs. spread).

Another important advantage of WGS is that SNV analysis can reveal mutations that were not previously observed in the global database and could also be used to monitor novel mutations. These newly observed mutations could (1) be the

result of technical errors, like PCR mistakes or sequencing noise, (2) belong to minority (or even defective) genomes that are overlooked in clinical samples when the consensus sequence is generated, (3) be specific of the intestinal shedding of SARS-CoV-2 while most data are derived from nasopharyngeal swabs, (4) simply not persist in the population due to genetic drift or fitness disadvantage, (5) arise from non-human reservoirs also shedding into sewage (Smyth et al., 2022). Additional work on SARS-CoV-2 genetic diversity in different compartments of infected individuals, and in commensal animals, are still needed to better interpret the vast amount of information provided by WW sequencing. Yet, beside the monitoring of known VOC, this approach may contribute to discover novel viral mutations that are threatening for vaccine efficacy.

CONCLUSION

Here, we described the successful adaptation of a SARS-CoV-2 whole-genome sequencing approach for wastewater samples. This technique has the advantages of being (1) time-efficient, providing sequencing data within 3–4 days of sewage samples arriving in the laboratory and (2) cost-efficient as it gives information at a community level, (3) reliable in a range of SARS-CoV-2 RNA concentration of 10^4 – 10^5 cRNA/L. Our study also underlines the value of wastewater-based SARS-CoV-2 WGS, which detected the circulation of the Alpha VOC in a French city earlier than a specific qRT-PCR, and identified shifts in variant predominance. Nevertheless, as multiple strains of SARS-CoV-2 are mixed in sewage samples, the sequencing approach in this matrix only detects mutations in association with a genome position instead of strains in association with an individual, providing indirect proof for the presence of a lineage. Therefore, thorough comparisons with clinical data are needed in order to identify the degree and limits to which environmental surveillance could be used as an early-detection tool to support public health decision-making. Within this frame, wastewater-based SARS-CoV-2 sequencing can contribute to monitor epidemiologically or clinically relevant mutations or variants within an unbiased population.

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DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the online repository DataRef from the SeaDataCloud Network: <https://data-dataref.ifremer.fr/bioinfo/ifremer/obepine/lsem/data/dna-sequence-raw/>, 20210210-run-1, 20210303-run-2, 20210324-run-3, 20210413-run-4, 20210505-run-5, 20210507-run-6, 20210708-run-7, and 20210727-run-8.

AUTHOR CONTRIBUTIONS

LB and MD conceived and designed the study and wrote the manuscript. JS, SJ, and LB performed the experiments. SW and LM designed the Alpha VOC qRT-PCR assay. LB, JS, AB, and MD analyzed the data. MD and FL obtained the funding. LB, JS, AB, SW, LM, FL, MD, and the OBEPINE Consortium reviewed the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

- Agrawal, S., Orschler, L., and Lackner, S. (2021). Metatranscriptomic analysis reveals SARS-CoV-2 mutations in wastewater of the frankfurt metropolitan area in southern Germany. *Microbiol. Resour. Announc.* 10:e00280–21. doi: 10.1128/MRA.00280-21
- Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J. W., et al. (2020). First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. *Sci. Total Environ.* 728:138764. doi: 10.1016/j.scitotenv.2020.138764
- Ahmed, W., Bivins, A., Smith, W. J. M., Metcalfe, S., Stephens, M., Jennison, A. V., et al. (2022). Detection of the Omicron (B.1.1.529) variant of SARS-CoV-2 in aircraft wastewater. *Sci. Total Environ.* 820:153171. doi: 10.1016/j.scitotenv.2022.153171
- Ai, Y., Davis, A., Jones, D., Lemeshow, S., Tu, H., He, F., et al. (2021). Wastewater SARS-CoV-2 monitoring as a community-level COVID-19 trend tracker and variants in ohio, united states. *Sci. Total Environ.* 801:149757. doi: 10.1016/j.scitotenv.2021.149757
- Alamin, M., Tsuji, S., Hata, A., Hara-Yamamura, H., and Honda, R. (2022). Selection of surrogate viruses for process control in detection of SARS-CoV-2 in wastewater. *Sci. Total Environ.* 823:153737. doi: 10.1016/j.scitotenv.2022.153737
- Amereh, F., Negahban-Azar, M., Isazadeh, S., Dabiri, H., Masihi, N., Jahangiri-rad, M., et al. (2021). Sewage systems surveillance for SARS-CoV-2: identification of knowledge gaps, emerging threats, and future research needs. *Pathogens* 10:946. doi: 10.3390/pathogens10080946
- Bar-Or, I., Weil, M., Indenbaum, V., Bucris, E., Bar-Ilan, D., Elul, M., et al. (2021). Detection of SARS-CoV-2 variants by genomic analysis of wastewater samples in Israel. *Sci. Total Environ.* 789:148002. doi: 10.1016/j.scitotenv.2021.148002
- Barril, P. A., Pianciola, L. A., Mazzeo, M., Ousset, M. J., Jaureguiberry, M. V., Alessandrello, M., et al. (2021). Evaluation of viral concentration methods for SARS-CoV-2 recovery from wastewaters. *Sci. Total Environ.* 756:144105. doi: 10.1016/j.scitotenv.2020.144105

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.889811/full#supplementary-material>

- Bibby, K., and Peccia, J. (2013). Identification of viral pathogen diversity in sewage sludge by metagenome analysis. *Environ. Sci. Technol.* 47, 1945–1951. doi: 10.1021/es305181x
- Brić, D., Lojčić, I., Skoko, I., Kresic, N., Simic, I., Keros, T., et al. (2022). SARS-CoV-2 circulation in Croatian wastewaters and the absence of SARS-CoV-2 in bivalve molluscan shellfish. *Environ. Res.* 207:112638. doi: 10.1016/j.envres.2021.112638
- Chang, J. J. M., Ip, Y. C. A., Bauman, A. G., and Huang, D. (2020). MinION-in-ARMS: nanopore sequencing to expedite barcoding of specimen-rich macrofaunal samples from autonomous reef monitoring structures. *Front. Mar. Sci.* 7:448. doi: 10.3389/fmars.2020.00448
- Crits-Christoph, A., Kantor, R. S., Olm, M. R., Whitney, O. N., Al-Shayeb, B., Lou, Y. C., et al. (2021). Genome sequencing of sewage detects regionally prevalent SARS-CoV-2 variants. *mBio* 12, e02703–e02720. doi: 10.1128/mBio.02703-20
- Davis, J. J., Long, S. W., Christensen, P. A., Olsen, R. J., Olson, R., Shukla, M., et al. (2021). Analysis of the ARTIC version 3 and version 4 SARS-CoV-2 primers and their impact on the detection of the G142D amino acid substitution in the spike protein. *Microbiol. Spectr.* 9:e0180321. doi: 10.1128/Spectrum.01803-21
- Desdouts, M., Piquet, J. C., Wacrenier, C., Le Mennec, C., Parnaudeau, S., Jousse, S., et al. (2021). Can shellfish be used to monitor SARS-CoV-2 in the coastal environment? *Sci. Total Environ.* 778:146270. doi: 10.1016/j.scitotenv.2021.146270
- Farkas, K., Hillary, L. S., Malham, S. K., McDonald, J. E., and Jones, D. L. (2020). Wastewater and public health: the potential of wastewater surveillance for monitoring COVID-19. *Curr. Opin. Environ. Sci. Health Environ. Health* 17, 14–20. doi: 10.1016/j.coesh.2020.06.001
- Fontenele, R. S., Krabberger, S., Hadfield, J., Driver, E. M., Bowes, D., Holland, L. R. A., et al. (2021). High-Throughput sequencing of SARS-CoV-2 in wastewater provides insights into circulating variants. *medRxiv* [Preprint] doi: 10.1101/2021.01.22.21250320
- Gaymard, A., Bosetti, P., Feri, A., Destras, G., Enouf, V., Andronico, A., et al. (2021). Early assessment of diffusion and possible expansion of SARS-CoV-2 lineage 201/501Y.V1 (B.1.1.7, variant of concern 202012/01) in france, January to March 2021. *Eurosurveillance* 26:2100133. doi: 10.2807/1560-7917.ES.2021.26.9.2100133

- Hadfield, J., Megill, C., Bell, S. M., Huddleston, J., Potter, B., Callender, C., et al. (2018). Nextstrain: real-time tracking of pathogen evolution. *Bioinformatics* 34, 4121–4123. doi: 10.1093/bioinformatics/bty407
- He, J., Guo, Y., Mao, R., and Zhang, J. (2021). Proportion of asymptomatic coronavirus disease 2019: a systematic review and meta-analysis. *J. Med. Virol.* 93, 820–830. doi: 10.1002/jmv.26326
- Hillary, L. S., Farkas, K., Maher, K. H., Lucaci, A., Thorpe, J., Distaso, M. A., et al. (2021). Monitoring SARS-CoV-2 in municipal wastewater to evaluate the success of lockdown measures for controlling COVID-19 in the UK. *Water Res.* 200:117214. doi: 10.1016/j.watres.2021.117214
- Hordel, V., Kwasiborski, A., Balière, C., Matheus, S., Batéjat, C. F., Manuguerra, J.-C., et al. (2020). Rapid genomic characterization of SARS-CoV-2 by direct amplicon-based sequencing through comparison of MinION and Illumina iSeq100TM system. *Front. Microbiol.* 11:571328. doi: 10.3389/fmicb.2020.571328
- Izquierdo-Lara, R., Elsinga, G., Heijnen, L., Munnink, B. B. O., Schapendonk, C. M. E., Nieuwenhuijse, D., et al. (2021). Monitoring SARS-CoV-2 circulation and diversity through community wastewater sequencing, the Netherlands and Belgium. *Emerg. Infect. Dis.* 27, 1405–1415. doi: 10.3201/eid2705.204410
- Jahn, K., Dreifuss, D., Topolsky, I., Kull, A., Ganesanandamoorthy, P., Fernandez-Cassi, X., et al. (2021). Detection of SARS-CoV-2 variants in Switzerland by genomic analysis of wastewater samples. *medRxiv* [Preprint] doi: 10.1101/2021.01.08.21249379
- Le Guyader, F. S., Parnaudeau, S., Schaeffer, J., Bosch, A., Loisy, F., Pommepuy, M., et al. (2009). Detection and quantification of noroviruses in shellfish. *Appl. Environ. Microbiol.* 75, 618–624. doi: 10.1128/AEM.01507-08
- Lin, X., Glier, M., Kuchinski, K., Mierlo, T. R. V., McVea, D., Tyson, J. R., et al. (2021). Assessing multiplex tiling PCR sequencing approaches for detecting genomic variants of SARS-CoV-2 in municipal wastewater. *mSystems* 6:e0106821. doi: 10.1128/mSystems.01068-21
- Lodder, W. J., Buisman, A. M., Rutjes, S. A., Heijne, J. C., Teunis, P. F., and de Roda Husman, A. M. (2012). Feasibility of quantitative environmental surveillance in poliovirus eradication strategies. *Appl. Environ. Microbiol.* 78, 3800–3805. doi: 10.1128/AEM.07972-11
- Madoux-Humery, A. S., Dorner, S., Sauvé, S., Aboulfadl, K., Galarneau, M., Servais, P., et al. (2016). The effects of combined sewer overflow events on riverine sources of drinking water. *Water Res.* 92, 218–227. doi: 10.1016/j.watres.2015.12.033
- Manor, Y., Shulman, L. M., Kaliner, E., Hindiyeh, M., Ram, D., Sofer, D., et al. (2014). Intensified environmental surveillance supporting the response to wild poliovirus type 1 silent circulation in Israel, 2013. *Euro Surveill.* 19:20708. doi: 10.2807/1560-7917.es2014.19.7.20708
- Mao, K., Zhang, K., Du, W., Ali, W., Feng, X., and Zhang, H. (2020). The potential of wastewater-based epidemiology as surveillance and early warning of infectious disease outbreaks. *Curr. Opin. Environ. Sci. Health Environ. Health* 17, 1–7. doi: 10.1016/j.coesh.2020.04.006
- Martin, J., Klapsa, D., Wilton, T., Zambon, M., Bentley, E., Bujaki, E., et al. (2020). Tracking SARS-CoV-2 in sewage: evidence of changes in virus variant predominance during COVID-19 pandemic. *Viruses* 12:1144. doi: 10.3390/v12101144
- Martin, L. R., Duke, G. M., Osorio, J. E., Hall, D. J., and Palmenberg, A. C. (1996). Mutational analysis of the mengovirus poly(C) tract and surrounding heteropolymeric sequences. *J. Virol.* 70, 2027–2031. doi: 10.1128/JVI.70.3.2027-2031.1996
- Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., and Brouwer, A. (2020). Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. *Environ. Sci. Technol. Lett.* 7, 511–516. doi: 10.1021/acs.estlett.0c00357
- Michael-Kordatou, I., Karaolia, P., and Fatta-Kassinos, D. (2020). Sewage analysis as a tool for the COVID-19 pandemic response and management: the urgent need for optimised protocols for SARS-CoV-2 detection and quantification. *J. Environ. Chem. Eng.* 8:104306. doi: 10.1016/j.jece.2020.104306
- Miura, T., Lhomme, S., Le Saux, J. C., Le Mehaute, P., Guillois, Y., Couturier, E., et al. (2016). Detection of hepatitis E virus in sewage after an outbreak on a French Island. *Food Environ. Virol.* 8, 194–199. doi: 10.1007/s12560-016-9241-9
- Mondal, S., Feirer, N., Brockman, M., Preston, M. A., Teter, S. J., Ma, D., et al. (2021). A direct capture method for purification and detection of viral nucleic acid enables epidemiological surveillance of SARS-CoV-2. *Sci. Total Environ.* 795:148834. doi: 10.1016/j.scitotenv.2021.148834
- Nemudryi, A., Nemudraia, A., Wiegand, T., Surya, K., Buyukyoruk, M., Vanderwood, K. K., et al. (2020). Temporal detection and phylogenetic assessment of SARS-CoV-2 in municipal wastewater. *medRxiv* [Preprint] doi: 10.1101/2020.04.15.20066746
- Pater, A. A., Bosmeny, M. S., Pararasmpuria, M., Eddington, S. B., Ovington, K. N., Barkau, C. L., et al. (2021). High throughput nanopore sequencing of SARS-CoV-2 viral genomes from patient samples. *BioRxiv* [Preprint] doi: 10.1101/2021.02.09.430478
- Polo, D., Quintela-Balaja, M., Corbishley, A., Jones, D. L., Singer, A. C., Graham, D. W., et al. (2020). Making waves: wastewater-based epidemiology for COVID-19 - approaches and challenges for surveillance and prediction. *Water Res.* 186:116404. doi: 10.1016/j.watres.2020.116404
- Polo, D., Schaeffer, J., Teunis, P., Buchet, V., and Le Guyader, F. S. (2018). Infectivity and RNA persistence of a norovirus surrogate, the tulane virus, in oysters. *Front. Microbiol.* 9:716. doi: 10.3389/fmicb.2018.00716
- Prado, T., Fumian, T. M., Mannarino, C. F., Resende, P. C., Motta, F. C., Eppinghaus, A. L. F., et al. (2021). Wastewater-based epidemiology as a useful tool to track SARS-CoV-2 and support public health policies at municipal level in Brazil. *Water Res.* 191:116810. doi: 10.1016/j.watres.2021.116810
- Rios, G., Lacoux, C., Leclercq, V., Diamant, A., Lebrigand, K., Lazuka, A., et al. (2021). Monitoring SARS-CoV-2 variants alterations in nice neighborhoods by wastewater nanopore sequencing. *Lancet Reg. Health* 10:100202. doi: 10.1016/j.lanepe.2021.100202
- Rouchka, E. C., Chariker, J. H., Saurabh, K., Waigel, S., Zacharias, W., Zhang, M., et al. (2021). The rapid assessment of aggregated wastewater samples for genomic surveillance of SARS-CoV-2 on a city-wide scale. *Pathogens* 10:1271. doi: 10.3390/pathogens10101271
- Rubio-Acero, R., Beyerl, J., Muenchhoff, M., Roth, M. S., Castelletti, N., Paunovic, I., et al. (2021). Spatially resolved qualified sewage spot sampling to track SARS-CoV-2 dynamics in munich - one year of experience. *Sci. Total Environ.* 797:149031. doi: 10.1016/j.scitotenv.2021.149031
- Santé Publique France (2021). *Taux D'incidence de L'épidémie de COVID-19*. Available online at: <https://www.data.gouv.fr/fr/datasets/taux-d-incidence-de-lepidemie-de-covid-19/> (accessed March 2, 2022).
- Smyth, D. S., Trujillo, M., Gregory, D. A., Cheung, K., Gao, A., Graham, M., et al. (2022). Tracking cryptic SARS-CoV-2 lineages detected in NYC wastewater. *Nat. Commun.* 13:635. doi: 10.1038/s41467-022-28246-3
- Strubbia, S., Schaeffer, J., Oude Munnink, B. B., Besnard, A., Phan, M. V. T., Nieuwenhuijse, D. F., et al. (2019). Metavirome sequencing to evaluate norovirus diversity in sewage and related bioaccumulated oysters. *Front. Microbiol.* 10:2394. doi: 10.3389/fmicb.2019.02394
- Tyson, J. R., James, P., Stoddart, D., Sparks, N., Wickenhagen, A., Hall, G., et al. (2020). Improvements to the ARTIC multiplex PCR method for SARS-CoV-2 genome sequencing using nanopore. *BioRxiv* [Preprint] doi: 10.1101/2020.09.04.283077
- Wang, X. W., Li, J. S., Guo, T. K., Zhen, B., Kong, Q. X., Yi, B., et al. (2005). Concentration and detection of SARS coronavirus in sewage from Xiao Tang Shan Hospital and the 309th Hospital. *J. Virol. Methods* 128, 156–161. doi: 10.1016/j.jviromet.2005.03.022
- Wilton, T., Bujaki, E., Klapsa, D., Majumdar, M., Zambon, M., Fritzsche, M., et al. (2021). Rapid increase of SARS-CoV-2 variant B.1.1.7 detected in sewage samples from england between october 2020 and january 2021. *mSystems* 6:e0035321. doi: 10.1128/mSystems.00353-21
- World Health Organisation [Who] (2020). *SARS-CoV-2 Variant - United Kingdom of Great Britain and Northern Ireland*. Available online at: <https://www.who.int/emergencies/disease-outbreak-news/item/2020-DON304> (accessed March 2, 2022).
- Wu, F., Xiao, A., Zhang, J., Moniz, K., Endo, N., Armas, F., et al. (2022). SARS-CoV-2 RNA concentrations in wastewater foreshadow dynamics and clinical presentation of new COVID-19 cases. *Sci. Total Environ.* 805:150121. doi: 10.1016/j.scitotenv.2021.150121
- Wu, F., Zhang, J., Xiao, A., Gu, X., Lee, W. L., Armas, F., et al. (2020). SARS-CoV-2 titers in wastewater are higher than expected from clinically confirmed cases. *mSystems* 5, e614–e620. doi: 10.1128/mSystems.00614-20
- Wurtz, N., Revol, O., Jardot, P., Giraud-Gatineau, A., Houhamdi, L., Soumagnac, C., et al. (2021). Monitoring the circulation of SARS-CoV-2 variants by genomic

- analysis of wastewater in Marseille, South-East France. *Pathogens* 10:1042. doi: 10.3390/pathogens10081042
- Wurtzer, S., Marechal, V., Mouchel, J. M., Maday, Y., Teyssou, R., Richard, E., et al. (2020). Evaluation of lockdown effect on SARS-CoV-2 dynamics through viral genome quantification in waste water, greater Paris, France, 5 March to 23 April 2020. *Euro Surveill.* 25:2000776. doi: 10.2807/1560-7917.ES.2020.25.50.2000776
- Wurtzer, S., Waldman, P., Levert, M., Mouchel, J. M., Gorgé, O., Boni, M., et al. (2021a). Monitoring the propagation of SARS CoV2 variants by tracking identified mutation in wastewater using specific RT-QPCR. *medRxiv* [Preprint] doi: 10.1101/2021.03.10.21253291
- Wurtzer, S., Waldman, P., Ferrier-Rembert, A., Frenois-Veyrat, G., Mouchel, J. M., Boni, M., et al. (2021b). Several forms of SARS-CoV-2 RNA can be detected in wastewaters: implication for wastewater-based epidemiology and risk assessment. *Water Res.* 198:117183. doi: 10.1016/j.watres.2021.117183
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Systematic Approach to Address Early Pandemic's Diagnostic Unmet Needs

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During the first few months of the global Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) pandemic, the medical research community had to expeditiously develop, select, and deploy novel diagnostic methods and tools to address the numerous testing challenges presented by the novel virus. Integrating a systematic approach to diagnostic selection with a rapid validation protocol in a clinical setting can shorten the timeline to bring new technologies to practice. In response to the urgent need to provide tools for identifying SARS-CoV-2-positive individuals, we developed a framework for assessing technologies against a set of prioritized performance metrics to guide device selection. We also developed and proposed clinical validation frameworks for the rapid screening of new technologies. The rubric described here represents a versatile approach that can be extended to future technology assessments and can be implemented in preparation for future emerging pathogens.

Keywords: COVID-19, point-of-service, diagnostic, SARS-CoV-2, rubric system

INTRODUCTION

The novel coronavirus, now designated Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), responsible for the Coronavirus Disease of 2019 (COVID-19), has reportedly infected over 416 million people as of February 17, 2022 (Dong et al., 2020; Worldometer, 2021). The need to effectively triage patients, inform treatment decisions, perform contact tracing to control infectious outbreaks, and collect epidemiological data about infection spread to inform national and state-level policies have highlighted the critical importance of diagnostic testing (Binnicker, 2020). Early in the pandemic, the need for diagnostic testing was quickly recognized in resource-constrained healthcare settings having limited hospital staff, personal protective equipment (PPE) shortages, and insufficient negative pressure rooms (Ferretti et al., 2020). The ability to accurately triage SARS-CoV-2-infected patients with testing was essential to protecting

healthcare workers and patients alike. In countries with extensive contact-tracing programs such as South Korea, high volume testing paired with quarantine efforts was found to dramatically slow viral spread (Shim et al., 2020; Wrighton and Lawrence, 2020). Finally, as countries pushed to reopen their economies, it became evident that diagnostic testing would be critical not only for mass scale asymptomatic testing to enable institutions to resume operating, but also for generating the epidemiological data to closely monitor the spread of infection and inform decisions around closing and reopening businesses (Cheng et al., 2020).

As the need for diagnostics grew, the challenges and uncertainties associated with obtaining such diagnostics emerged. Some of these challenges were rooted in deconstructing the biological mechanisms mediating susceptibility to infection and disease, such as a lack of understanding of tissue and cell-specific compartmentalization during different phases of infection (Bourgonje et al., 2020; Hoffmann et al., 2020), the time course of infection and infectivity, and the nature and time course of the immune response to the virus. Other challenges were more logistical or operational; for example, what pre-existing diagnostic systems could be adapted or repurposed to detect SARS-CoV-2 to confirm COVID-19 diagnosis, what pre-existing supply chains could be leveraged or redirected in support of this effort, what sensitivity and specificity levels were required, and what infrastructure and personnel support were required and available in different kinds of locations (Frisch et al., 2021). Of particular concern was the lack of options for point-of-care/point-of-need environments, areas where trained staff and time are limited (relative to centralized laboratories), but demand was (and remains) very high.

While the critical role of diagnostics has been made clear, testing capacity and turnaround time have been significant barriers to more effective testing strategies (Clipman et al., 2021). One of the major problems faced during the first months of the pandemic was the shortage of molecular testing assays in general (Ward et al., 2020) and the absence of diagnostics that were appropriate for point-of-care settings in particular; as the diagnostic devices that were initially available were both too large and too complex to be used in decentralized patient care settings (Giri et al., 2021). More importantly, numerous factors hindered the testing capacity even when the diagnostic devices were available. As noted in the analyst report by McKinsey & Co. (Behnam et al., 2020), even when the diagnostic devices were available, there often was a shortage of sample collection supplies, required reagents, and qualified personnel to perform the tests. These supply chain challenges exacerbated the challenge posed by the inherent urgency of the need to identify infected individuals at point-of-care during a global pandemic caused by a novel pathogen.

This paper summarizes efforts developed by a diverse team of subject matter experts to rapidly address these uncertainties, provide actionable guidance to decision-makers, and create a framework that could be used to support similar analyses in the event of future pandemics. The scope of the effort was limited to early detection of COVID-19 and how to

address challenges with limited clinical indicators to minimize the time to clinical validation of the diagnostic technology. The paper aims to address the following considerations: (1) Develop a framework for the broader diagnostics and healthcare provider communities to evaluate new testing methodologies and ease future technology assessment efforts; (2) Catalyze a discussion within this research community on how to prepare for the next emerging pathogen; and (3) Propose necessary clinical validation frameworks and lessons learned from this process to inform and improve subsequent analyses.

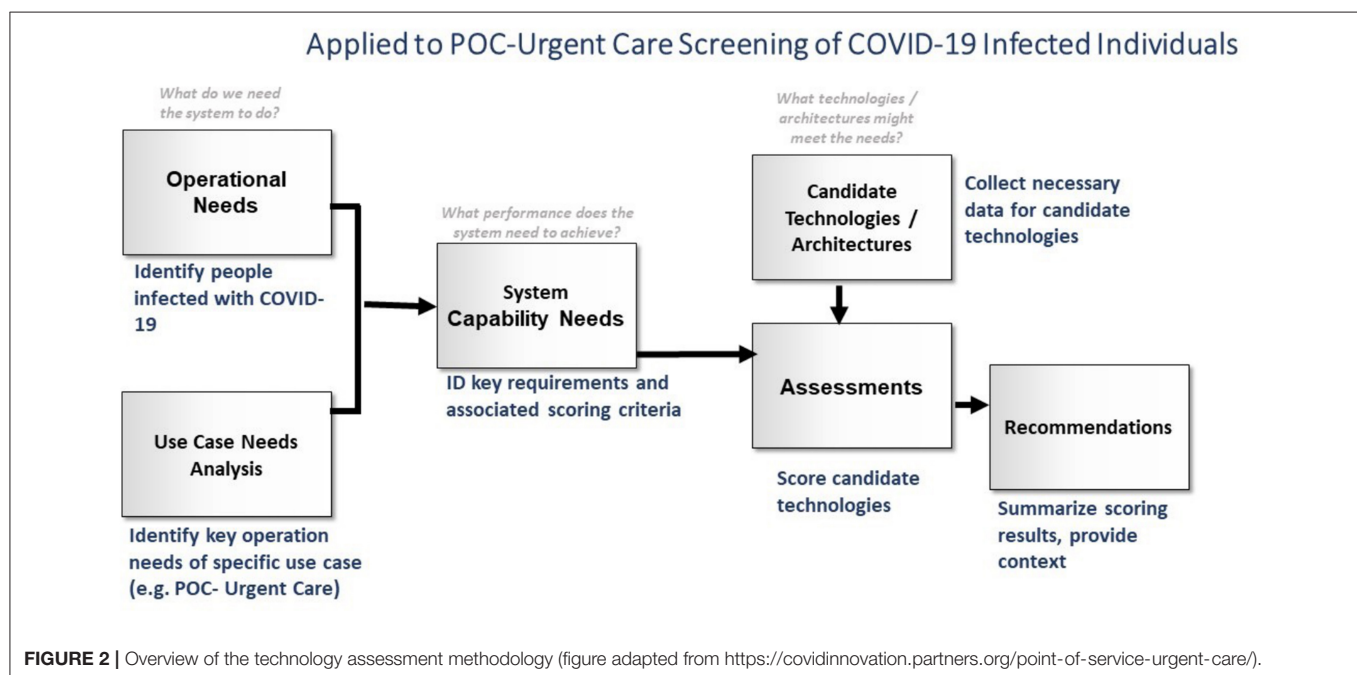
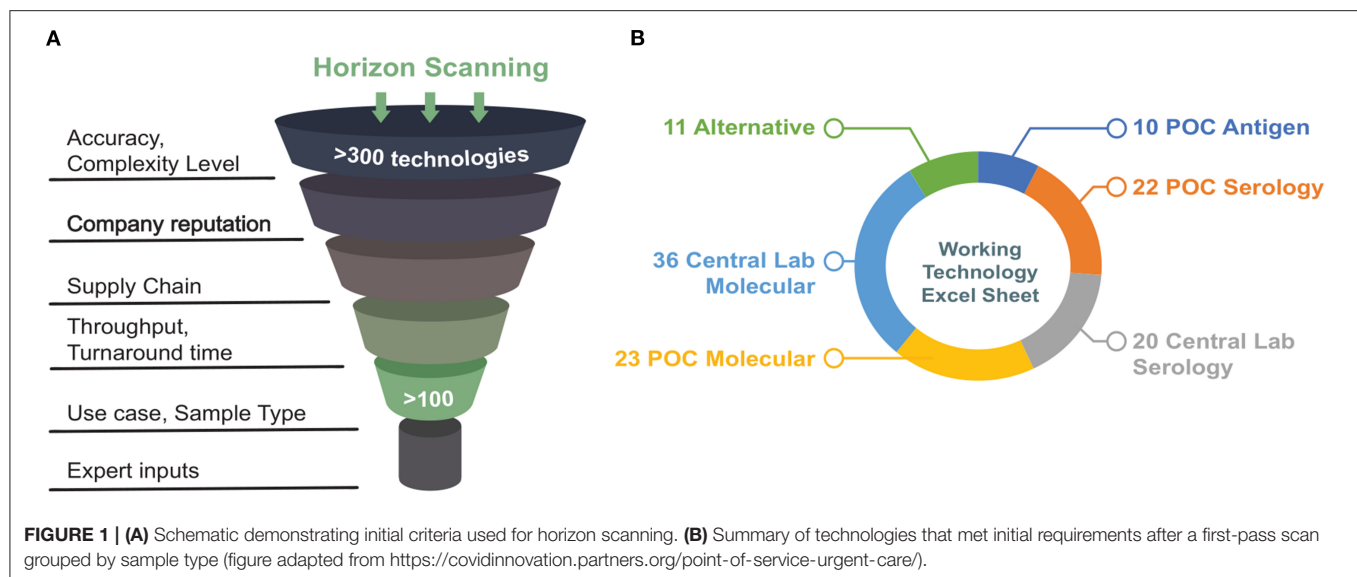
METHODS

Horizon Scanning and Acquisition of Information

A deep horizon scan of commercially available viral RNA and serology tests was performed as a first step. The results were stored in a database comprising technologies in different phases of development. The database was populated using the FDA's list of emergency use authorization (EUA)-approved and EUA-pending tests, diagnostics industry newsletters, press releases, and professional networks and online repositories. An example of one of those repositories is from the Foundation for Innovative New Diagnostics (FIND), a non-profit collaborating center of the World Health Organization (WHO). This database is an up-to-date resource of manufacturer-independent evaluation data gathered from many international laboratories for point-of-care molecular and rapid antigen tests for SARS-CoV-2, as well as serological tests to detect antibodies against SARS-CoV-2 (FIND, 2020). We developed a set of initial inclusion criteria based on sensitivity, specificity, and supply-chain logistics, formalized them into a questionnaire to consolidate information for initial assessment (**Supplementary Figure 1**). In addition to in-house evaluations from what became the Diagnostic Accelerator (DA) working group, these public evaluation results were used to guide the selection of test platforms. **Figure 1A** represents the initial criteria used for horizon scanning performed in April 2020.

Early Assessment Criteria

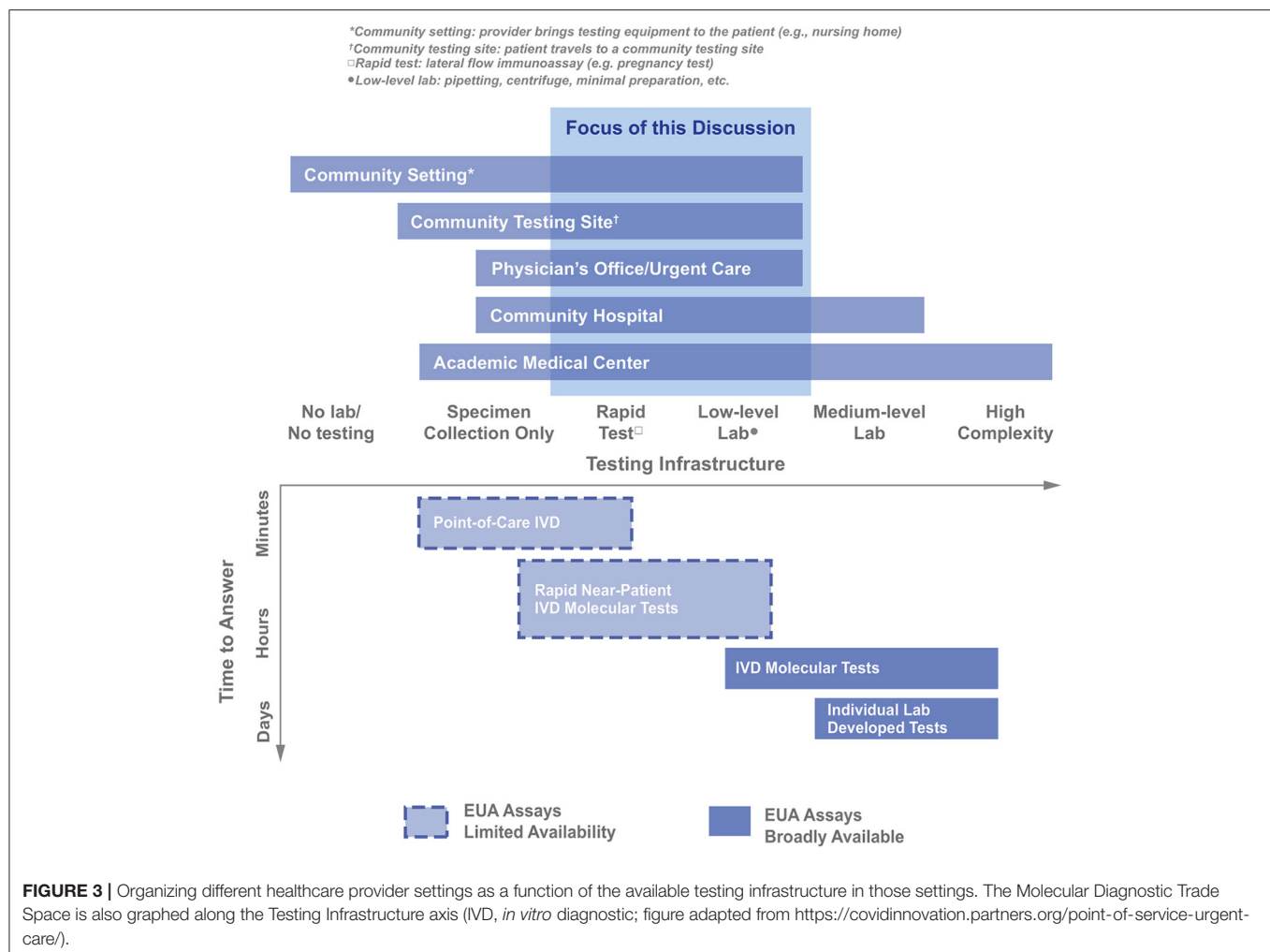
A systems analysis approach (Delaney et al., 2015) was applied to assess emerging diagnostics that might be suitable for point-of-care use. The overall goal was to provide recommendations for technologies that could be acquired, evaluated, and ideally be deployed as quickly as possible to support diagnostic needs in a clinical setting. More specifically, the focus was to review and recommend diagnostics that could be used in Point-of-Care (POC) and/or urgent care settings, and that directly sensed the presence of SARS-CoV-2 through assays targeting viral RNA. Initially, a broader range of targets was considered, the most significant of which were viral protein antigens (**Figure 1B**; **Supplementary Table 1**). However, when this review was being conducted (April-June 2020), antigen- and serology-based assays were not mature enough to be deployed immediately and did not yet provide the same degree of confidence as molecular (RNA) assays. Therefore,



this discussion will be focused exclusively on molecular assays targeting viral RNA.

The analysis focused on assessing tests that could be performed in settings, such as point-of-care (POC) environments, with fewer resources than regional hospitals and central laboratories. Testing in these settings would reduce the centralized diagnostic burden and provide more immediate responses to medical care professionals. This diagnostics assessment effort had two goals. The first and most critical was to rapidly identify the most promising technologies to address the urgent needs to counter the COVID-19 pandemic through point-of-service diagnostics that provide timely

and reliable information. The second goal was to develop a formalized way to structure, execute, and document this assessment process to inform the medical community (and others) by making this process transparent, comprehensible, and supportive of similar decision-making efforts in the future. A systems analysis-based approach, which is well-suited to identifying possible technical solutions to a challenging and complex problem, was adapted to these specific goals. While the process (**Figure 2**) is displayed linearly, feedback loops were developed between boxes to refine efforts and strengthen the final analysis. Experts in the clinical, industry, and research spheres, including hospital leadership, were consulted



frequently to ensure the recommendations would suit the clinical need.

RESULTS

Adapted Systems Analysis Approach

Systems analysis is an approach to understanding and addressing complex challenges (Delaney et al., 2015). It provides a framework for conceptualizing the assessment and decision-making process. Through a formalized, step-by-step methodology, a diverse team can reach consensus on the problems to be addressed and the solution options, rank/prioritize those options, and generate a set of consensus recommendations (Delaney et al., 2015). Formalizing and documenting this process enables the effective inclusion of new perspectives, data, and requirements, which enables the generation of updated recommendations in response to changing conditions. This formalized, documented process also provides a transparent roadmap to how recommendations were generated, which should, in theory, allow the broader community to easily understand the decision-making process

and facilitate solicitation and incorporation of feedback from those community members.

Applying this systems analysis approach to the challenges of selecting point-of-care diagnostics for COVID-19, including supply chain constraints, required two parallel efforts—(1) Understanding and defining the operational need (in this case, enabling diagnosis of SARS-CoV-2 infection in POC settings) and (2) Determining which technical options are available to meet that need (in this case, diagnostics). These parallel efforts were brought together in an assessment phase, in which “what-is-needed” is compared to “what-is-possible”, and that evaluation informs the ultimate recommendations of technologies to pursue. Ideally, these parallel efforts are described with the same terminology, enabling a clear consideration of how candidate technologies meet operational goals. However, during the early pandemic response, information about disease pathogenesis and symptomology was unclear, new diagnostic technologies appeared daily, and supply chains quickly went from a topic rarely discussed to a vital part of all decision-making processes. Supply chain measures how quickly core reagents could be obtained to perform the diagnostic tests. Such

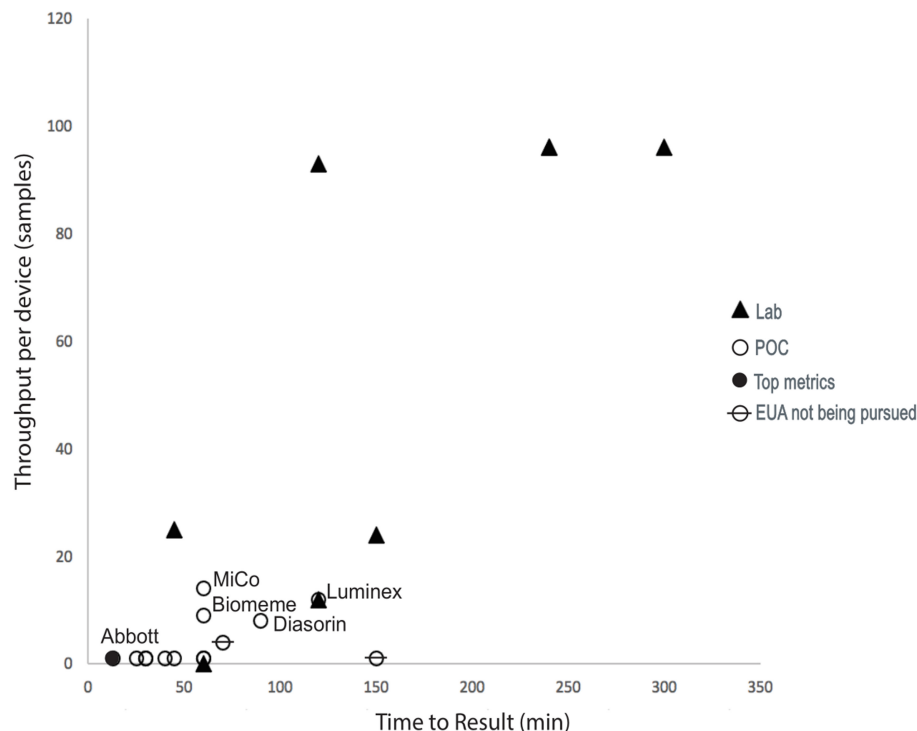


FIGURE 4 | COVID-19 assay landscape during this diagnostics assessment effort. Device throughput and time-to-result for diagnostics available in April 2020. Technologies labeled in the graph were scored with the highest metrics, including supply chain considerations. “Lab” refers to assays that required significant analysis infrastructure (e.g., PCR machines) to be available and were not therefore compatible with operation at point-of-care (POC) settings, which were assumed to have no pre-existing analysis capabilities.

reagents included pipette tips, polymerases, RNA extraction kits, specialized swabs and viral transport media, and other reagents. As a rule of thumb, we prioritized technologies where the tests could be obtained within a week. However, supply chain ebbed and flowed during different SARS-CoV-2 surges, and no single technology could fulfill the unmet need (Humble et al., 2021). Hence, we aimed to diversify the tests in evaluation, and predicted that technologies that had simpler workflows and required fewer specialized reagents to pose fewer supply chain obstacles, such as the Fluxergy CoVID-19 Sample-to-Answer RT-PCR (Rawlings et al., 2021). It became clear that the need to provide actionable information quickly precluded a complete, formal, and deliberate systems analysis. This process was supported by the redeployment of dozens of administrative and research staff throughout the Mass General Brigham (MGB) system and beyond to assist with screening and evaluation of new diagnostics as they were developed and brought to market. Key components of this process were retained as necessary to facilitate communication, optimize time spent researching technologies, and enable documentation of this fast-moving effort such that it could be readily revised as new information became available and could be leveraged by other groups facing similar challenges during this and future pandemics. Efforts were therefore focused on the aspects of the methodology that were most critical to the primary analysis—assessing diagnostic technologies for use in

POS settings—and directed toward parallel creation of both an assessment rubric and a technology summary table. Updates and preliminary findings from each group were shared daily and used to guide the work of both groups.

Operational and Use-Case Needs Analysis

The use case motivating this assessment was detecting SARS-CoV-2 infections in individuals at the point-of-care, to inform medical and public health decisions (e.g., further treatment, isolation, and patient triage). Other use cases, such as population-level surveillance, travel, and return-to-work consideration, were outside the scope of our efforts. To achieve the goal of determining which diagnostic technologies were best-suited for use in POC settings, both terminology and scope had to be defined. It became clear that the working group members had varying definitions of “point-of-care” and “diagnostic”. It was found that working within the systems analysis framework, which provides a formalized process and tools for defining key terms, allowed the team to both reach consensus and clearly document our process and terms.

It was apparent that the logistical constraints of the POC environment (e.g., infrastructure and staffing availability) would drive the analysis (Figure 3, X-axis). While there are exceptions to the organization shown above, it was agreed that this analysis described most facilities within the scope and would provide

Metrics Table for Near-Term Development

Technical		Operational					
		Logistics			Supply Chain		
Regulatory	Lower Limit of Detection (copies/mL)	Complexity*	Throughput Per Device (# samples processed in parallel)	Time to Perform (minutes)	Supply Chain/ Vendor	Supply Chain Issues – Hardware	Supply Chain Issues – Consumables
Granted	<125	Small Lab	24+	<15	Platform already in use by MGB	Leveraging Existing, already-approved Platform	1000+/week
Submitted/ Pending	125–250	Moderate-High	2–24	15–60	Ready for Validation	Available	100+/week
Planned	>250	High	1	60+	New Technology	<6 months	Backordered
Not Planned						6+ months	In Progress

Criteria Match ■ High ■ Medium ■ Low ■ Not Assessed

* Based on FDA EUA letter if available; CLIA-waived status not relevant at this time because of the inclusion of NP swabs as part of assay

FIGURE 5 | Attributes and Ranking System for POC Molecular Diagnostics. Metrics Table for Near-Term Development. (Table intended to serve as an example, criteria weighting should be adjusted to the specific use-case as needed).

a helpful framework going forward. **Figure 3** summarizes the different testing locations that may be needed to deploy COVID-19 diagnostics, highlighting the range of testing infrastructure (e.g., power, controlled environment, and equipment) that may be available. The available testing infrastructure also determined, broadly, which classes of diagnostics may be successfully administered on-site (**Figure 3**). It should be noted that, during the time of the working group's activity (April–June 2020), there were very few diagnostics with emergency use authorization (EUA) status for SARS-CoV-2 and even fewer that were compatible with use in lower-resource settings (**Figure 3**). In addition to understanding the resources available, there was a need to understand the relative advantages/drawbacks of different classes of molecular diagnostics. No single diagnostic is perfect in all ways; the group spent a significant fraction of its time discussing what “good enough” could be for different metrics and which diagnostic metrics could be relaxed so that others could be optimized. For example, as shown in **Figure 3**, if speed (minimal time-to-answer) is a top priority, then POC *in vitro* diagnostics are the most promising category; however, this class of diagnostics had limited EUA assays available.

Some aspects of the complex trade space associated with molecular diagnostics and other key metrics (not shown in the figure), such as desired time-to-answer, cost, device

throughput, and positive predictive value (PPV)/negative predictive value (NPV), were considered and are captured in the assessment rubric developed during this effort. In April 2020, clear trade-offs existed in the trade space. **Figure 4** demonstrates an inverse relationship between throughput and time-to-result POC devices and highlights those assays that were ranked highest when the assessment rubric was applied.

System Capability Needs

System capability needs refers to the capacity within the hospital or health care infrastructure to adapt and use the diagnostic devices and tests (Walton and Ivers, 2020). Based on the needs of the POC use case, the essential requirements and associated ranking criteria were identified, summarized, and prioritized to determine if the given technologies were well suited to address the operational needs. The critical categories were broadly grouped into two main areas:

- (1) Technical: Meet the diagnostic needs. This category initially included several metrics, including limit of detection (LoD), swab type, specificity, and sensitivity. However, as the analysis progressed, it was evident that three main characteristics (“assay type,” “regulatory status,” and “LoD”) were the critical categories:

- a. As noted earlier, this category initially included “type of test”—RNA vs. antigen vs. antibody; however, it was decided that, at the time this analysis was being performed, only the assays that were directed toward sensing viral RNA would provide sufficient diagnostic confidence to enable further clinical decision-making.
 - b. Regulatory status captured the EUA status of each technology.
 - c. LoD was the sole metric/category that captured assay performance. Please note that the subgroup clearly understood that assay performance is important and that “sensitivity,” “specificity,” and other associated metrics were tracked in the data table. However, for a quick assessment, those metrics were not helpful at the time: for most technologies, it was difficult to obtain this information, and, when reported, the information was rarely reported objectively to allow for ranking or assessment. It was decided that tests that had obtained EUA status would be considered to have adequate performance parameters for this near-term assessment.
- (2) Operational: Meet the logistical and supply chain requirements. The assessment began with a high-level understanding of the operational requirements of these settings and as the assessment continued, this understanding was refined and clarified. The logistical and supply chain requirements were further broken down into more specific categories, as described below:
- a. Logistics: This set of parameters was most directly impacted by the focus on POC and urgent-care settings. If the operational requirements of those locations were further refined, or if this analysis were applied to other locations, the assessment criteria in these categories would be expected to vary significantly. Within the set of logistics characteristics, the critical categories were as follows:
 - i. Assay Complexity: This category was initially defined as “CLIA-waived” but it was evident that, at least during the 2 weeks when this assessment was performed, the FDA was not assessing any assay as CLIA-waived if it included sample collection by a nasopharyngeal (NP) swab. Since during the time of this review all the technologies under consideration did include an NP swab, this category was redefined to capture the minimum necessary lab complexity required to perform a given assay. When available, this information was collected from the FDA EUA approval letter.
 - ii. Throughput-per-device and Time-to-Perform: Together, these two parameters combine to provide a first-pass estimate of the number of assays that could be performed per hour/day/shift. Since different testing locations are expected to have various limitations in terms of space, labor, and other resources, it was determined that it would be more useful to separate throughput-per-device and time-to perform as categories for this, and future, assessments.
 - b. Supply chain: Pragmatically, the best assay in the world is useless if it cannot be reliably obtained. This set of parameters focused on finding quantitative or semi-standardized ways to describe how available and reliable the supply chain was for each technology under evaluation.
 - i. Vendor: This category was the most subjective and relied upon the expertise of workgroup members in identifying established, reputable vendors. It was assumed that the more familiar the MGB, or broader, medical community was with the vendor, the more likely it was that the vendor was reliable.
 - ii. Hardware: This category captures the degree to which the hardware necessary to run a given assay was already available within the MGB community. Several assays under consideration were designed to be compatible with POC devices already commercially available and, of those, some were already in use within the MGB community. It was assumed that the more integrated these hardware platforms were within the MGB community, the more likely they could be readily available for COVID-19 screening.
 - iii. Consumables: The criteria for this category were revised several times to reflect updated feedback from different vendors. Ultimately, assays requiring the use of proprietary buffers, reagents, storage media, or swabs were examined critically concerning supply chain robustness, and assays using more widely available consumables with redundant supply chains were viewed favorably.

Assessing Candidate Technologies

A multi-pronged data collection and assessment process was developed to identify POC tests for further validation. In addition to the existence of supply chain challenges at the time of this effort, it was also clear that the use case for POC tests was rapidly evolving to include more non-traditional settings (e.g., nursing homes, drive-through testing sites, and airports). For more accurate reporting, a data collection and assessment process was developed to be adapted for different use cases by varying the weighting assigned to test characteristics of interest. Existing and emerging technologies of interest were typically those with high sensitivity and specificity. However, consideration was also given to the form factor of instruments, company reputation, throughput, turnaround time, and type of readout. Company reputation measured the credibility of companies based on their prior success in deploying diagnostics, market penetration and obtaining quality system certifications for medical devices, such as International Standards Organization (ISO) certifications. We predicted that these companies would more efficiently repurpose their existing platforms for SARS-CoV-2 diagnosis, especially if their systems had been previously validated for different diagnostic applications. Several technologies that ranked high were not available for several months and were therefore excluded from the analysis. A questionnaire with information about the technology, the parent company, and its manufacturing processes was completed for promising candidate technologies. The information was entered into a shared spreadsheet created for this purpose (**Supplementary Figure 1**). The POS working

group prioritized molecular tests at the time (April 2020), since these technologies were immediately available for deployment to meet the health care need. The group also recognized the need for evaluating and deploying rapid antigen tests, especially for decentralized and home testing. However, the supply chain of rapid tests was severely limited until the end of 2020. We were subsequently able to access some platforms to evaluate in Massachusetts (Suliman et al., 2021), and in collaboration with global partners who relied more on rapid tests to expand decentralized testing in resource-constrained settings (Kawser et al., 2022; Muthamia et al., 2022). These rapid tests necessitate different parameters in our evaluation rubric since they are known to have lower sensitivities than molecular tests, but can be powerful tools for screening highly infectious individuals with high SARS-CoV-2 viral loads (Guglielmi, 2020; Ricco et al., 2022).

If a large amount of information was missing from publicly available sources, companies were contacted *via* phone or email for additional information. Initial discussions with companies closely followed the questionnaire, and further follow-up was conducted in the case of particularly promising technologies. Working group members were briefed on appropriate questions and how to proceed with obtaining sample assays or additional information *via* a formal agreement, if applicable.

Scoring Technologies With a Rubric

As mentioned earlier, there are numerous criteria to be assessed when determining which assay system (reagents + hardware) is well-suited for a specific use case. A rubric/assessment metric system was used to assess the suitability of candidate technologies for use in POC/urgent-care settings. Documenting these decision metrics clearly and systematically facilitating discussion helped in reaching consensus. Using terminology and criteria already part of the systems needs assessment and technology assessment simplified the use of this rubric to assess technologies and drive recommendations.

At the time of this assessment, new molecular diagnostics were being announced weekly, if not daily. Top-tier criteria were identified and used as a first-cut of candidate technologies to a short-list of most promising candidates to efficiently manage limited resources and accelerate the timeline to finalize recommendations. These criteria related to pragmatic considerations of regulatory status, the possibility of acquisition, and compatibility with resources available at POS locations, including pharmacies, ambulatory services and urgent care settings.

First and foremost was emergency use authorization (EUA) status; only technologies that had submitted an EUA were considered for further assessment; while only those diagnostics that had obtained an EUA could be administered, those that had at least submitted an EUA were still kept in the appraisal because, at the time of this assessment, EUA determinations for diagnostics were progressing rapidly. It seemed possible that technologies could shift from “submitted” to “approved” within a reasonable time frame. The prioritization of key metrics was also strongly informed by the technology assessment; as it became clear that a challenging supply chain was a common

concern, the metrics for high/medium/low were modified, and it became a top-tier metric (no matter how otherwise perfect a technology option might be, if it cannot be purchased, it is not helpful). The final top-tier criterion was complexity. Given that the goal was identifying diagnostics for POC use, technology had to be usable (and approved by FDA) in a setting other than a high-complexity laboratory which is not available in most POC use cases such as Urgent Care settings. While initially this criterion was assessed based on CLIA-waived status, that had to be adjusted since, at the time of this assessment, all molecular assays required a nasopharyngeal (NP) swab and could not, therefore, be designated as CLIA-waived. Instead, we deferred to the subject matter experts on the assessment team to provide a subjective assessment of the relative complexity of the laboratory requirements necessary to a given diagnostic. We defined “complexity level” as the additional reagents and equipment needed outside the supplied system to complete the test, e.g., heat blocks and vortexes. Increasing system complexity would increase the reliance on specialized central labs and trained personnel, whereas POS testing aims to simplify and decentralize access to these diagnostics, so they can be used by health care providers outside of specialized clinical microbiology labs, such as in pharmacies, ambulatory services and urgent care settings. **Figure 5** highlights the criteria developed to accelerate, analyze, and collect information to focus on high-probability technologies.

Please note there were many other criteria collected for these technologies; this extensive data table remains a valuable resource for more in-depth analysis. One key criterion not shown in the table is cost; while certainly a priority that must be considered, at the time of this assessment there were relatively few technologies that passed the top-tier criteria and those that did were in very high demand. In situations other than a global, rapidly progressing pandemic, it is expected that cost would become a higher priority.

If a technology did not meet all these criteria at any level, it was not assessed further. Still, it did remain on a watch list so that it could be re-assessed if the criteria changed or the technology characteristics changed. It is also important to emphasize that the rubric system is adaptable to meet the testing demands in different contexts. For instance, we can adjust the rubric to assign a higher importance to low cost, and low complexity in rural resource-constrained settings, which face additional challenges (Naidoo et al., 2022).

Evaluation of Diagnostic Technologies

With the influx of POC technologies to diagnose SARS-CoV-2 infections during the COVID-19 pandemic, including those described in this paper, rigorous criteria to independently evaluate the accuracy and usability of these tests are crucial. Many of these POC tests (e.g., the Accula SARS-CoV-2 test from Mesa Biotech Inc. and the BD Veritor System for Rapid Detection of SARS-CoV-2 from BD Biosciences) obtained EUAs under the condition that they would be used in regulated settings by certified personnel in moderate to high complexity testing labs with CLIA compliance, and would need to show a Certificate of Waiver, Certificate of Compliance, or Certificate

of Accreditation, which would allow their use in some but not all health care settings. Ideally, POC tests could include non-accredited technologies that can also be used outside CLIA-compliant settings. This aspect is essential for mass screening and triaging infected individuals in the community during a pandemic. However, decentralized administration of POC tests raised concerns about the accuracy of these platforms and subsequent interpretation of test results by both providers and end-users (Syal, 2021). The pandemic necessitated expedited approvals of diagnostics by the FDA through the EUA process. Thus, the data used to obtain EUA were generally based on small and restricted sample sizes that are often not reflective of the entire population, particularly asymptomatic SARS-CoV-2 infected carriers (Oran and Topol, 2020; Pray et al., 2021; Suliman et al., 2021). Therefore, it is critical that a standardized and rigorous evaluation of the performance characteristics of these diagnostic tests be performed on samples from diverse sources, preferably by third parties with no conflicts of interest regarding the outcome of these evaluations, who can objectively recommend tests for implementation.

Our rubric system highlighted both established manufacturers (e.g., Abbott ID NowTM), and new technologies from startup companies (e.g., Fluxergy CoVID-19 Sample-to-Answer RT-PCR). Both technologies have successfully progressed in the diagnostic market, where Abbott ID NowTM has been deployed as a primary diagnostic tool by the US government, and several health care centers, with a pooled sensitivity of Abbott ID NowTM was shown to be 0.79 (95% CI, 0.69–0.86) and 1.00 (95% CI, 0.98–1.00) (Lee and Song, 2021). On the other hand, the Fluxergy COVID-19 Sample-to-Answer RT-PCR have filled a different niche, where it was used in the USA outside of health care settings, in a pooled testing back-to-work application (Rawlings et al., 2021). The company successfully obtained a CE mark, which allows for its deployment in the European market.

DISCUSSION

The emergence of SARS-CoV-2 variants harboring mutations will directly impact the performance of several diagnostics. If mutations in the SARS-CoV-2 genome impact primer binding sites for molecular tests, the rates of amplification drop-outs will increase, thereby decreasing the sensitivity of these tests. In addition, coding mutations that result in amino acid substitutions may impact the performance of rapid antigen tests that rely on antibodies that recognize the intact viral protein antigens. Therefore, evaluation frameworks that enable rapid evaluation of the performance characteristics of molecular and rapid antigen tests against SARS-CoV-2 variants remain critical.

The shifting landscape of the COVID-19 pandemic challenges our ability to define priorities for validating diagnostic platforms, as newer platforms and technologies are continually developed, rendering former ones obsolete. For instance, more sensitive rapid antigen tests may soon replace PCR platforms for certain applications such as mass surveillance of students and workers currently taking place in many college campuses and organizations, where the goal is to identify infectious individuals,

not necessarily everyone who is infected (see, for example, Larremore et al., 2021). Furthermore, access to tests with dwindling supply chain availability and prioritization of tests for immediate implementation has limited test availability for third-party researchers to conduct thorough evaluations. Earlier in the summer of 2020, the US Department of Health and Human Services (HHS) bought millions of rapid antigen tests from Becton Dickinson (BD Veritor) (Young et al., 2020; Kilic et al., 2021; Muthamia et al., 2021), Quidel (Sofia2) (Pray et al., 2021; Smith et al., 2021) and more recently, Abbott (BinaxNOW) (Okoye et al., 2021; Pilarowski et al., 2021; Pollock et al., 2021) as soon as they received EUAs based on limited samples of symptomatic individuals (HHS.gov, 2020a,b). Furthermore, the rapid changes in approval status of tests and the shifting political appetite for different testing modalities meant that the FDA priorities had to accommodate these changes accordingly. To this effect, we intend to maintain a flexible and adaptable pipeline to accommodate evaluations of different types of platforms and technologies as they arise.

The work summarized in this paper was conducted early in the pandemic and focused on assessing diagnostics that could identify if an individual was infected with SARS-CoV-2. As the pandemic enters its third year, other applications for diagnostics, such as “is this individual infectious?” or “is this individual susceptible to SARS-CoV-2 infection (or re-infection)?”, are increasingly important but remain largely unaddressed. Debates regarding the use of rapid tests (see, for example, Guglielmi, 2021) are part of a growing awareness that tests that assess, in an individual, the presence of a pathogen, pathogen component, or evidence of prior pathogen exposure, have a broader scope of use than solely informing subsequent medical decisions for that individual. These other applications, such as informing return-to-work status, may impose a different set of requirements than the more traditional diagnostics applications that are the focus of this paper. The analysis framework presented here can still be applied to facilitate discussion and consensus-building, derive appropriate requirements and prioritization, and assess available technologies against those requirements.

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

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REFERENCES

- Behnam, M., Dey, A., Gambell, T., and Talwar, V. (2020). *COVID-19: Overcoming Supply Shortages for Diagnostic Testing*. McKinsey and Co. Available online at: <https://www.mckinsey.com/industries/pharmaceuticals-and-medical-products/our-insights/covid-19-overcoming-supply-shortages-for-diagnostic-testing> (accessed August 11, 2021).
- Binnicker, M. J. (2020). Emergence of a novel coronavirus disease (COVID-19) and the importance of diagnostic testing: why partnership between clinical laboratories, public health agencies, and industry is essential to control the outbreak. *Clin. Chem.* 66, 664–666. doi: 10.1093/clinchem/hvaa071
- Bourgonje, A. R., Abdulle, A. E., Timens, W., Hillebrands, J. L., Navis, G. J., Gordijn, S. J., et al. (2020). Angiotensin-converting enzyme 2 (ACE2), SARS-CoV-2 and the pathophysiology of coronavirus disease 2019 (COVID-19). *J. Pathol.* 251, 228–248. doi: 10.1002/path.5471
- Cheng, M. P., Papenburg, J., Desjardins, M., Kanjilal, S., Quach, C., Libman, M., et al. (2020). Diagnostic testing for severe acute respiratory syndrome-related coronavirus 2: a narrative review. *Ann. Intern. Med.* 172, 726–734. doi: 10.7326/M20-1301
- Clipman, S. J., Wesolowski, A., Mehta, S. H., Cobey, S., Cummings, D. A. T., and Solomon, S. S. (2021). Improvements in SARS-CoV-2 testing cascade in the US: data from serial cross-sectional assessments. *Clin. Infect. Dis.* 74, 1534–1542. doi: 10.1093/cid/ciab683
- Delaney, B. C., Curcin, V., Andreasson, A., Arvanitis, T. N., Bastiaens, H., Corrigan, D., et al. (2015). Translational medicine and patient safety in Europe: TRANSFoRM—architecture for the learning health system in Europe. *Biomed. Res. Int.* 2015, 961526. doi: 10.1155/2015/961526
- Dong, E., Du, H., and Gardner, L. (2020). An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infect. Dis.* 20, 533–534. doi: 10.1016/S1473-3099(20)30120-1
- Ferretti, L., Wymant, C., Kendall, M., Zhao, L., Nurtay, A., Abeler-Dörner, L., et al. (2020). Quantifying SARS-CoV-2 transmission suggests epidemic control with digital contact tracing. *Science* 368, 6491. doi: 10.1126/science.abb6936
- FIND (2020). *FIND (Foundation for Innovative New Diagnostics) Evaluation Update: SARS-CoV-2 Immunoassays*. Access to COVID-19 Tools (ACT) Accelerator. Available online at: <https://www.finddx.org/sarscov2-eval-antibody/> (accessed March 28, 2021).
- Frisch, M. F., Scott, K. W., and Binagwaho, A. (2021). An implementation research approach to re-orient health supply chains toward an equity agenda in the COVID-19 era. *Ann. Glob. Health* 87, 42. doi: 10.5334/aogh.3209
- Giri, B., Pandey, S., Shrestha, R., Pokharel, K., Ligler, F. S., and Neupane, B. B. (2021). Review of analytical performance of COVID-19 detection methods. *Anal. Bioanal. Chem.* 413, 35–48. doi: 10.1007/s00216-020-02889-x
- Guglielmi, G. (2020). The explosion of new coronavirus tests that could help to end the pandemic. *Nature* 583, 506–509. doi: 10.1038/d41586-020-02140-8
- Guglielmi, G. (2021). Rapid coronavirus tests: a guide for the perplexed. *Nature* 590, 202–205. doi: 10.1038/d41586-021-00332-4
- HHS.gov (2020a). *Trump Administration Announces Initiative for More and Faster COVID-19 Testing in Nursing Homes*. Available online: <https://www.hhs.gov/about/news/2020/07/14/trump-administration-announces-initiative-more-faster-covid-19-testing-nursing-homes.html> (accessed August 11, 2021).
- HHS.gov (2020b). *Trump Administration Will Deploy 150 Million Rapid Tests in 2020*. Available online at: <https://public3.pagefreezer.com/browse/HHS%20%E2%80%9393%C2%A0About%20News/20-01-2021T12:29/https://www.hhs.gov/about/news/2020/08/27/trump-administration-will-deploy-150-million-rapid-tests-in-2020.html> (August 11, 2021).
- Hoffmann, M., Kleine-Weber, H., Schroeder, S., Kruger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181, 271–280.e278. doi: 10.1016/j.cell.2020.02.052
- Humble, R. M., Merrill, A. E., Ford, B. A., Diekema, D. J., and Krasowski, D. M. (2021). Practical considerations for implementation of SARS-CoV-2 serological testing in the clinical laboratory: experience at an academic medical center. *Acad. Pathol.* 8, 23742895211002802. doi: 10.1177/23742895211002802
- Kawser, Z., Hossain, M., Suliman, S., Lockman, S., Gitaka, J., Bandawe, G., et al. (2022). An assessment of a rapid SARS-CoV-2 antigen tests in Bangladesh. *IJTMH*. (in press). doi: 10.1101/2021.10.05.21264551
- Kilic, A., Hiestand, B., and Palavecino, E. (2021). Evaluation of performance of the BD veritor SARS-CoV-2 chromatographic immunoassay test in COVID-19 symptomatic patients. *J. Clin. Microbiol.* 59, e00260–21. doi: 10.1128/JCM.00260-21
- Larremore, D. B., Wilder, B., Lester, E., Shehata, S., Burke, J. M., Hay, J. A., et al. (2021). Test sensitivity is secondary to frequency and turnaround time for COVID-19 screening. *Sci. Adv.* 7, eabd5393. doi: 10.1126/sciadv.abd5393
- Lee, J., and Song, J. U. (2021). Diagnostic accuracy of the Cepheid Xpert Xpress and the Abbott ID NOW assay for rapid detection of SARS-CoV-2: a systematic review and meta-analysis. *J. Med. Virol.* 93, 4523–4531. doi: 10.1002/jmv.26994
- Muthamia, E., Mungai, S., Mungai, M., Bandawe, G., Qadri, F., Kawser, Z., et al. (2022). JGJ. assessment of performance and implementation characteristics of rapid point of care SARS-CoV-2 antigen testing. *AAS Open Res.* 5, 10. doi: 10.12688/aasopenres.13323.1
- Muthamia, E., Mungai, S., Mungai, M., Bandawe, G., Qadri, F., Kawser, Z., et al. (2021). JGJ. assessment of performance and implementation characteristics of rapid point of care SARS-CoV-2 antigen testing. *MedRxiv*. doi: 10.1101/2021.06.03.21258290
- Naidoo, S., Gitaka, J., Suliman, S., Baptista, S., Oyedemi, B. M., Nepolo, E., et al. (2022). Coronavirus disease 2019 diagnostics: key to Africa's recovery. *DNA Cell Biol.* 41, 30–33. doi: 10.1089/dna.2021.0540
- Okoye, N. C., Barker, A. P., Curtis, K., Orlandi, R. R., Snively, E. A., Wright, C., et al. (2021). Performance characteristics of BinaxNOW COVID-19 antigen card for screening asymptomatic individuals in a university setting. *J. Clin. Microbiol.* 59, e03282–20. doi: 10.1128/JCM.03282-20
- Oran, D. P., and Topol, E. J. (2020). Prevalence of asymptomatic SARS-CoV-2 infection: a narrative review. *Ann. Intern. Med.* 173, 362–367. doi: 10.7326/M20-3012
- Pilarowski, G., Lebel, P., Sunshine, S., Liu, J., Crawford, E., Marquez, C., et al. (2021). Performance characteristics of a rapid SARS-CoV-2 antigen detection assay at a public plaza testing site in San Francisco. *J. Infect. Dis.* 223, 1139–1144. doi: 10.1093/infdis/jiaa802
- Pollock, N. R., Jacobs, J. R., Tran, K., Cranston, A. E., Smith, S., O'Kane, C. Y., et al. (2021). Performance and implementation evaluation of the Abbott BinaxNOW rapid antigen test in a high-throughput drive-through community testing site in Massachusetts. *J. Clin. Microbiol.* 59, e00083–21. doi: 10.1128/JCM.00083-21
- Pray, I. W., Ford, L., Cole, D., Lee, C., Bigouette, J. P., Abedi, G. R., et al. (2021). Performance of an antigen-based test for asymptomatic and symptomatic SARS-CoV-2 testing at two University Campuses - Wisconsin,

- September–October 2020. *MMWR Morb. Mortal. Wkly. Rep.* 69, 1642–1647. doi: 10.15585/mmwr.mm695152a3
- Rawlings, S. A., Scott, B., Layman, L., Naranatt, P., Heltsley, R., Ignacio, C., et al. (2021). Can't work from home: pooled nucleic acid testing of laboratory workers during the COVID-19 pandemic. *Open Forum. Infect. Dis.* 8, ofab129. doi: 10.1093/ofid/ofab129
- Ricco, M., Ranzieri, S., Peruzzi, S., Valente, M., Marchesi, F., Bragazzi, N. L., et al. (2022). Antigen detection tests for SARS-CoV-2: a systematic review and meta-analysis on real world data. *Acta Biomed.* 93, e2022036. doi: 10.23750/abm.v93i2.11031
- Shim, E., Tariq, A., Choi, W., Lee, Y., and Chowell, G. (2020). Transmission potential and severity of COVID-19 in South Korea. *Int. J. Infect. Dis.* 93, 339–344. doi: 10.1016/j.ijid.2020.03.031
- Smith, R. L., Gibson, L. L., Martinez, P. P., Ke, R., Mirza, A., Conte, M., et al. (2021). Longitudinal assessment of diagnostic test performance over the course of acute SARS-CoV-2 infection. *medRxiv.* 224, 976–982. doi: 10.1093/infdis/jiab337
- Suliman, S., Matias, W. R., Fulcher, I. R., Molano, F. J., Collins, S., Uceta, E., et al. (2021). Evaluation of the Access Bio CareStart™ rapid SARS-CoV-2 antigen test in asymptomatic individuals tested at a community mass-testing program in Western Massachusetts. *Submitted.* doi: 10.1101/2021.06.17.21259109
- Syal, K. (2021). Guidelines on newly identified limitations of diagnostic tools for COVID-19 and consequences. *J. Med. Virol.* 93, 1837–1842. doi: 10.1002/jmv.26673
- Walton, D. A., and Ivers, L. C. (2020). Facility-level approaches for COVID-19 when caseload surpasses surge capacity. *Am. J. Trop. Med. Hyg.* 103, 605–608. doi: 10.4269/ajtmh.20-0681
- Ward, S., Lindsley, A., Courter, J., and Assa'ad, A. (2020). Clinical testing for COVID-19. *J. Allergy Clin. Immunol.* 146, 23–34. doi: 10.1016/j.jaci.2020.05.012
- Worldometer (2021). *United States Coronavirus: 7,833,763 Cases and 217,738 Deaths.* Available online: <https://www.worldometers.info/coronavirus/country/us/> (accessed August 11, 2021).
- Wrighton, M. S., and Lawrence, S. J. (2020). Reopening colleges and universities during the COVID-19 pandemic. *Ann. Intern. Med.* 173, 664–665. doi: 10.7326/M20-4752
- Young, S., Taylor, S. N., Cammarata, C. L., Varnado, K. G., Roger-Dalbert, C., Montano, A., et al. (2020). Clinical evaluation of BD Veritor SARS-CoV-2 point-of-care test performance compared to PCR-based testing and versus the Sofia 2 SARS Antigen point-of-care test. *J. Clin. Microbiol.* 59, e02338–20. doi: 10.1128/JCM.02338-20

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Combination of Isothermal Recombinase-Aided Amplification and CRISPR-Cas12a-Mediated Assay for Rapid Detection of Major Severe Acute Respiratory Syndrome Coronavirus 2 Variants of Concern

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Coronavirus disease 2019 (COVID-19) pandemic caused by SARS-CoV-2 variants is a new and unsolved threat; therefore, it is an urgent and unmet need to develop a simple and rapid method for detecting and tracking SARS-CoV-2 variants. The spike gene of SARS-CoV-2 was amplified by isothermal recombinase-aided amplification (RAA) followed by the cleavage of CRISPR-Cas12a in which five allele-specific crRNAs and two Omicron-specific crRNAs were designed to detect and distinguish major SARS-CoV-2 variants of concerns (VOCs), including alpha, beta, delta variants, and Omicron sublineages BA.1 and BA.2. The whole reaction can be carried out in one tube at 39°C within 1.5–2 h, and the results can be read out by a fluorescence meter or naked eyes. Our results show that the RAA/CRISPR-Cas12a-based assay could readily distinguish the signature mutations, i.e., K417N, T478K, E484K, N501Y, and D614G, with a sensitivity of 100.0% and a specificity of 94.9–100.0%, respectively. The assay had a low limit of detection (LOD) of 10⁴ copies/reaction and a concordance of 92.59% with Sanger sequencing results when detecting 54 SARS-CoV-2 positive clinical samples. The two Omicron-specific crRNAs can readily and correctly distinguish Omicron BA.1 and BA.2 sublineages with a LOD of as low as 20 copies/reaction. Furthermore, no cross-reaction was observed for all crRNAs analyzed when detecting clinical samples infected with 11 common respiratory pathogens. The combination of isothermal amplification and CRISPR-Cas12a-mediated assay is suitable for rapid detection of major SARS-CoV-2 variants in point-of-care testing and in resource-limiting settings. This simple assay could be quickly updated for emerging variants and implemented to routinely monitor and track the spread of SARS-CoV-2 variants.

Keywords: SARS-CoV-2, isothermal amplification, CRISPR-Cas12a, variants of concern, variant genotyping

INTRODUCTION

The ongoing pandemic of coronavirus disease 2019 (COVID-19) caused by the emerging variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a great challenge for the prevention and control of the COVID-19 epidemic (Tao et al., 2021; Dong et al., 2022). According to the relevant biological properties and public health concerns, these emerged variants have been classified by the World Health Organization (WHO) into variants of concern (VOCs, including alpha, beta, gamma, delta, and recently identified Omicron), variants of interest (VOIs, including lambda and mu), or variants under monitoring (VUMs, including kappa, iota, and eta; World Health Organization [WHO], 2022). The SARS-CoV-2 variants are mainly characterized by the signature mutations in the spike protein, which are proved to be associated with higher transmissibility and virulence (Dong et al., 2021; Khandia et al., 2022), and compromise the efficacy of COVID-19 vaccines (Brown et al., 2021; Hacisuleyman et al., 2021; Kroidl et al., 2021; Rovida et al., 2021; Vignier et al., 2021). Therefore, the ability to rapidly screen and monitor the spread of SARS-CoV-2 variants is essential to control the COVID-19 pandemic and to timely adjust vaccination strategy.

There are several methods to identify and detect SARS-CoV-2 mutations and variants, such as viral whole-genome sequencing, although the cost and complexity may limit its accessibility (Chiara et al., 2021), reverse transcription-polymerase chain reactions (RT-PCR)-based nucleic acid tests, which are the gold-standard technology for diagnosis of SARS-CoV-2 infection and have been developed to distinguish SARS-CoV-2 variants (Vega-Magaña et al., 2021; Vogels et al., 2021; Wang et al., 2021; Zelyas et al., 2021). However, the above two methods are time-consuming and labor-intensive and are difficult to be widely implemented. At present, only a few methods for rapidly detecting SARS-CoV-2 variants have been reported (de Puig et al., 2021; Welch et al., 2022).

In the past decades, the CRISPR-Cas-based detection platform has emerged as the next-generation of molecular diagnostics and has become a powerful tool for pathogen detection or genotyping by using specific CRISPR RNAs (crRNAs). The Cas13a-based SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) platform was able to identify subtypes of Zika virus and dengue virus (Gootenberg et al., 2017, 2018), whereas the Cas12a-based DETECTR (DNA endonuclease-targeted CRISPR *trans* reporter) platform could discriminate between genotypes 16 and 18 of HPV (Chen et al., 2018). The detection sensitivity can be further enhanced by combining it with a pre-amplification step such as isothermal enzymatic reaction to fulfill clinical requirements. CRISPR-Cas-based assays have been developed for detecting SARS-CoV-2 (Broughton et al., 2020; Joung et al., 2020).

For the purpose of characterizing and differentiating the major VOCs of SARS-CoV-2 by using CRISPR-based assays, it is required to carefully design and select specific crRNAs that can discriminate single-nucleotide mutations in the target sequences. We have previously reported a system to combine RT-PCR and CRISPR-Cas12a-mediated assay to detect major VOCs

of SARS-CoV-2 with high sensitivity and specificity (Liang et al., 2021), because mismatches between the crRNAs and the target sequences would inhibit the cleavage activity of Cas12a proteins and could be adapted to distinguish SARS-CoV-2 variants. Although the aforementioned RT-PCR/CRISPR-Cas12a-based approach is affordable, simple, and rapid, it is still not feasible for point-of-care testing (POCT) because qPCR equipment and facilities are required. Multiple reactions increase the complexity of testing and the risk of contamination by PCR products. Herein, we refined the system by integrating isothermal recombinase-aided amplification (RAA) technology with a CRISPR-Cas12a-mediated assay to develop a one-tube genotyping assay for major SARS-CoV-2 VOCs, including Omicron sub-lineages BA.1 and BA.2. In our RAA/CRISPR-Cas12a-mediated assay, nucleic acid amplification and CRISPR-Cas12a-mediated cleavage could be processed in one tube at 39°C within 1.5–2 h without the need of high-end facilities or trained technicians. The results can be read out by fluorescence meter or judged by naked eyes.

MATERIALS AND METHODS

Clinical Samples

A total of 54 SARS-CoV-2 positive samples, including 4 nucleic acid samples and 50 oropharyngeal specimens, were included in this study. A total of 50 oropharyngeal specimens were confirmed by real-time quantitative reverse transcription PCR assay (Easydiagnosis Biomedicine Co., Ltd., Wuhan, China) targeting both ORF1a/b and nucleocapsid (NP) genes of SARS-CoV-2 and by Sanger sequencing in the Guangdong Provincial Center for Diseases Prevention and Control between March 2020 and December 2021. Demographic data including sampling date, age, gender, infection sources, and disease stages, but no patient identification information, were collected (**Supplementary Table 1**). In addition, a total of 19 SARS-CoV-2 negative clinical samples infected with various respiratory pathogens collected before the COVID-19 pandemic were used as negative controls and for the evaluation of assay specificity. These respiratory pathogens include common human coronavirus (HCoV) 229E, OC43, and HKU1 as well as rhinovirus (HRV), adenovirus (ADV), respiratory syncytial virus (RSV) A and B, human bocavirus (HBoV), human metapneumovirus (HMPV), and human parainfluenza virus one (HPIV-1) and four (HPIV-4). Written informed consent was obtained from all subjects enrolled in this study. Research protocols were in accordance with the Declaration of Helsinki.

Cas12a Proteins

The LbCas12a gene of the Lachnospiraceae bacterium (Addgene #69988) and AsCas12a of *Acidaminococcus* sp. (Addgene #114073) were cloned into expression vector pET-28a (+) and transformed into DE3 competent cells (TransGen Biotech, Beijing, China), respectively, to express LbCas12a and AsCas12a proteins in our laboratory. Expressed proteins were purified on HisTrap HP columns (Marlborough, MA,

United States), and eluted proteins were dialyzed in storage buffer (600 mM NaCl, 5% glycerol, 2 mM DTT, 50 mM Tris-HCl, pH 7.5; **Supplementary Figure 1**). The concentration of purified proteins was further quantitated using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States). Aliquots of purified proteins were stored at -80°C until use. In addition, we also purchased LbCas12a (New England Biolabs, Ipswich, MA, United States) and LbCas12a (Bio-lifesci, Guangzhou, China) to develop a CRISPR-Cas12a assay.

Construction of the Plasmids of Severe Acute Respiratory Syndrome Coronavirus 2 Spike Gene

The full-length genomic fragment (nt21,563–25,384) of the spike (S) protein of the SARS-CoV-2 wild-type strain (GenBank accession no. MN908947) and the mutant S gene containing mutations of L5F, D80A, D215G, R246I, K417N, L452R, Y453F, T478K, E484Q, N501Y, A570D, D614G, P681H, A701V, T716I, S982A, D1118H, P1263L, and the gene fragment of Omicron sublineages BA.1 and BA.2 spike proteins were synthesized and inserted into the vector pUC57 (Sangon Biotech Co., Ltd., Shanghai, China) to be used as templates for developing the CRISPR-Cas12a assay. The detailed information on the plasmids used in this study is available in **Supplementary Table 2**.

Design and Preparation of the Primers and crRNAs

The primers used for isothermal amplification were designed to target the conserved sequences of the SARS-CoV-2 spike gene according to the manufacturer's instructions of the recombinase-aided amplification nucleic acid amplification kit (Qitian, Jiangsu, China). The length of the forward and reverse primers was 32–37 nucleotides (nt), and the melting temperatures were around $54\text{--}67^{\circ}\text{C}$. The expected amplicon size was 209–523 bp. Since a T-rich protospacer adjacent motif (PAM, 5'-TTTN-3', where N refers to A/G/C) sequence at the 5' terminus of the target sequence is necessary for the activation of Cas12a protein (Zetsche et al., 2015), an artificial PAM sequence was inserted into the primers to produce amplified products with a PAM motif when necessary.

We downloaded from the GenBank database, the sequences of spike protein of wild-type and major SARS-CoV-2 variants were collected from different countries or regions and conducted alignment analysis (**Supplementary Figure 2**), and 5 signature mutations in the spike protein (K417N, T478K, E484K, N501Y, and D614G) were identified and selected for developing a CRISPR-Cas12a-based assay (**Supplementary Table 3**). A total of 5 allele-specific crRNAs targeting the aforementioned 5 signature mutations were designed according to the working principle of the CRISPR-Cas12a system (Zetsche et al., 2015). In addition, two Omicron-specific crRNAs were designed, i.e., an Omicron sublineage BA.1-specific crRNA (crRNA-S-49X) covering Q493R, G496S, and Q498R mutations and an Omicron-specific crRNA (crRNA-S-50X) covering Q498R

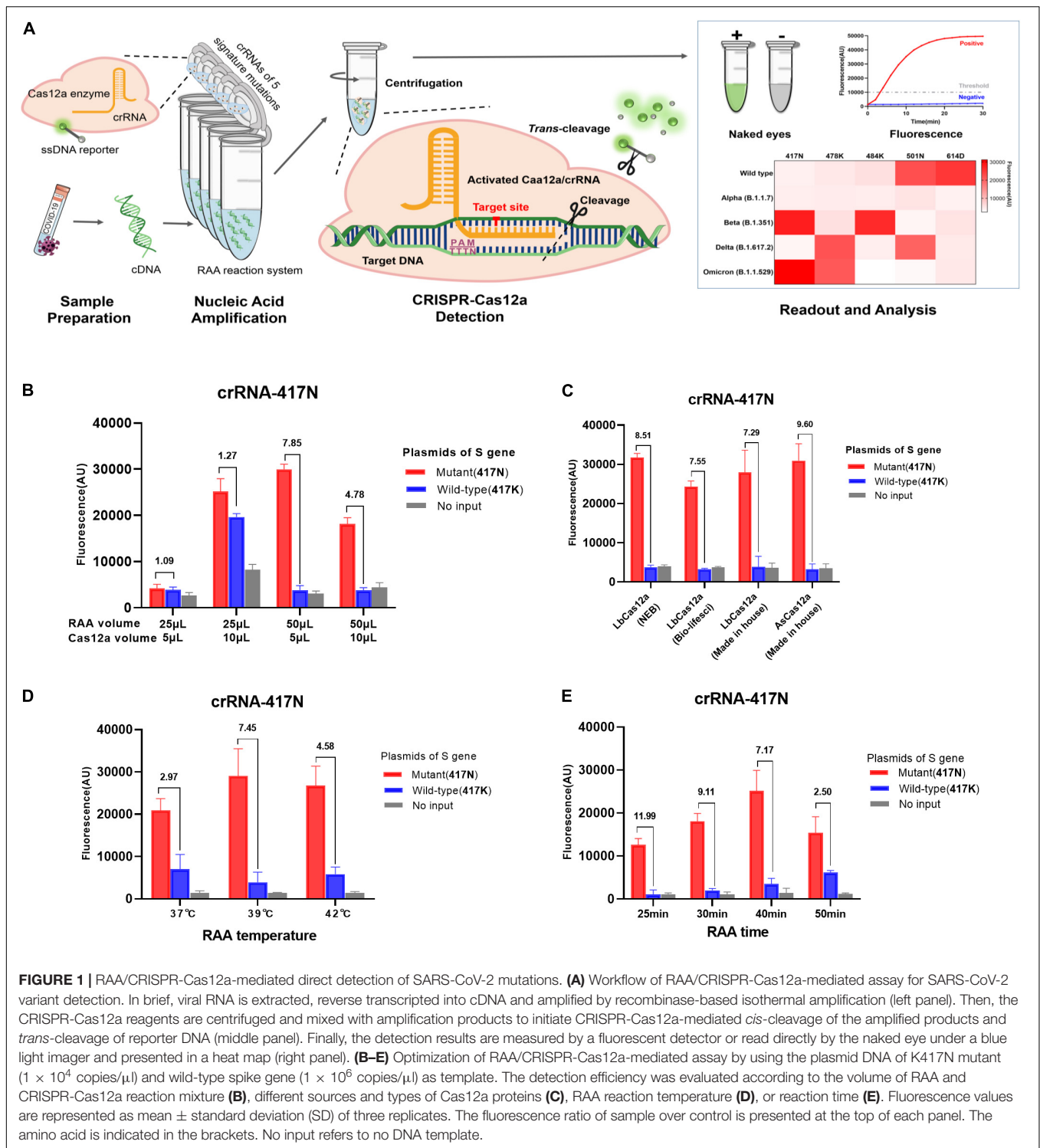
and N501Y mutations. For the preparation of crRNAs, DNA oligonucleotides containing T7 promoter, conserved stem-loop sequences, and guide sequences and the completely complementary single-stranded DNAs were synthesized and denatured at 95°C for 10 min and annealed from 95 to 25°C with a temperature reduction of 2°C every minute. Afterward, 1 μg purified dsDNA was transcribed at 37°C for 4 h using HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, Ipswich, MA, United States). The transcription product was treated with 4 units of DNase I (New England Biolabs, Ipswich, MA, United States) at 37°C for 40 min and then purified using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany). The concentration of crRNAs was quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). All the primer and crRNA sequences are listed in **Supplementary Table 4**, and all the oligonucleotides were synthesized using the Ruiboxingke Biotechnology (Beijing, China).

Recombinase-Aided Amplification/CRISPR-Cas12a-Mediated Assay

Viral RNA was extracted from oropharyngeal swab samples of confirmed COVID-19 patients by using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed to cDNA using Oligo(dT) or random primer (Roche Diagnostics, Indianapolis, IN, United States). The recombinase-aided amplification reaction (RAA) was performed according to the instructions of the RAA basic kit (Qitian, Jiangsu, China) with slight modifications. Briefly, 25 μl of rehydration buffer, 2 μl of each primer (10 μM), 2 μl of target DNA template, and 16.5 μl of nuclease-free water were added into the tube containing a dried enzyme pellet (including recombinase, single-stranded DNA binding protein, and strand-displacing DNA polymerase) and 2.5 μl of magnesium acetate (280 mM). Subsequently, 5 μl of CRISPR reaction mixture [6 μM of crRNA, 0.8 μM of AsCas12a, 3 μl of NEB buffer 2.1 and 2 μM probe reporter (5'-6-FAM-TTATT-BHQ-1-3')] were transferred to the lid of the RAA reaction tube and incubated at 39°C for amplification (25 min for crRNA-S-49X and 50X while 40 min for other 5 specific crRNAs). After that, the tube was centrifuged to move the CRISPR-Cas12a reagents to the bottom of tube and incubated at 39°C for 30–40 min for detection (**Figure 1A**). The fluorescence signal was measured by a fluorescent detector (Qitian, Jiangsu, China) in real-time or judged by the naked eye under a portable blue light imager (Sangon Biotech, Shanghai, China).

Statistical Analysis

Data analysis was conducted using the IBM SPSS software, version 22 (IBM Corporation, Armonk, NY, United States). The two-tailed Mann-Whitney *U* test and Fisher's exact test were used to analyze the difference detected by the CRISPR-Cas12a-mediated assay. The receiver-operating characteristic curve (ROC) and the area under the ROC curve (AUC) were



calculated to assess the performance of the CRISPR-Cas12a-mediated assay, while the cutoff value was estimated according to the Youden index. Positive predictive value (PPV) and negative predictive value (NPV), as well as 95% binomial confidence intervals, were calculated according to Clopper–Pearson score.

The concordance between the CRISPR-Cas12a-based assay and Sanger sequencing was calculated according to the kappa value. P -value < 0.05 was considered statistically significant. Data plotting was performed using the GraphPad Prism software (Version 8.0, La Jolla, CA, United States).

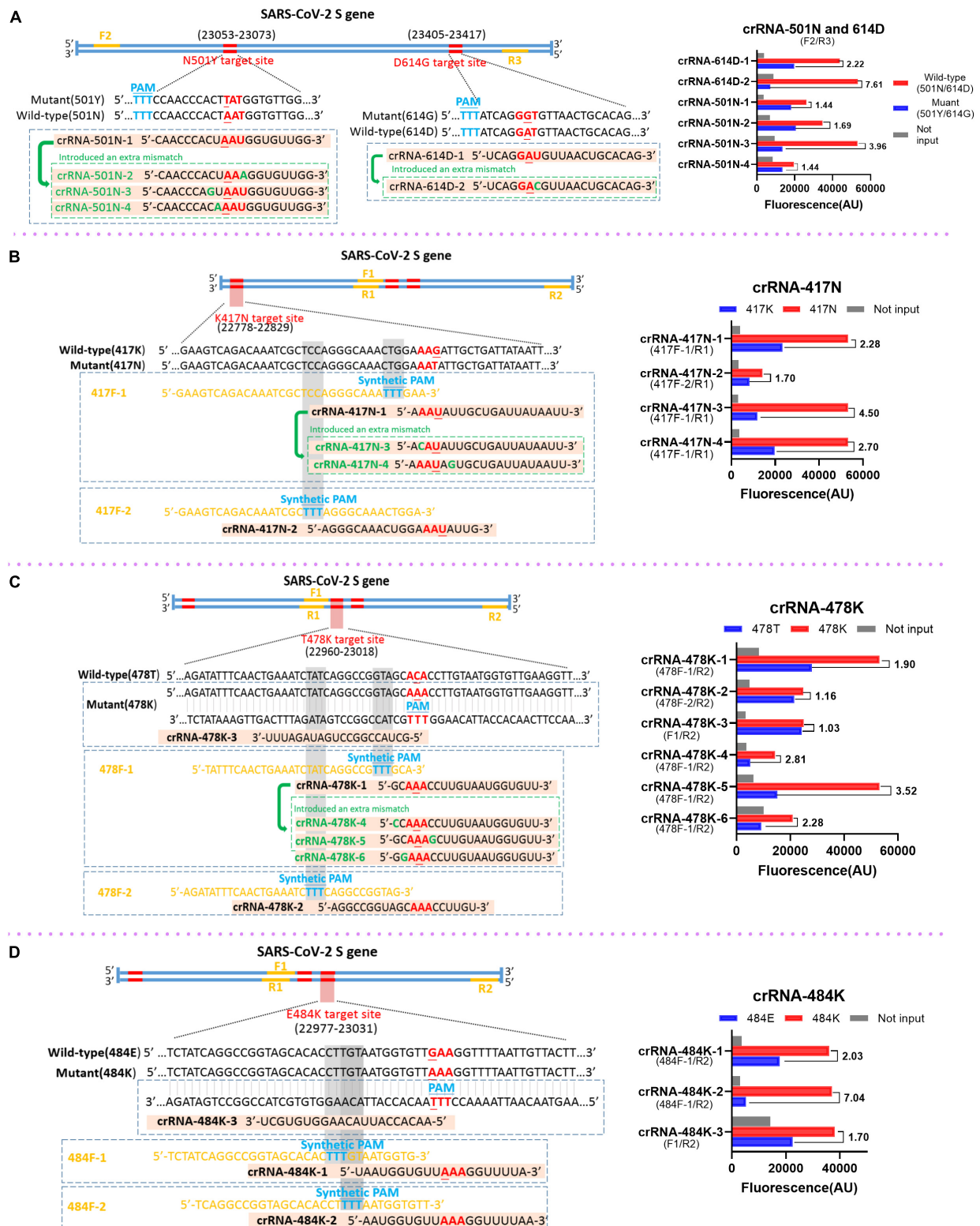


FIGURE 2 | Design and selection of RAA primers and crRNAs. The schematic of the sequences and positions of RAA primers (yellow), the crRNAs with the specific mutations (red), the protospacer adjacent motif PAM (blue) for the mutations of SARS-CoV-2 spike gene (left panel), and the performance of the crRNAs (right panel) for the mutation of N501Y and D614G (A), K417N (B), T478K (C), and E484K (D), respectively. In the right panels, the wild-type and mutant template were labeled in red and blue, respectively. No input means negative control and was labeled in gray. The RAA primer sets for the corresponding crRNAs are presented within the parenthesis.

RESULTS

Optimization of Recombinase-Aided Amplification/CRISPR-Cas12a-Mediated Assay

The purpose of this study was to develop a rapid and simple system by integrating RAA and CRISPR-Cas12a reaction in one tube as shown in **Figure 1A**. We adapted a strategy of two separate reactions of RAA and CRISPR-Cas12a cleavage in the same tube to avoid invalid amplification caused by the early cleavage of the target template by activated Cas12a when RAA and Cas12a-mediated digestion reacted simultaneously. We optimized the reaction conditions by detecting K417N mutation and found that the volume of RAA and CRISPR-Cas12a reaction as well as the ratio of the two mixtures significantly affected the amplification efficiency of RAA and the *trans*-cleavage efficiency of CRISPR-Cas12a for ssDNA reporter (**Figure 1B**). As shown in **Figure 1B**, the combination of 50 μ l RAA and 5 μ l CRISPR-Cas12a reaction mixture exhibited the greatest fluorescence ratio of 7.85 for the positive (417N) over the negative control (417K), suggesting that the final concentration of the reagents and templates are critical for the efficiency and specificity of both RAA and CRISPR-Cas12a reactions. However, the types and sources of Cas12a proteins did not significantly affect the cleavage activity since the fluorescence ratio for detecting 417N over 417K was quite similar when using the in-house-made AsCas12a and LbCas12a or commercially available LbCas12a (**Figure 1C**).

Furthermore, the highest efficiency of isothermal amplification was obtained when the RAA reaction was conducted at 39°C, where the signal ratio was 7.45, greater than at 37°C (ratio = 2.97) or 42°C (4.58), respectively (**Figure 1D**). Interestingly, we found that the extended isothermal amplification could increase the strength of fluorescence signal but decrease the fluorescence ratio since the signal ratio was 11.99, 9.11, 7.17, and 2.50 when the RAA lasted for 25, 30, 40, and 50 min, respectively (**Figure 1E**). Considering the relatively low fluorescence signal at 25 and 30 min, we decided that the optimized condition for RAA was at 39°C for 40 min.

Next, we tested the efficiency of the CRISPR reaction at 37°C and 39°C using the optimized conditions as above since the Cas detection reaction had only been tested at 37°C so far, although a recent study reported that AsCas12a was robust to temperature (Ooi et al., 2021). We observed that the fluorescence readout even increased slightly at 39°C (**Supplementary Figure 3**). Therefore, we conclude that our RAA/CRISPR-Cas12a assay could be performed at the same temperature of 39°C.

Design and Evaluation of Recombinase-Aided Amplification Primers and crRNAs

Different strategies were applied in this study to design and select the RAA primers and crRNAs. For the mutations N501Y and D614G in which the target sequences already have the PAM motif that is required for the recognition and cleavage of Cas12a protein, the principles for designing and selecting RAA

primers just follow the criteria of RAA reaction. Our results indicated that the primer sets F2 and R3 could efficiently amplify the templates with N501Y and D614G mutations (data not shown) and were used in the subsequent analysis (**Figure 2A**). Interestingly, we found that the original crRNAs crRNA-614D-1 and crRNA-501N-1 that are specific for 614D and 501N mutations did not distinguish between D614G and N501Y very well, respectively (**Figure 2A**). We then designed a crRNA-614D-2 and another three crRNAs, i.e., crRNA-501N-2, -3, and -4 by introducing extra mutations around the 614D or 501N mutation. Our results indicated that the crRNA-614D-2 and crRNA-501N-3 distinguished D614G and N501Y more efficiently and specifically than other crRNAs (**Figure 2A**). A more complicated strategy was adopted for K417N, T478K, and E484K mutations, which do not contain suitable PAM motifs around these mutations. We first designed the RAA forward primers by inserting an artificial PAM motif (5'-TTTN-3') into the 3' end of the forward RAA primers in order to introduce the PAM sequences into the amplified fragments (**Figures 2B–D**). A series of crRNAs with extra mutations were designed to evaluate their performance in distinguishing K417N, T478K, and E484K mutations, respectively. Our results identified the best combination of the appropriate RAA primers and crRNAs, i.e., the primer set of 417F-1/R1 and crRNA-417N-3 for K417N (**Figure 2B**); 478F-1/R2 and crRNA-478K-5 for T478K (**Figure 2C**); and 484F-1/R2 and crRNA-484K-2 for E484K (**Figure 2D**). Therefore, the above optimized RAA primer sets and crRNAs were included for the following evaluation of our RAA-CRISPR-Cas12a-mediated genotyping assay.

Detection Limit and Specificity of Recombinase-Aided Amplification/CRISPR-Cas12a Assay

According to the aforementioned optimized conditions as well as the RAA primers and crRNAs, we determined the low limit of detection (LOD) of the RAA/CRISPR-Cas12a-mediated assay using 10-fold serial dilutions of the target DNA templates, which ranged from 10^3 to 10^6 copies/ μ l. We found that there was a very good correlation between the reaction time and fluorescence intensity, and a linear relationship was observed in the presence of 10^4 copies/ μ l of the target templates (**Figure 3**). Our results indicated that the 5 signature mutations could be readily detected by fluorescent detector or by naked eyes under blue light by using crRNA-417N-3, crRNA-478K-5, crRNA-484K-3, crRNA-501N-3, and crRNA-614D-2 when the templates were as low as 10^4 copies/ μ l (**Figures 3A–E**), indicating the LOD of 10^4 copies for our RAA/CRISPR-Cas12a-mediated assay.

Moreover, the specificity of the RAA/CRISPR-Cas12a assay was validated with clinical samples infected with 11 common respiratory viruses, including common human coronavirus (HKU1, 229E, and OC43), respiratory syncytial virus (RSV) A and B, parainfluenza virus (HPIV) 1 and 4, rhinovirus (HRV), adenovirus (AdV), human bocavirus (HBoV), and human metapneumovirus (HMPV). As shown in **Figure 4A**, robust fluorescence signal was observed when the plasmid of the SARS-CoV-2 S gene was included as positive control,

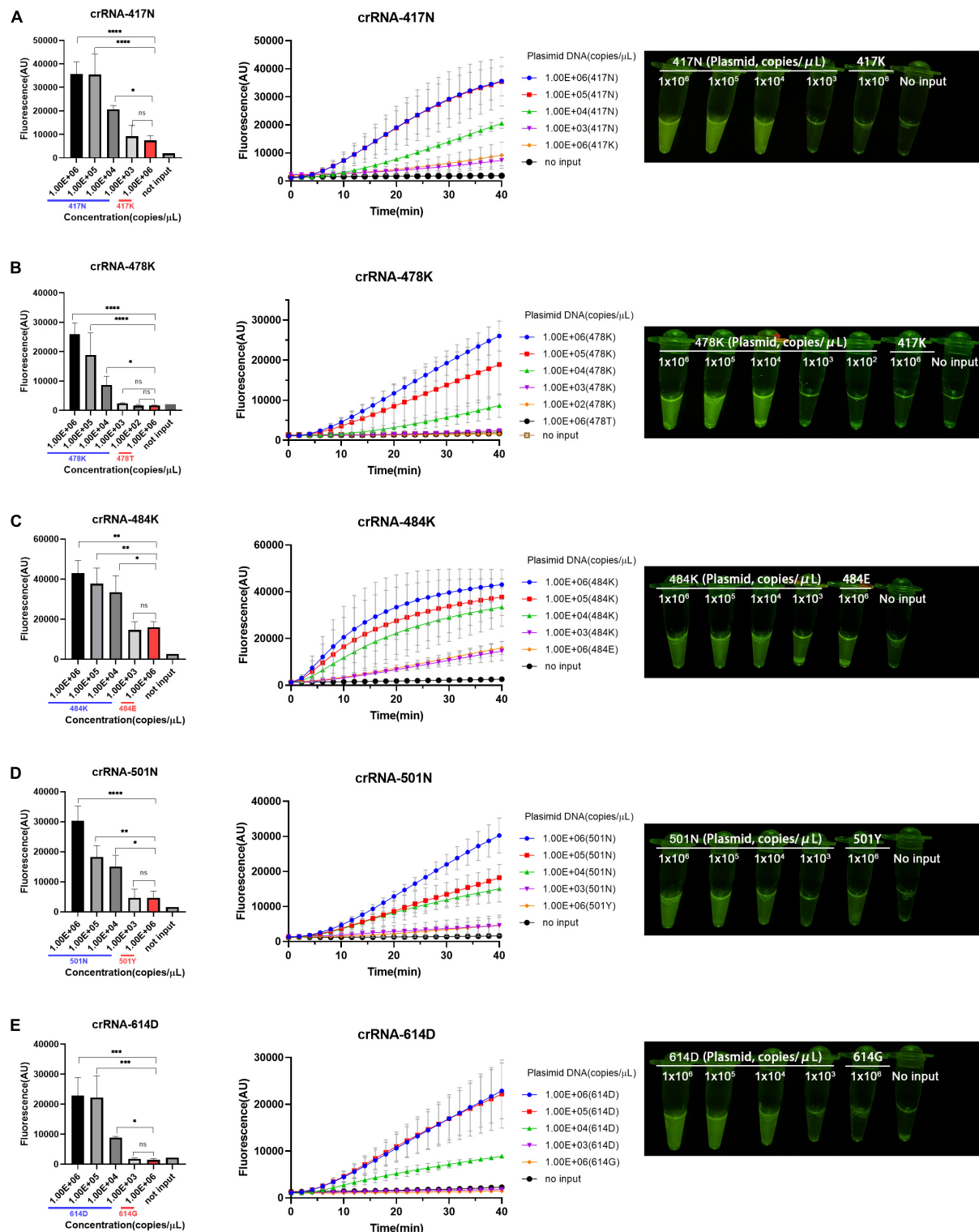


FIGURE 3 | Limit of detection (LOD) of RAA/CRISPR-Cas12a-mediated detection. The LOD was determined and presented for the 5 crRNAs specific for the amino acid 417N (A), 478K (B), 484K (C), 501N (D), and 614D (E) of SARS-CoV-2 spike protein, respectively. A series of 10-fold diluted synthetic SARS-CoV-2 plasmid DNAs of wild-type (10^2 – 10^6 copies/ μ L) and mutant S gene (10^6 copies/ μ L) were used as the templates for RAA followed by the detection of CRISPR-Cas12a-mediated assay. Fluorescence intensity are represented as mean $\pm$ standard deviation (SD) of three replicates or read out directly by the naked eye under a blue light imager. No input refers to no DNA template. Two-tailed Mann–Whitney *U* test was used to analyze the fluorescence difference between on-target and off-target template detected by the RAA/Cas12a-mediated assay. ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

but no cross-reaction was found when detecting SARS-CoV-2 negative samples.

Performance of Recombinase-Aided Amplification/CRISPR-Cas12a Assay in Detecting Severe Acute Respiratory Syndrome Coronavirus 2 Variants of Concerns

We examined 54 SARS-CoV-2 positive clinical samples, including 4 samples infected with wild-type strains, 16 with alpha variant, 14 with beta variant, 15 with delta variant, and 5 with Omicron variant, and compared them with Sanger sequencing results (Figure 4B). There was no significant difference as to baseline characteristics between the samples infected with wild-type or different SARS-CoV-2 variants (Supplementary Table 5). In general, all the allele-specific crRNAs could specifically identify the corresponding signature mutations, and the comprehensive results of all the allele-specific crRNAs could accurately distinguish SARS-CoV-2 strains with or without the corresponding mutations (Figure 4B). For example, a strong fluorescence signal was observed in the clinical samples infected with the wild-type strain when using 614D-specific crRNA-614D since only the wild-type strain contains the original 614D amino acid, while a very weak signal was detected in the clinical samples infected with alpha, delta, and Omicron variant since they all carry the D614G substitution (Figure 4B). Similar results were obtained for other specific crRNAs (Figure 4B). According to the cutoff values for each crRNA determined by the ROC curves (Supplementary Figure 4), a sensitivity of 100.0% and a specificity of 94.9–100% were obtained for the crRNAs tested when compared with Sanger sequencing results (Table 1). Furthermore, our RAA/CRISPR-Cas12a-mediated assay showed a concordance of 92.59% (50/54) with Sanger sequencing. The positive and negative predictive values were 100% and 92.9–100.0%, respectively (Table 2). Of note, our RAA/CRISPR-Cas12a-mediated assay characterized the virus in one sample 2021A-XG08905 as delta plus variant (AY.1), which is a delta variant with an extra mutation of K417N (Kannan et al., 2021). The results are consistent with Sanger sequencing data. However, our CRISPR-Cas12a-mediated assay also showed a false positive signal of an extra 484K mutation in one sample (2021A-XG04560) infected with the alpha variant and failed to detect the N501Y mutation in 2 samples (2021A-XG09089 and 2021A-XG08715) that were infected with the delta variant (Figure 4B). All the testing results, as measured by fluorescence meter and judged by naked eyes under blue light, were consistent and presented in Supplementary Figure 5.

Detection of Omicron Sublineages Using Single Omicron-Specific crRNA

Unlike other SARS-CoV-2 VOCs, the Omicron variant carries multiple mutations at the S protein and RBD regions due to its high rate of mutation (Yu et al., 2022). We noticed that Omicron sublineages BA.1 and BA.2 have 37 and 31 mutations in the spike protein, respectively, and share multiple common mutations except for their unique mutations, which makes it possible

to design Omicron-specific crRNAs to specifically diagnose Omicron infection and Omicron sublineage-specific crRNA to differentiate Omicron sublineages. After careful alignment analysis of SARS-CoV-2 Omicron sequences, we identified and designed two Omicron-specific crRNAs, i.e., crRNA-S-49X to cover Q493R, G496S, and Q498R mutations, and crRNA-S-50X to cover Q498R and N501Y mutations, respectively (Figure 5A). We predicted that the crRNA-S-49X can specifically detect the BA.1 variant because the G496S mutation is unique to Omicron sublineage BA.1, whereas the crRNA-S-50X can specifically diagnose Omicron infection. We also designed an RAA forward primer to cover the Omicron unique mutation S477N based on the target sequences to further increase the assay specificity.

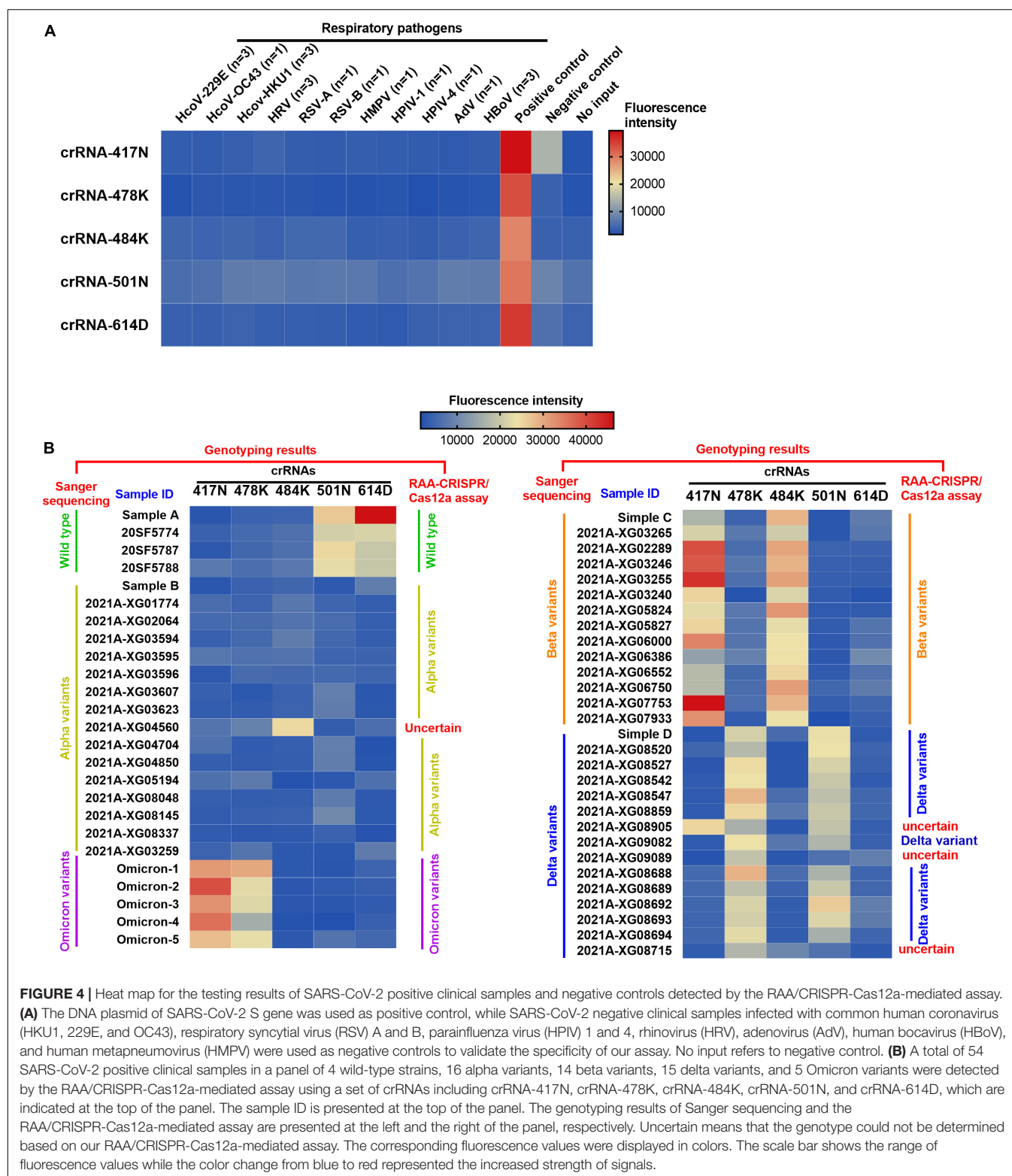
By using crRNA-S-49X, our assay could specifically detect as low as 20 copies of Omicron BA.1 plasmid DNA per reaction without cross-reaction with 2×10^6 copies of wild-type (Wuhan, China) or other SARS-CoV-2 VOCs plasmids (Figure 5B). Furthermore, the crRNA-S-49X could distinguish 200 copies of BA.1 plasmid DNA per reaction from 2×10^6 copies of BA.2 plasmid DNA (Figure 5B). Both the quantitative results (Figure 5C) and the testing results judged by the naked eye (Figure 5D) proved the super specificity of crRNA-S-49X for detecting the Omicron BA.1 template and for distinguishing it from Omicron BA.2, other SARS-CoV-2 VOC plasmids or wild-type plasmid templates.

As expected, the crRNA-S-50X could readily detect both Omicron BA.1 and BA.2 plasmids with a LOD of 200 copies per reaction based on the reaction curves (Figures 5E,H), the quantitative results (Figures 5F,I), and the results judged by the naked eye (Figures 5G,J). Of note, a stronger fluorescence signal was obtained for detecting BA.1 plasmid than BA.2 by using the crRNA-S-50X (Figures 5E,H), probably due to the two unique extra mutations (G496S and T547K) of BA.2 in the amplification products, especially the G496S mutation located at the PAM motifs of crRNA-S-50X, which may affect the PAM identification and the efficiency of crRNA-S-50X to trigger collateral cleavage capability of Cas proteins (Tang et al., 2019).

Furthermore, both crRNA-S-49X and crRNA-S-50X could specifically detect Omicron variant in 5 clinical samples infected with BA.1 sublineage and verified by NGS and distinguish Omicron variant from other SARS-CoV-2 strains including wild-type strain and the variants of alpha, beta, and delta isolated from COVID-19 patients according to the reaction curves (Figures 5K,M) or the results visualized by eyes (Figures 5L,N). No cross-reaction was found when detecting SARS-CoV-2 negative clinical samples infected with common respiratory pathogens (Figures 5O,P).

DISCUSSION

The continuous emergence and spread of SARS-CoV-2 variants manifest the importance of simple and rapid SARS-CoV-2 genotyping methods. We have previously reported a PCR/CRISPR-Cas12a-based approach to distinguish between SARS-CoV-2 wild-type and major VOCs (Liang et al., 2021). In this study, we further refined the genotyping platform by



replacing PCR with isothermal amplification, optimizing the crRNAs and primer sequences for detecting major SARS-CoV-2 VOCs, including the Omicron variant and its two major sublineages BA.1 and BA.2, and integrating all reactions in one

tube. The refined assay is more feasible for rapid detection and tracking of SARS-CoV-2 variants.

Compared to other isothermal amplification methods such as loop-mediated isothermal amplification (LAMP), RAA appears

TABLE 1 | Performance of allele-specific crRNAs in RAA/CRISPR-Cas12a-mediated assay compared with Sanger sequencing.

RAA/CRISPR testing results	Sequencing results		ROC curve area	P value	cut-off value	Sensitivity (% , 95% CI)	Specificity (% , 95% CI)	Positive predictive value (% , 95% CI)	Negative predictive value (% , 95% CI)	Kappa value
	Detected	Not detected								
crRNA-417N			1	<0.0001	>10437	100.0 (78.0–100.0)	100.0 (87.4–100.0)	100.0 (78.0–100.0)	100.0 (87.4–100.0)	1.000
Detected	20	0								
Not detected	0	34								
crRNA-478K			1	<0.0001	>11758	100.0 (78.0–100.0)	100.0 (87.4–100.0)	100.0 (78.0–100.0)	100.0 (87.4–100.0)	1.000
Detected	20	0								
Not detected	0	34								
crRNA-484K			99.33	<0.0001	>14935	100.0 (73.2–100.0)	97.50 (85.3–99.87)	93.33 (66.0–99.7)	100.0 (88.8–100.0)	0.953
Detected	14	1								
Not detected	0	39								
crRNA-501N			97.87	<0.0001	>12792	100.0 (74.7–100.0)	94.87 (81.4–99.11)	88.23 (62.3–97.9)	100.0 (88.3–100.0)	0.911
Detected	15	2								
Not detected	0	37								
crRNA-614D			1	<0.001	>14238	100.0 (39.6–100.0)	100.0 (91.1–100.0)	100.0 (39.6–100.0)	100.0 (91.1–100.0)	1.000
Detected	4	0								
Not detected	0	50								

TABLE 2 | Concordance between RAA/CRISPR-Cas12a-based assay and Sanger sequencing.

RAA/CRISPR testing results	Sequencing results		Sensitivity (% , 95% CI)	Specificity (% , 95% CI)	Positive predictive value (% , 95% CI)	Negative predictive value (% , 95% CI)	Kappa value
	Detected	Not detected					
Wild-type strain			100.0 (39.6–100.0)	100.0 (91.1–100.0)	100.0 (39.6–100.0)	100.0 (91.1–100.0)	1.000
Detected	4	0					
Not detected	0	50					
Alpha variant			93.8 (67.7–99.7)	100.0 (88.6–100.0)	100.0 (74.7–100.0)	97.4 (82.9–99.9)	0.955
Detected	15	0					
Not detected	1	38					
Beta variant			100.0 (73.2–100.0)	100.0 (89.1–100.0)	100.0 (73.2–100.0)	100.0 (89.1–100.0)	1.000
Detected	14	0					
Not detected	0	40					
Delta variant			80.0 (51.4–94.7)	100.0 (88.8–100.0)	100.0 (69.9–100.0)	92.9 (79.4–98.1)	0.852
Detected	12	0					
Not detected	3	39					
Omicron variant			100.0 (46.3–100.0)	100.0 (90.9–100.0)	100.0 (46.3–100.0)	100.0 (90.9–100.0)	1.000
Detected	5	0					
Not detected	0	49					

to be an appropriate technology for rapid detection (20–40 min) with relatively simple primer design and selection (**Supplementary Table 6**). The most important fact is that the low-amplification temperature (37–42°C) makes it possible to integrate RAA with CRISPR-Cas12a-based detection in one tube to simplify the assay operation and to avoid the inactivation of Cas protein during thermal cycling or potential contamination caused by amplification products. In addition, the results can be read out directly by the naked eye under a portable blue light imager. These new features of our RAA/CRISPR-Cas12a-based

assay make it more feasible to be implemented as point-of-care testing, which is more suitable for use in resource-limited settings. Compared to previously reported RAA or RPA/CRISPR-based nucleic acid detection methods (Ai et al., 2019; Bai et al., 2019; Jiao et al., 2021), our assay showed a comparable detection time and a single-base specificity.

Different from the PCR/CRISPR-Cas12a-mediated assay, our RAA/CRISPR-Cas12a-mediated assay shows a wide range of LOD from 10 to 10⁴ copies/μl of plasmid DNA according to the crRNAs. Our results indicated that relatively high LOD was

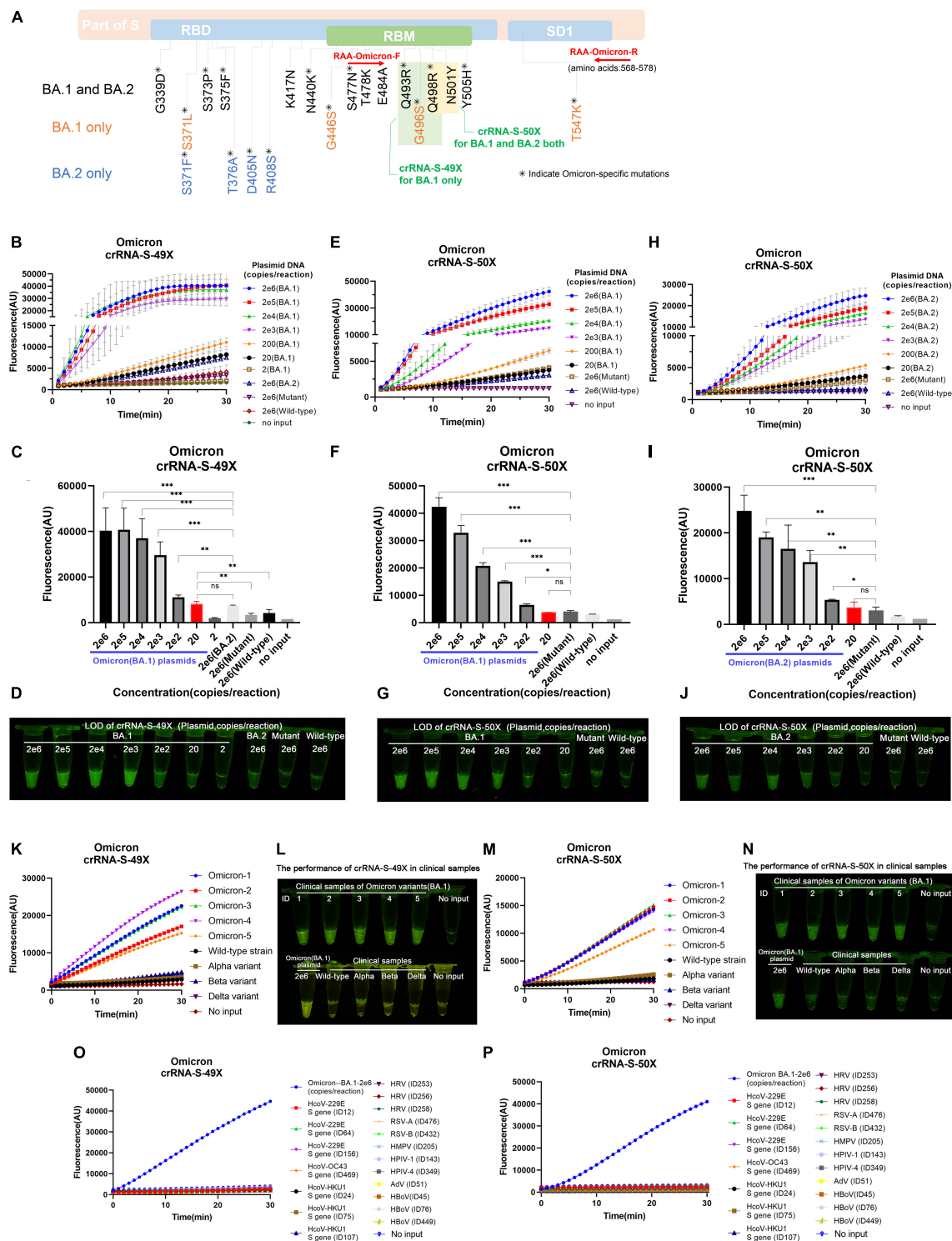


FIGURE 5 | Detection of Omicron BA.1 and BA.2 sublineages via optimized RAA/CRISPR-Cas12a-mediated assay by using crRNA-S-49X and crRNA-S-50X.

(A) The schematic of the specific mutations and the RAA primers. The shared mutations of BA.1 and BA.2 (black) and the mutations specific for BA.1 (yellow) or BA.2 (blue) were presented, while the RAA primers were labeled in red. A series of 10-fold diluted synthetic SARS-CoV-2 plasmid DNAs of wild-type, SARS-CoV-2 mutant, and Omicron BA.1 and BA.2 sublineages were used as the templates for RAA/CRISPR-Cas12a-mediated assay. The low limit of detection was determined and quantitatively analyzed for BA.1 template by using crRNA-S-49X (B,C) and crRNA-S-50X (E,F), whereas the LOD of crRNA-S-50X was analyzed using BA.2 template (H,I). Testing results were visualized by the naked eyes under blue light at 30 min post-reaction (D,G,J). Five clinical samples infected with BA.1 sublineage could be specifically distinguish from other SARS-CoV-2 VOCs-infected samples by using crRNA-S-49X (K,L) and crRNA-S-50X (M,N). Both crRNA-S-49X (K) and

(Continued)

FIGURE 5 | crRNA-S-50X (**M**) could specifically detect Omicron variant, but not wild-type strain and variant alpha, beta, and delta. The testing results were visualized by the naked eye under blue light (**L,N**). The DNA plasmid of Omicron variant were used as positive control, while SARS-CoV-2 negative clinical samples infected with common human coronavirus (HCoV) 229E, HCoV OC43, and HCoV HKU1 as well as various other respiratory pathogens, including rhinovirus (HRV), respiratory syncytial virus (RSV) A and B, human metapneumovirus (HMPV), human parainfluenza virus (HPIV-1 and HPIV-4), human adenovirus (HAdV), and human bocavirus (HBoV) were used as negative controls to validate the specificity of our assay (**O,P**). In all panels, error bars represent the mean $\pm$ standard deviation (SD) from three replicates of experiments. A two-tailed Mann-Whitney *U* test was used to analyze the fluorescence difference between on-target and off-target templates detected by CRISPR-Cas12a-based assay. ****P* < 0.001; ***P* < 0.01; **P* < 0.05; ns, *P* > 0.05.

observed for the 5 crRNAs with a single signature mutation, whereas low LOD was obtained for the two Omicron-specific crRNAs in which multiple mutations are included, suggesting that crRNA sequences and the number of mismatches between crRNAs and the target sequences play an important role in determining the detection sensitivity. In addition, artificial PAM motifs may affect the efficiency of RAA amplification, which in turn decreases the detection sensitivity of RAA/CRISPR-Cas12a-mediated assay. This may explain the relatively lower LOD of the RAA/CRISPR-Cas12a-mediated assay than the PCR/CRISPR-Cas12a-mediated assay. However, our results indicated that the relatively high LOD of our RAA/CRISPR-Cas12a-mediated assay did not affect its sensitivity when detecting major SARS-CoV-2 variants (**Table 1**). Previous studies showed that virus titers ranged from 10^4 to 10^8 copies/ μ l for the majority of SARS-CoV-2 positive samples (Jones et al., 2020; Pan et al., 2020; Pujadas et al., 2020), suggesting that our assay is suitable for detecting most of the clinical samples.

Sequencing is still the gold standard technology to identify mutations and to determine genotypes. In this study, we further evaluated our assay performance by comparing the results with Sanger sequencing data, and observed a positive predictive value of 100.0% and a negative predictive value of 92.9–100.0% (**Table 2**). The preliminary data showed a concordance of 92.59% with the Sanger sequencing method. Meanwhile, 100.0% specificity was achieved by our assay since no cross-reaction was found when detecting other common respiratory pathogens (**Figure 4B**). Of note, when detecting the same panels of SARS-CoV-2 positive and negative clinical samples, the RAA-CRISPR-Cas12a-mediated assay is slightly better than our PCR-CRISPR-Cas12a-mediated assay (Liang et al., 2021). That could be due to the further optimization of crRNAs used in this study to improve their performance (see below).

Genotyping based on CRISPR-Cas technology is due to the specific binding of crRNAs and the target sequences to activate Cas enzymes for both sequence-specific cutting (in cis) and non-specific sequence cleavage (in trans). In other words, mismatches between the crRNAs and the target sequences will affect the *trans*-cleavage efficiency and the strength of detection signals. In our study, we noticed the relatively high background and inefficiency of some crRNAs in differentiating single point mutations when using crRNAs that only contain one mismatched nucleotide (**Figure 2**). Previous studies indicate that the efficiency of crRNAs to trigger the collateral cleavage capability of CRISPR-Cas proteins could be affected by the extra substitutions in crRNAs (Creutzburg et al., 2020; Huang et al., 2021), especially the mismatches adjacent to the target mutations or in the PAM proximal regions (Kang et al.,

2020). Therefore, we designed a series of crRNAs with one extra additional mutation at the upstream or downstream of the original signature mutation of SARS-CoV-2 variants, and confirmed their capability to enhance the specific detection signal and decrease the non-specific reaction, which in turn improves the detection sensitivity and specificity. The selection of the primer sets and crRNAs will depend on the performance evaluation. This new strategy to design the primers and crRNAs makes our system more feasible to be improved for the detection of SARS-CoV-2 variants.

CONCLUSION

We successfully developed an RAA/CRISPR-Cas12a-mediated assay to specifically distinguish major SARS-CoV-2 variants, including the prevalent delta and Omicron sublineages BA.1 and BA.2. All the reactions were conducted in one sealed tube without the need for complex equipment and facilities. The simple and rapid assay could be set up and implemented routinely in resource-limited settings. In the future, this assay can be further simplified and used for high-throughput multiplex screening combined with sophisticated microfluidic devices.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

ST, XD, HL, and YL contributed to conception and design of the study, participated in interpretation of the data, and critical revisions of the manuscript. LZ, BL, and JS collected and sorted the materials. JZ and HW contributed with literature support. HL and YL performed the statistical analysis. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

- Ai, J.-W., Zhou, X., Xu, T., Yang, M., Chen, Y., He, G.-Q., et al. (2019). CRISPR-based rapid and ultra-sensitive diagnostic test for *Mycobacterium tuberculosis*. *Emerg. Microbe. Infect.* 8, 1361–1369. doi: 10.1080/22221751.2019.1664939
- Bai, J., Lin, H., Li, H., Zhou, Y., Liu, J., Zhong, G., et al. (2019). Cas12a-Based On-Site and Rapid Nucleic Acid Detection of African Swine Fever. *Front. Microbiol.* 10:2830. doi: 10.3389/fmicb.2019.02830
- Broughton, J. P., Deng, X., Yu, G., Fasching, C. L., Servellita, V., Singh, J., et al. (2020). CRISPR-Cas12-based detection of SARS-CoV-2. *Nat. Biotechnol.* 38, 870–874. doi: 10.1038/s41587-020-0513-4
- Brown, C. M., Vostok, J., Johnson, H., Burns, M., Gharpure, R., Sami, S., et al. (2021). Outbreak of SARS-CoV-2 Infections, Including COVID-19 Vaccine Breakthrough Infections, Associated with Large Public Gatherings - Barnstable County, Massachusetts, July 2021. *MMWR Morb. Mortal. Wkly. Rep.* 70, 1059–1062. doi: 10.15585/mmwr.mm7031e2
- Chen, J. S., Ma, E., Harrington, L. B., Da Costa, M., Tian, X., Palefsky, J. M., et al. (2018). CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science* 360, 436–439.
- Chiara, M., D'archia, A. M., Gissi, C., Manzari, C., Parisi, A., Resta, N., et al. (2021). Next generation sequencing of SARS-CoV-2 genomes: challenges, applications and opportunities. *Brief Bioinform.* 22, 616–630. doi: 10.1093/bib/bbaa297
- Creutzburg, S. C. A., Wu, W. Y., Mohanraju, P., Swartjes, T., Alkan, F., Gorodkin, J., et al. (2020). Good guide, bad guide: spacer sequence-dependent cleavage efficiency of Cas12a. *Nucleic Acids Res.* 48, 3228–3243. doi: 10.1093/nar/gkz1240
- de Puig, H., Lee, R. A., Najjar, D., Tan, X., Soeknsen, L. R., Angenent-Mari, N. M., et al. (2021). Minimally instrumented SHERLOCK (miSHERLOCK) for CRISPR-based point-of-care diagnosis of SARS-CoV-2 and emerging variants. *Sci. Adv.* 7:eabh2944. doi: 10.1126/sciadv.abh2944
- Dong, E., Du, H., and Gardner, L. (2022). *An Interactive Web-Based Dashboard to Track COVID-19 in Real Time*. Available online at: <https://coronavirus.jhu.edu/map.html> (accessed on May 10, 2022, 2021).
- Dong, Y., Dai, T., Wang, B., Zhang, L., Zeng, L. H., Huang, J., et al. (2021). The way of SARS-CoV-2 vaccine development: success and challenges. *Signal Transduct. Target Ther.* 6:387. doi: 10.1038/s41392-021-00796-w
- Gootenberg, J. S., Abudayyeh, O. O., Kellner, M. J., Joung, J., Collins, J. J., and Zhang, F. (2018). Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science* 360, 439–444. doi: 10.1126/science.aaq0179
- Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., et al. (2017). Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* 356, 438–442. doi: 10.1126/science.aam9321
- Hacisuleyman, E., Hale, C., Saito, Y., Blachere, N. E., Bergh, M., Conlon, E. G., et al. (2021). Vaccine Breakthrough Infections with SARS-CoV-2 Variants. *N Engl. J. Med.* 384, 2212–2218.
- Huang, X., Zhang, F., Zhu, K., Lin, W., and Ma, W. (2021). dsmCRISPR: Dual synthetic mismatches CRISPR/Cas12a-based detection of SARS-CoV-2 D614G mutation. *Virus Res.* 304:198530. doi: 10.1016/j.virusres.2021.198530
- Jiao, J., Kong, K., Han, J., Song, S., Bai, T., Song, C., et al. (2021). Field detection of multiple RNA viruses/viroids in apple using a CRISPR/Cas12a-based visual assay. *Plant Biotechnol. J.* 19, 394–405. doi: 10.1111/pbi.13474
- Jones, T. C., Mühlemann, B., Veith, T., Biele, G., Zuchowski, M., Hofmann, J., et al. (2020). An analysis of SARS-CoV-2 viral load by patient age. *medRxiv* [Preprint]. 2020.2006.2008.20125484. doi: 10.1101/2020.06.08.20125484
- Joung, J., Ladha, A., Saito, M., Kim, N. G., Woolley, A. E., Segel, M., et al. (2020). Detection of SARS-CoV-2 with SHERLOCK One-Pot Testing. *N Engl. J. Med.* 383, 1492–1494. doi: 10.1056/NEJMc2026172
- Kang, S.-H., Lee, W.-J., An, J.-H., Lee, J.-H., Kim, Y.-H., Kim, H., et al. (2020). Prediction-based highly sensitive CRISPR off-target validation using target-specific DNA enrichment. *Nat. Commun.* 11:3596.
- Kannan, S. R., Spratt, A. N., Cohen, A. R., Naqvi, S. H., Chand, H. S., Quinn, T. P., et al. (2021). Evolutionary analysis of the Delta and Delta Plus variants of the SARS-CoV-2 viruses. *J. Autoimmun.* 124:102715. doi: 10.1016/j.jaut.2021.102715
- Khandia, R., Singhal, S., Alqahtani, T., Kamal, M. A., El-Shall, N. A., Nainu, F., et al. (2022). Emergence of SARS-CoV-2 Omicron (B.1.1.529) variant, salient features, high global health concerns and strategies to counter it amid ongoing COVID-19 pandemic. *Environ. Res.* 209:112816. doi: 10.1016/j.envres.2022.112816
- Kroidl, I., Mecklenburg, I., Schneiderat, P., Müller, K., Grl, P., Wölfel, R., et al. (2021). Vaccine breakthrough infection and onward transmission of SARS-CoV-2 Beta (B.1.351) variant, Bavaria, Germany, February to March 2021. *Euro. Surveill.* 26:2100673. doi: 10.2807/1560-7917.ES.2021.26.30.2100673
- Liang, Y., Lin, H., Zou, L., Zhao, J., Li, B., Wang, H., et al. (2021). CRISPR-Cas12a-Based Detection for the Major SARS-CoV-2 Variants of Concern. *Microbiol. Spectr.* 9:e0101721. doi: 10.1128/Spectrum.01017-21
- Ooi, K. H., Liu, M. M., Tay, J. W. D., Teo, S. Y., Kaewsapsak, P., Jin, S., et al. (2021). An engineered CRISPR-Cas12a variant and DNA-RNA hybrid guides enable robust and rapid COVID-19 testing. *Nat. Commun.* 12:1739. doi: 10.1038/s41467-021-21996-6
- Pan, Y., Zhang, D., Yang, P., Poon, L. L. M., and Wang, Q. (2020). Viral load of SARS-CoV-2 in clinical samples. *Lancet Infect. Dis.* 20, 411–412.
- Pujadas, E., Chaudhry, F., McBride, R., Richter, F., Zhao, S., Wajenberg, A., et al. (2020). SARS-CoV-2 viral load predicts COVID-19 mortality. *Lancet Respirat. Med.* 8:e70.
- Rovida, F., Cassaniti, I., Paolucci, S., Percivalle, E., Sarasini, A., Piralla, A., et al. (2021). SARS-CoV-2 vaccine breakthrough infections with the alpha variant are asymptomatic or mildly symptomatic among health care workers. *Nat. Commun.* 12:6032. doi: 10.1038/s41467-021-26154-6
- Tang, L., Yang, F., He, X., Xie, H., Liu, X., Fu, J., et al. (2019). Efficient cleavage resolves PAM preferences of CRISPR-Cas in human cells. *Cell Regen.* 8, 44–50. doi: 10.1016/j.cr.2019.08.002
- Tao, K., Tzou, P. L., Nouhin, J., Gupta, R. K., De Oliveira, T., Kosakovsky Pond, S. L., et al. (2021). The biological and clinical significance of emerging SARS-CoV-2 variants. *Nat. Rev. Genet.* 22, 757–773. doi: 10.1038/s41576-021-00408-x
- Vega-Magaña, N., Sánchez-Sánchez, R., Hernández-Bello, J., Venancio-Landeros, A. A., Peña-Rodríguez, M., Vega-Zepeda, R. A., et al. (2021). RT-qPCR Assays for Rapid Detection of the N501Y, 69-70del, K417N, and E484K SARS-CoV-2 Mutations: A Screening Strategy to Identify Variants With Clinical Impact. *Front. Cell Infect. Microbiol.* 11:672562. doi: 10.3389/fcimb.2021.672562
- Vignier, N., Bérot, V., Bonnave, N., Peugny, S., Ballet, M., Jacoud, E., et al. (2021). Breakthrough Infections of SARS-CoV-2 Gamma Variant in Fully Vaccinated Gold Miners, French Guiana, 2021. *Emerg. Infect. Dis.* 27, 2673–2676. doi: 10.3201/eid2710.211427
- Vogels, C. B. F., Breban, M. I., Ott, I. M., Alpert, T., Petrone, M. E., Watkins, A. E., et al. (2021). Multiplex qPCR discriminates variants of concern to enhance global surveillance of SARS-CoV-2. *PLoS Biol.* 19:e3001236. doi: 10.1371/journal.pbio.3001236
- Wang, H., Miller, J. A., Verghese, M., Sibai, M., Solis, D., Mfuh, K. O., et al. (2021). Multiplex SARS-CoV-2 Genotyping RT-PCR for Population-Level Variant Screening and Epidemiologic Surveillance. *J. Clin. Microbiol.* 59:e0085921. doi: 10.1128/JCM.00859-21
- Welch, N. L., Zhu, M., Hua, C., Weller, J., Mirhashemi, M. E., Nguyen, T. G., et al. (2022). Multiplexed CRISPR-based microfluidic platform for clinical testing of

SUPPLEMENTARY MATERIAL

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- respiratory viruses and identification of SARS-CoV-2 variants. *Nat. Med.* 28, 1083–1094. doi: 10.1038/s41591-022-01734-1
- World Health Organization [WHO] (2022). *Tracking SARS-CoV-2 Variants*. Available online at: <https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/> (accessed on May 10, 2022).
- Yu, J., Collier, A.-R. Y., Rowe, M., Mardas, F., Ventura, J. D., Wan, H., et al. (2022). Neutralization of the SARS-CoV-2 Omicron BA.1 and BA.2 Variants. *N Engl. J. Med.* 386, 1579–1580.
- Zelyas, N., Pabbaraju, K., Croxen, M. A., Lynch, T., Buss, E., Murphy, S. A., et al. (2021). Precision Response to the Rise of the SARS-CoV-2 B.1.1.7 Variant of Concern by Combining Novel PCR Assays and Genome Sequencing for Rapid Variant Detection and Surveillance. *Microbiol. Spectr.* 9:e0031521. doi: 10.1128/Spectrum.00315-21
- Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., et al. (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163, 759–771. doi: 10.1016/j.cell.2015.09.038
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A Visual Assay of a Loop-Mediated Isothermal Amplification Based Vertical Immunoassay for SARS-CoV-2 RNA Detection

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SARS-CoV-2 is a novel coronavirus that has caused a global pandemic. To date, 504,907,616 people have been infected and developed coronavirus disease 2019 (COVID-19). A rapid and simple diagnostic method is needed to control this pandemic. In this study, a visual nucleic acid detection method combining reverse transcription loop-mediated isothermal amplification and a vertical flow visualization strip (RT-LAMP-VF) was successfully established and could detect 20 copies/ μ l of SARS-CoV-2 RNA transcript within 50 min at 61°C. This assay had no cross-reactivity with a variety of coronaviruses, including human coronavirus OC43, 229E, HKU1, NL63, severe acute respiratory syndrome-related coronavirus (SARSr-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and bat coronavirus HKU4, exhibiting very high levels of diagnostic sensitivity and specificity. Most strikingly, this method can be used for detecting multiple SARS-CoV-2 variants, including the Wuhan-Hu-1 strain, Delta, and Omicron variants. Compared with the RT-qPCR method recommended by the World Health Organization (WHO), RT-LAMP-VF does not require special equipment and is easy to perform. As a result, it is more suitable for rapid screening of suspected SARS-CoV-2 samples in the field and local laboratories.

Keywords: SARS-CoV-2, reverse transcription loop-mediated isothermal amplification, detection, visualization, variants

INTRODUCTION

In the past 50 years, numerous novel coronaviruses with the ability to infect humans have emerged in succession, causing significant losses and serious threats to human health and even entire public health systems. In particular, with the emergence of severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), coronavirus has attracted the attention of researchers around the world.

SARS-CoV-2 is the seventh coronavirus found to infect humans. Infection with SARS-CoV-2 can cause coronavirus disease 2019 (COVID-19). SARS-CoV-2 has been reported not only in

humans but also in dogs, ferrets, cats, tigers, and lions (Gollakner and Capua, 2020; Shi et al., 2020). As of 20 April 2022, SARS-CoV-2 has caused more than 504 million cases of COVID-19 and more than 6.21 million deaths. The World Health Organization (WHO) also classified COVID-19 as a public health emergency of international concern (PHEIC). SARS-CoV-2 is more transmissible than SARS-CoV and MERS-CoV (Chu et al., 2020; Sanche et al., 2020). However, unlike SARS-CoV and MERS-CoV, which were always spread in hospitals, SARS-CoV-2 can also spread rapidly in communities, increasing the difficulty of pandemic prevention and control (Munster et al., 2020). At present, although there are many approved vaccines against SARS-CoV-2, the mutation of the virus reduces their protective effects (Planas et al., 2021). Therefore, the work of pandemic prevention and control must continue.

The laboratory testing strategies for COVID-19 recommended by the WHO include pathogen detection, serological detection (IgG/IgM antibody detection), and nucleic acid detection; among these, nucleic acid detection is the most widely used. The nucleic acid detection for SARS-CoV-2 includes real-time RT-PCR, metagenomics sequencing, and gene editing detection based on CRISPR-Cas12 and CRISPR-Cas13 (Broughton et al., 2020; Corman et al., 2020; Di et al., 2020; Wang et al., 2021), etc. Nucleic acid detection has high sensitivity and accuracy, but there are some shortcomings. For instance, equipment that can perform precise temperature changes is needed, and the assay design methods are complicated. Virus isolation and culture is the internationally recognized gold standard for virological detection. This method has good specificity, but the process is cumbersome and time-consuming. Serological tests, such as enzyme-linked immunosorbent assays (ELISAs) and immunochromatographic strips, have good feasibility, but antibody detection has a certain lag, as antibodies can be detected only after an immune response has been initiated in the host. Therefore, the above methods are not applicable for rapid detection during a pandemic outbreak.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method invented by Notomi et al. (2000) that generally requires two outer primers and two inner primers. This method has high sensitivity and strong specificity. It is particularly attractive due to its convenience and quick operation. The LAMP requires only a portable metal heat block or thermostat water bath to complete the amplification. To date, the RT-LAMP-VF method has been widely used in the detection of various viruses, such as MERS-CoV, Ebola virus, and Rift Valley Fever virus (Oloniniyi et al., 2017; Huang et al., 2018; Han et al., 2020). And this method showed a high sensitivity and specificity (Fu et al., 2011). Conventional LAMP requires electrophoresis to observe the amplification product, and it is easy for false positives to arise because the need to open the lid during operation makes the operation vulnerable to aerosol pollution (Notomi et al., 2000). Some researchers have added magnesium ions to the reaction system, relying on the magnesium ions and pyrophosphate ions in the reaction system to form white magnesium pyrophosphate precipitates, thus achieving visual detection. However, the introduction of magnesium ions reduces the amplification efficiency, and the

visual interpretation of magnesium pyrophosphate precipitation is also prone to subjective judgment errors (Mori et al., 2001).

To compensate for the deficiencies of existing methods, we established a nucleic acid visualization detection method based on the N gene of SARS-CoV-2. The SARS-CoV-2 RNA was amplified by RT-LAMP, and the amplification products were detected by a closed vertical flow visualization strip (VF). In addition, two-loop primers were added based on the four primers of the traditional LAMP method to enhance amplification efficiency and specificity (Nagamine et al., 2002). Compared with PCR, the RT-LAMP-VF method does not require precise temperature-changing equipment (Corman et al., 2020) and also has the advantages of simple and rapid operation. Compared with other isothermal amplification technologies, such as nucleic acid sequence amplification, self-sustaining sequence replication, and chain replacement amplification, RT-LAMP-VF is rapid, accurate, and efficient and is suitable for use in local laboratories or at community medical sites (Tomita et al., 2008). This method can provide technical support for the rapid diagnosis of COVID-19.

MATERIALS AND METHODS

Primer Design

To establish an RT-LAMP-VF method for the detection of SARS-CoV-2, we aligned the whole-genome sequences of 20 SARS-CoV-2 pandemic strains, including the Wuhan-Hu-1 strain, Delta, and Omicron variants, published in the GenBank and GISAID databases from 2019 to 2022 with MEGALIGN 8.0. The alignment showed that the N gene was highly conserved. Therefore, the N gene sequence of SARS-CoV-2 was compared with those of the highly homologous MERS-CoV, SARS-CoV, and SARSr-CoV viruses, and conserved gene fragments were selected as targets. Six primers suitable for SARS-CoV-2 RT-LAMP were designed by using Primer Explorer V5¹ with the conserved region of the N gene as the template (**Figure 1**). The RT-LAMP-VF assay requires three sets of primers, including two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LF and LB). The outer and inner primers are conventional primers without labels, and fluorescein isothiocyanate (FITC) and biotin were labeled at the 5' ends of LF and LB, respectively (**Table 1**). All primers were synthesized by Bao Biological, Co., Ltd. (Dalian, China).

Cloning of Recombinant Plasmid and Viral RNA

The recombinant plasmid pUC57-N containing the SARS-CoV-2 N gene (GenBank number: MN908947.3) was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and transformed into *E. coli* DH5 α competent cells, which were then cultured at 37°C. The recombinant plasmid was purified using a plasmid rapid extraction kit (TIANGEN Company, Beijing, China) and stored at -20°C. The concentration of the purified pUC57-N was 297 ng/ μ l.

¹<http://primerexplorer.jp/lampv5e/index.html>

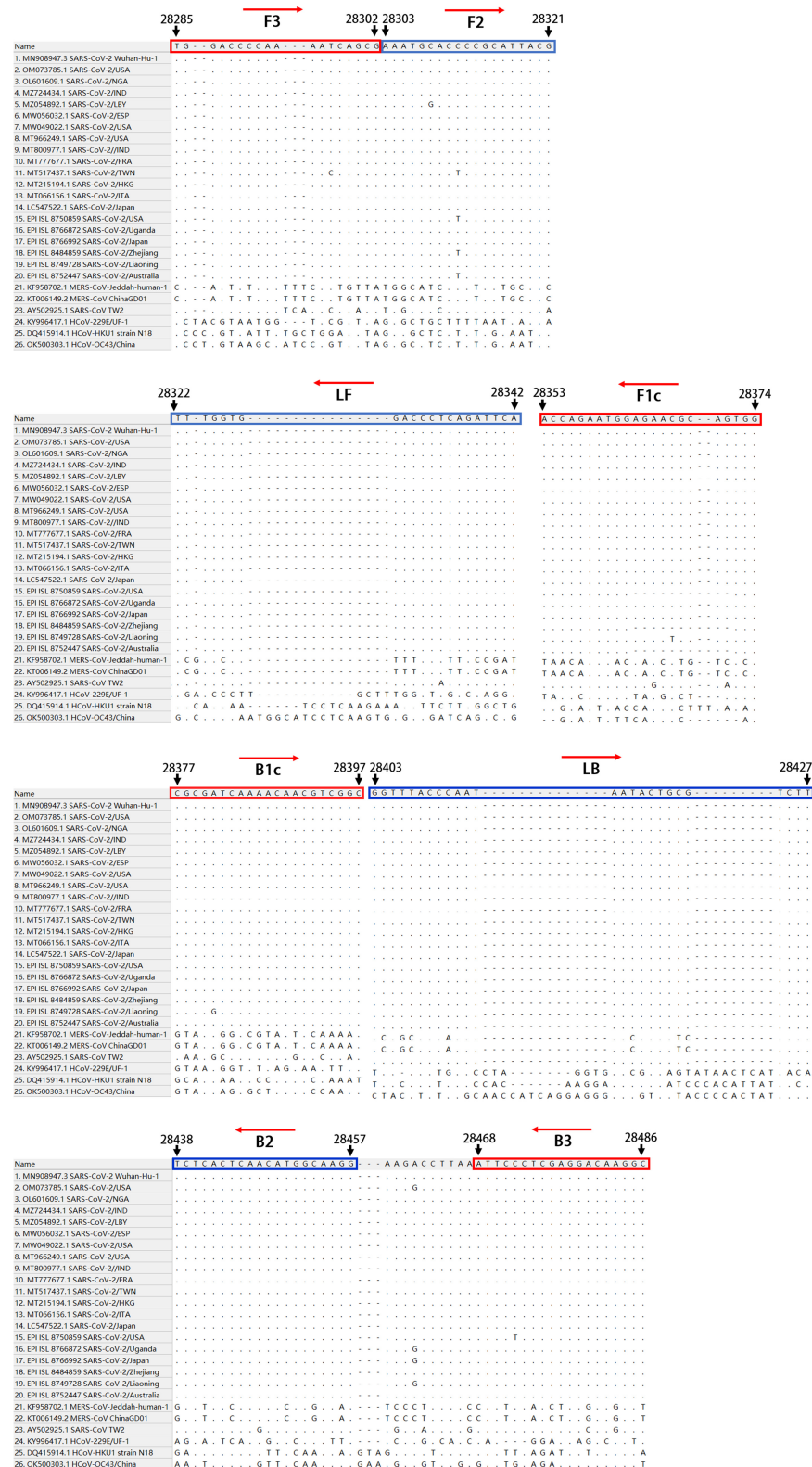


FIGURE 1 | The conserved and specific target were screened in SARS-CoV-2 N gene.

TABLE 1 | Primer and probe sequences for the SARS-CoV-2 RT-qPCR and RT-LAMP assays.

Method	Genomic target	Primer or probe	Primer Position	Sequence (5'-3')
RT-LAMP	N	F3	28285–28302	TGGACCCCAAAATCAGCG
		FIP(F1c + F2)	28353–28374	CCACTGCGTTCTCCATTCTGGT
			28303–28321	AAATGCACCCCGCATTACG
		B3	28468–28486	GCCTTGTCTCTGAGGGAAT
		BIP(B1c + B2)	28377–28397	CGCGATCAAAACAACGTCGGC
			28438–28457	CCTTGCCATGTTGAGTGAGA
		LF	28322–28342	FITC-TGAATCTGAGGGTCCACCAAA
		LB	28403–28427	Biotin-GGTTTACCCAATAACTGCGTCTT
RT-qPCR	N	Forward primer	28881–28902	GGGGAACCTTCTCTGCTAGAAT
		Reverse primer	28958–28979	CAGACATTTTGTCTCTCAAGCTG
		Probe	28934–28953	5'-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3'

TABLE 2 | Respiratory pathogens included in the NATrol RP multimarker controls kit.

RP1 Respiratory virus Strain		RP2 Respiratory virus Strain	
Influenza A H3N2	Brisbane/10/07	Influenza A H1	New Caledonia/20/99
Influenza A H1N1	NY/02/2009	Influenza B	Florida/02/06
Rhinovirus	Type 1A	RSV	Type A
Adenovirus	Type 3	Parainfluenza	Type 2
Parainfluenza	Type 1	Parainfluenza	Type 3
Parainfluenza	Type 4	Coronavirus	HKU1 (recombinant)
Metapneumovirus	Peru 6–2003	Coronavirus	OC43
C. pneumoniae	CWL-029	Coronavirus	NL63
M. pneumoniae	M129	Coronavirus	229E
Coxsackievirus	Type A1	Bordetella pertussis	A639

RNA transcripts of the N gene (from 28274 to 29533) of SARS-CoV-2 Wuhan-Hu-1 (GenBank No. MN908947.3), Delta variant (EPI_ISL_8038262), and Omicron (EPI_ISL_8752447) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Total RNA of SARS-related coronaviruses (SARSr-CoV JTMCI5 strain) and HKU4 was extracted from two intestinal tissue samples of bats infected with the different corresponding viruses and then stored in our laboratory. The RNA of respiratory secretions from BALB/c mice and cynomolgus monkeys infected with SARS-CoV-2 is stored at Changchun Veterinary Research Institute. Total RNA of MERS-CoV strain GD01 was stored in our laboratory.

The total nucleic acids of multiple respiratory pathogens, such as human coronavirus OC43, 229E, NL63, and HKU1 (Table 2), were purified from the NATrol RP Multimarker Controls kit (ZeptoMetrix Corporation, Franklin, United States) by the TIANamp Virus DNA/RNA Kit (TIANGEN Company, Beijing, China).

Establishment and Optimization of the RT-LAMP-VF Reaction System

Different concentrations of the recombinant plasmid pUC57-N were used as templates to estimate the RT-LAMP-VF method. The reaction solution with a total volume of 25 μ l was configured

and the main containing primer, AMV reverse transcriptase (Promega, Beijing, China), Bst2.0 WarmStart® DNA polymerase (New England Biolabs, Beijing, China), and the template was prepared, moreover, the other components and their information are presented in Table 3. The reaction mixture was mixed and amplified at 61°C for 50 min. All amplification products were detected with a disposable nucleic acid visualization detection device (Ustar Biotech, Co., Ltd., Hangzhou, China).

The recombinant plasmid pUC57-N was used as the amplification template, and five different amplification temperatures (59, 61, 63, 65, and 67°C) were tested to determine the optimal amplification temperature. The amplification was performed at constant temperature for 50 min, and the reaction results were analyzed. After determining the optimal amplification temperature, four different amplification times (30, 40, 50, and 60 min) were tested at the optimal temperature. Each amplification reaction was repeated three times.

SARS-CoV-2 RNA was used as the RT-LAMP-VF amplification template to verify the above-optimized conditions, and samples with different RNA copy numbers were detected under the optimal conditions. Each amplification reaction was repeated three times.

RT-LAMP-VF Assay Specificity and Sensitivity Evaluation

To evaluate the specificity of the RT-LAMP-VF method, we extracted SARSr-CoV and HKU4 RNA from the intestinal tissues of two bats with an RNA extraction kit and extracted the RNAs of various respiratory pathogens from the RP1 and RP2 kits of NATrol™ RP Multimarker control. Then, the above nucleic acids were tested to evaluate the specificity of the RT-LAMP-VF.

The RNA transcript samples were diluted to 2×10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , 10^0 , and 10^{-1} copies/ μ l by 10-fold serial dilution. The sensitivity of the RT-LAMP-VF method was evaluated by assaying samples with different RNA copy numbers to obtain a lower detection limit.

The synthetic RNA transcripts of SARS-CoV-2 mutant strains, including Wuhan-Hu-1 strain, Delta, and Omicron variants, were used to evaluate the RT-LAMP-VF assay, and the RNA concentration of each mutant strain was 20 copies/ μ l.

TABLE 3 | Reaction system of RT-LAMP-VF assay.

Composition	Final concentration	Manufacturer
dNTP	1.4 mM	Bao Biological, Dalian, China
MgSO ₄	4 mM	Sigma, Shanghai, China
10 × Buffer	2.5 μL	New England Biolabs, Beijing, China
Betaine	0.2 M	Sigma, Shanghai, China
Bst2.0 WarmStart DNA polymerase	8 U	New England Biolabs, Beijing, China
AMV reverse transcriptase	5 U	Promega, Beijing, China
FIP/BIP	0.2 μM	Bao Biological, Dalian, China
F3/B3	0.05 μM	Bao Biological, Dalian, China
LF/LB	0.1 μM	Bao Biological, Dalian, China
Template	5 μL	Sangon Biotech, Shanghai, China
DEPC-treated water	7.25 μL	Solarbio, Beijing, China

TABLE 4 | Reaction temperature optimization for RT-LAMP-VF.

Temperature/°C	Recombinant plasmids dilution (2 × copies/μl)								
	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰	N
59	+	+	—	—	—	—	—	—	—
61	+	+	+	+	+	—	—	—	—
63	+	+	+	+	+	—	—	—	—
65	+	+	+	+	+	+	+	—	—
67	+	+	+	+	+	—	—	—	—

Three replications were performed for each trial.

TABLE 5 | Reaction time optimization for the RT-LAMP-VF assay.

Time/min	Recombinant plasmids dilution (2 × copies/μl)								
	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰	N
30	+	+	+	+	+	—	—	—	—
40	+	+	+	+	+	+	—	—	—
50	+	+	+	+	+	+	+	—	—
60	+	+	+	+	+	+	+	—	—

Three replications were performed for each trial.

TABLE 6 | Reaction temperature optimization of RNA for RT-LAMP-VF assay.

Temperature/°C	Recombinant plasmids dilution (2 × copies/μl)								
	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰	N
59	+	+	—	—	—	—	—	—	—
61	+	+	+	+	+	+	+	—	—
63	+	+	+	+	+	+	—	—	—
65	+	+	+	+	+	+	—	—	—

Three replications were performed for each trial.

The Gold Standard for Detection of SARS-CoV-2 RNA

According to a protocol of the gold standard for SARS-CoV-2 recommended by the Chinese center for Disease Control and Prevention, the RT-qPCR was performed. The sequence of primers and probes was described in **Table 1**. Reactions were conducted in a 25 μl volume following the instructions of TaqManTM qPCR Master Mix (Applied Biosystems, Foster, CA, United States). The reaction cycle parameters were set as follows: reverse transcription at 50°C for 10 min, denaturation at 95°C for 5 min, and then 40 amplification cycles of 95°C for 10 s and 55°C for 40 s. After completion of amplification, if the cycle threshold (CT) value is lower than 37, the results are judged to be positive. The result is negative when the CT value is greater than 40. When the CT value is between 37 and 40, it is suggested to be detected again. Each group included one no-template control.

Detection of Clinical Samples by the RT-LAMP-VF Method

The RNA of respiratory secretions from BALB/c mice and cynomolgus monkeys infected with SARS-CoV-2 was collected, including 6 respiratory secretions from BALB/c mice, 5 throat swab samples from cynomolgus monkeys, and 20 respiratory secretions from healthy BALB/c mice and cynomolgus monkeys. The RNA of the above samples was detected by RT-LAMP-VF and RT-qPCR. Each amplification reaction was repeated three times.

RESULTS

RT-LAMP-VF Assay Development

In this assay, the amplification product is placed into a disposable nucleic acid detection device, and the detection device is closed. The closed vertical flow nucleic acid detection device consists of a test strip and diluent. In this device, the control line and detection line are labeled with anti-streptavidin antibody and anti-FITC antibody, respectively. Simultaneously, gold particles are incubated on the binding pad, which was coated with streptavidin. After the tube containing the amplification products is placed into the device, the amplicons that are labeled with biotin can bind to the colloidal gold particles conjugated with streptavidin to form a complex. Then, the complex labeled with FITC is captured by an anti-FITC antibody on the test line of the strip, and the test results are produced. Abundant gold particles gather to form visible lines. The results are observable by

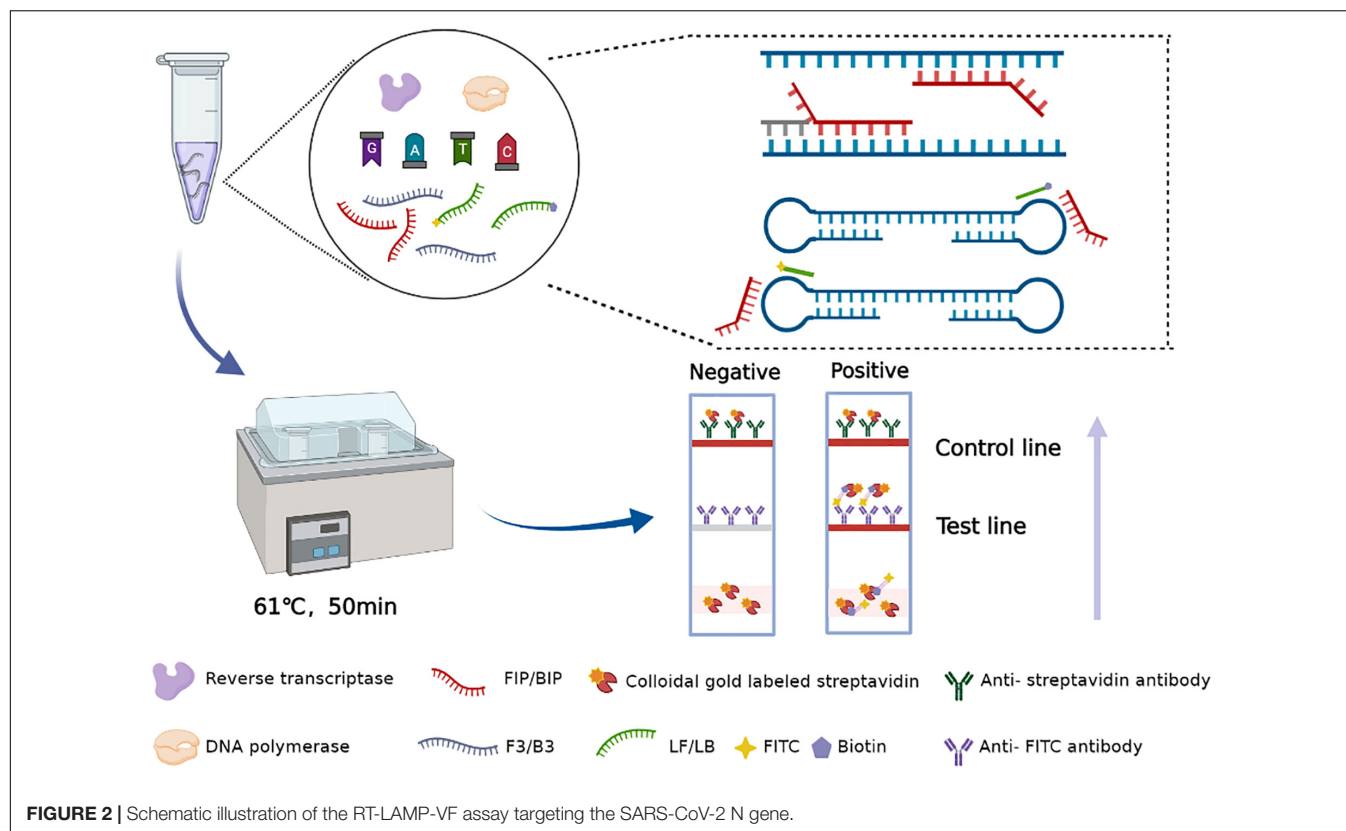


TABLE 7 | The sensitivity and specificity of the RT-LAMP-VF assay were evaluated in viral RNA specimens.

The RT-LAMP-VF assay panel	Two gold standard real time RT-PCR assays		Sensitivity	Specificity	Concordance rate (%)
	Positive samples (N = 11)	Negative samples (N = 20)	Concordance rate (95% CI)		
Positive	11	0	100% (67.8–100%)	100% (88–100%)	100%
Negative	0	20			

the naked eye within 5 min, without additional dyes or fluorescence signal acquisition equipment. When both the detection line and the control line show red bands, the results are judged to be positive; if only the control line appears as a red band, the result is negative; if no red band appears at the control line, the test result is considered invalid (Figure 2).

Optimizing the RT-LAMP-VF Reaction Conditions

The reaction conditions of the RT-LAMP-VF method were optimized with different concentrations of recombinant plasmid pUC57-N as the amplification template.

The RT-LAMP amplification reaction solution was tested at five different amplification temperatures (59, 61, 63, 65, and 67°C) with amplification at constant temperature for 50 min. According to the reaction results, the sensitivity of the RT-LAMP reaction was optimal at 65°C, at which temperature the recombinant plasmid pUC57-N could be detected at a minimum of 2×10^1 copies/ μ l (Table 4).

To determine the optimal amplification time, 25 μ l of the amplification reaction solution was amplified at 65°C for 30, 40, 50, and 60 min. The lowest concentration of recombinant plasmid pUC57-N, 2×10^1 copies/ μ l, could be detected when the amplification time was 50 min at 65°C. Therefore, 50 min was considered the optimal amplification time for the RT-LAMP reaction (Table 5).

Since SARS-CoV-2 is an RNA virus, RNA transcripts were used as amplification templates to verify that the optimized conditions were suitable for RT-LAMP-VF detection of SARS-CoV-2. At least 2×10^1 copies/ μ l of RNA transcripts were detected after amplification at 61°C for 50 min. Therefore, 61°C was considered the optimal amplification temperature (Table 6).

Specificity and Sensitivity of the RT-LAMP-VF Assay

The specificity of the RT-LAMP-VF method was evaluated by testing RNA samples of MERS-CoV, SARSr-CoV, HKU4, and various respiratory pathogens and the RNA transcripts of SARS-CoV-2 as templates. The results showed that

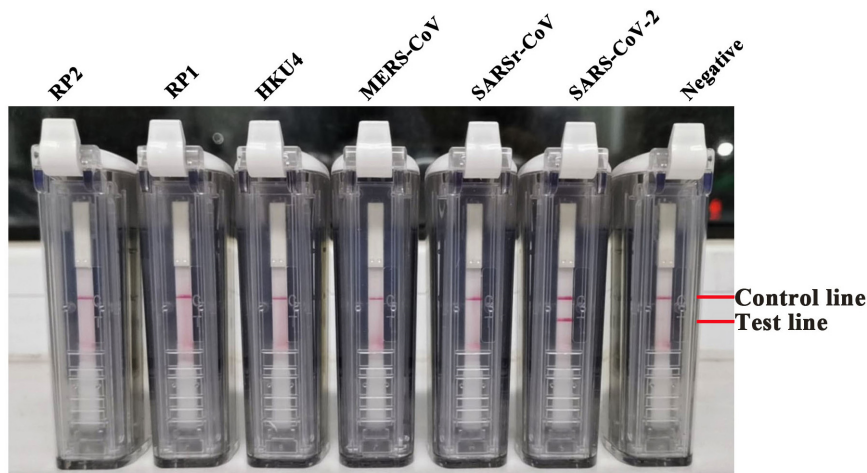


FIGURE 3 | The specificity of the RT-LAMP-VF assay has been examined through the respiratory pathogens. Nucleic acids from a variety of human coronaviruses and other respiratory pathogens were detected by the RT-LAMP-VF assay.

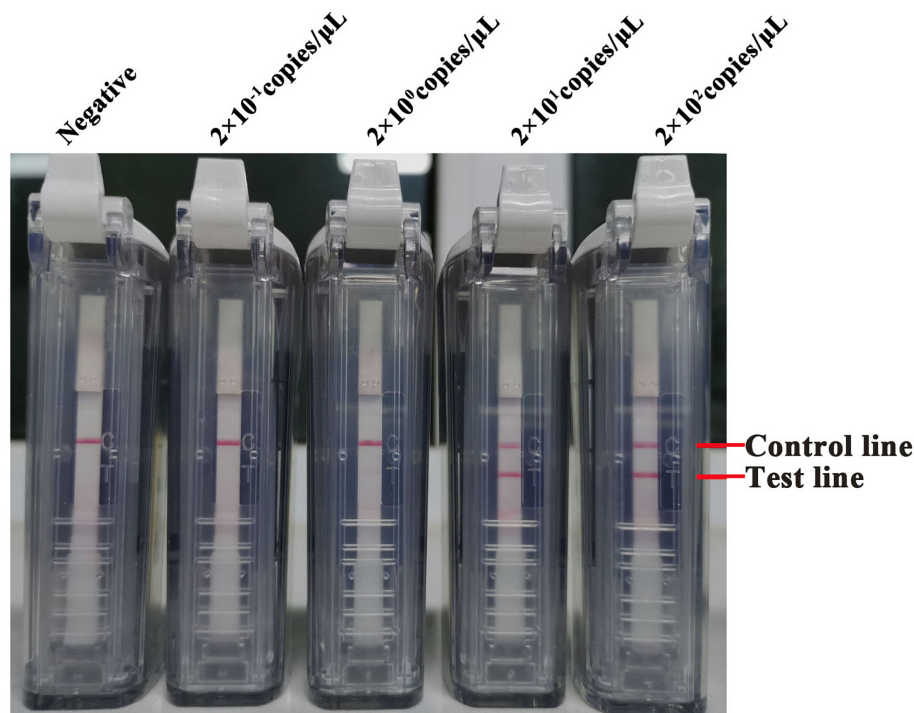


FIGURE 4 | The sensibility of the RT-LAMP-VF assay targeting the N gene. The limit of detection of the RT-LAMP-VF assay using 10-fold serially of SARS-CoV-2 RNA transcripts.

only the RNA transcript of SARS-CoV-2 produced a positive result, and the RT-LAMP-VF assay had no cross-reaction with SARSr-CoV, HKU4, HKU1, OC43, 229E, or others (Figure 3).

Tenfold serial dilutions of the synthesized RNA transcripts (ranging from 2×10^6 to 2×10^{-1} copies/ μ L) were subjected to the RT-LAMP-VF assay to assess its detection limit. Three replicates were performed for each trial. The

amplification was performed at 61°C for 50 min, and the method can detect SARS-CoV-2 RNA transcripts at as few as 20 copies/ μ L (Figure 4). For evaluating the applicability of the RT-LAMP-VF assay, multiple variants were used for evaluating this method, including the Wuhan-Hu-1 strain and the Delta and Omicron variants. As shown in Figure 5, RT-LAMP-VF can be used to detect Delta and Omicron variants, and the detection limit for all was 20

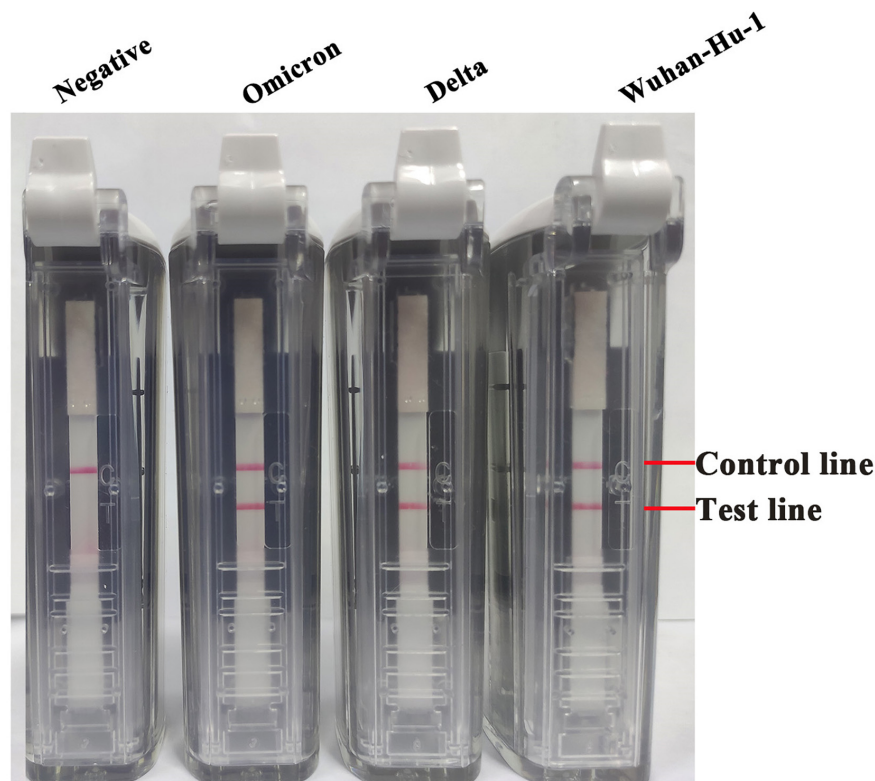


FIGURE 5 | The applicability of the RT-LAMP-VF assay detected multiple SARS-CoV-2 mutants. Multiple RNA transcripts of variants including Wuhan-Hu-1 strain, Delta, and Omicron were used to evaluate the RT-LAMP-VF assay, and the concentration of each RNA transcript was 20 copies/ μ l.

copies/ μ l RNA transcripts. This proves that the RT-LAMP-VF assay has good applicability in the detection of Delta and Omicron mutants.

Evaluation of the RT-LAMP-VF Method Using Clinical Samples

The RNA of respiratory secretions from BALB/c mice and cynomolgus monkeys infected with SARS-CoV-2 and from 20 healthy BALB/c mice and cynomolgus monkeys was also studied. Using the above method, all 11 positive samples were accurately detected. The results show that RT-LAMP-VF can be applied to the detection of clinical samples. The RT-LAMP-VF assay had a sensitivity of 100% (95% CI, 0.678–1.00) and a specificity of 100% (95% CI, 0.88–1.00). The coincidence rate between the RT-qPCR and RT-LAMP-VF assays was 100%, indicating that the assays showed high consistency (Table 7).

DISCUSSION

SARS-CoV-2 first emerged in December 2019, and it has since been spreading rapidly worldwide, causing the ongoing global COVID-19 pandemic. In some areas with large populations, once disease spread begins, there is a demand for immediate nucleic acid testing at various sites, even

multiple rounds of full nucleic acid testing, and there is insufficient testing personnel and equipment for the large amount of testing required. In addition, some localities have set up temporary sampling collection sites for nucleic acid detection, following which the collected samples are sent to a central laboratory for screening. To ensure the quality of samples, reduce the risk of personal infection caused by sample transportation, and meet the need for rapid on-site diagnosis of COVID-19, we established a rapid and simple RT-LAMP-VF detection method.

In this article, we established and characterized a RT-LAMP-VF detection method for the SARS-CoV-2 N gene. This assay takes only 50 min to detect an RNA transcript at 2×10^1 copies/ μ l. Compared with other SARS-CoV-2 molecular methods, such as PCR and RT-qPCR, the RT-LAMP-VF method is fast and can be performed at a constant temperature. Yan and colleagues developed an RT-LAMP method for detecting SARS-CoV-2 that targeted the ORF1ab gene and S gene (Yan et al., 2020). The amplification results were interpreted with a real-time turbidity meter or visually. The primers targeting the ORF1ab gene and S gene could detect 2×10^1 copies/ μ l and 2×10^2 copies/ μ l SARS-CoV-2 RNA, respectively. Huang and colleagues also developed RT-LAMP methods for SARS-CoV-2 targeting the ORF1ab, N, and S genes (Huang et al., 2020). The amplification results were generated by a colorimetric method based on

the pH indicator phenol red. The minimum detection level was 2 copies of SARS-CoV-2 RNA, but the assessment of the colorimetric results was not intuitive when the sample was weakly positive, and the results were easily affected by the pH of the buffer.

Unlike the RT-LAMP methods mentioned above, the RT-LAMP-VF method introduces additional loop primers into the amplification system, which improves the overall amplification efficiency. Furthermore, the method used FITC and biotin to label the 5' ends of loop primers LF and LB, which allowed the amplification results to be visualized on a closed vertical flow nucleic acid detection device. Conventional LAMP makes the operation vulnerable to aerosol pollution (Notomi et al., 2000). To make up for these deficiencies, we used the closed vertical flow nucleic acid detection device, it not only showed the RT-LAMP product but also avoided the problem of aerosol contamination of the LAMP reaction. However, the RT-LAMP-VF method also has some shortcomings. The container matched with the disposable nucleic acid detection device is a single PCR tube, so the detection amount of a single sample is limited, and high-throughput detection of a large number of samples cannot be realized.

With the continuation of the COVID-19 epidemic, SARS-CoV-2, similar to other RNA viruses, continues to mutate, and new variants continue to appear all over the world, including some variants with stronger infectivity and transmission potential, which further increases the difficulty of epidemic prevention and control. D614G was the earliest identified mutation of SARS-CoV-2, affecting the gene encoding the S protein (Corum and Zimmer, 2021). A variant named 01, which originated from N501y, was found in the United Kingdom in December 2020 and eventually called SARS-CoV-2 VOC 202012Accord 01 or B.1.1.7. Subsequently, the mutant B.1.351 was also found to have a variety of mutations (Madhi et al., 2021). Compared with the original SARS-CoV-2, many nucleotides have been replaced in these variants, leading to amino acid mutations, and most of these mutations were located in the S protein (Faria et al., 2021; Leung et al., 2021; Rambaut et al., 2021). The loss of certain sites in the S protein or nucleotide sequence can affect the performance of PCR detection and diagnostic methods that rely on the S gene as the target (Corum and Zimmer, 2021). Our RT-LAMP-VF detection method targeted a specific fragment of the N gene of SARS-CoV-2 and can be used for detecting many SARS-CoV-2 variants, including the original strain, the Delta variant, and the Omicron variant. To test whether the mutation of the virus strain affected the accuracy of RT-LAMP-VF detection, we tested our method on SARS-CoV-2 mutant RNA transcripts. The results showed that the sensitivity of this method was not significantly different for the Delta and Omicron mutants.

Since the outbreak of the pandemic in late 2019, multifarious detection kits have emerged to effectively control the spread of the pandemic, but the targets of different methods are different. The researchers analyzed and compared the sensitivity

and detection efficiency of RT-qPCR primers and probes for SARS-CoV-2 (Vogels et al., 2020). Among them, the primer sensitivity and detection efficiency for the N gene and ORF1a/b gene were the highest. A previous study showed that RT-LAMP for the detection of the SARS-CoV-2 N gene could specifically detect SARS-CoV-2 RNA and did not cross-react with related coronaviruses (Baek et al., 2020). In this paper, we also analyzed the RT-LAMP-VF detection method for the N gene of SARS-CoV-2. The specificity of the method was evaluated by application to samples of other coronaviruses, including SARSr-CoV, MERS-CoV, HKU4, HKU1, OC43, 229E, and NL63. Our results were consistent with the gold standard of RT-qPCR.

Since COVID-19 has the characteristics of rapid propagation, wide distribution, and repeated outbreaks, it is of great significance to develop a rapid and simple method to improve the control of COVID-19. In this paper, the RT-LAMP-VF method for the N gene of SARS-CoV-2 takes only 50 min and can detect RNA transcript at 20 copies/ μ l. The RT-LAMP-VF method established in this paper is suitable for the rapid detection of new or recurrent infectious diseases and can be considered the best alternative to RT-qPCR (Keikha, 2018). In summary, this method has broad applicability and is expected to achieve on-site real-time detection without the need to transport samples, making it especially useful for screening in airports and train stations.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

HW and PH designed the experiments. MY, PH, and HW wrote the manuscript. MY, YS, and XL performed the experiment. HJ and YB analyzed the data. HZ, YYL, YG, and HW reviewed the manuscript. YGL gave help in the field of RNA extraction. All authors have read and agreed to the published version of the manuscript.

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REFERENCES

- Baek, Y. H., Um, J., Antigua, K. J. C., Park, J. H., Kim, Y., Oh, S., et al. (2020). Development of a reverse transcription-loop-mediated isothermal amplification as a rapid early-detection method for novel SARS-CoV-2. *Emerg Microbes Infect* 9, 998–1007. doi: 10.1080/22221751.2020.1756698
- Broughton, J. P., Deng, X., Yu, G., Fasching, C. L., Servellita, V., Singh, J., et al. (2020). CRISPR-Cas12-based detection of SARS-CoV-2. *Nat Biotechnol* 38, 870–874. doi: 10.1038/s41587-020-0513-4
- Chu, H., Chan, J. F., Wang, Y., Yuen, T. T., Chai, Y., Hou, Y., et al. (2020). Comparative Replication and Immune Activation Profiles of SARS-CoV-2 and SARS-CoV in Human Lungs: An Ex Vivo Study With Implications for the Pathogenesis of COVID-19. *Clin Infect Dis* 71, 1400–1409. doi: 10.1093/cid/ciaa410
- Corman, V. M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D. K., et al. (2020). Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill* 25, doi: 10.2807/1560-7917.Es.2020.25.3.2000045
- Corum, J., and Zimmer, C. (2021). *Coronavirus variants and mutations*. The New York Times 26 April, 2021.
- Di, L., Fu, Y., Sun, Y., Li, J., Liu, L., Yao, J., et al. (2020). RNA sequencing by direct tagmentation of RNA/DNA hybrids. *Proc Natl Acad Sci U S A* 117, 2886–2893. doi: 10.1073/pnas.1919800117
- Faria, N. R., Mellan, T. A., Whittaker, C., Claro, I. M., Candido, D. D. S., Mishra, S., et al. (2021). Genomics and epidemiology of the P.1 SARS-CoV-2 lineage in Manaus, Brazil. *Science* 372, 815–821. doi: 10.1126/science.abh2644
- Fu, S., Qu, G., Guo, S., Ma, L., Zhang, N., Zhang, S., et al. (2011). Applications of loop-mediated isothermal DNA amplification. *Appl Biochem Biotechnol* 163, 845–850. doi: 10.1007/s12010-010-9088-8
- Gollakner, R., and Capua, I. (2020). Is COVID-19 the first pandemic that evolves into a panzootic? *Vet Ital* 56, 7–8. doi: 10.12834/VetIt.2246.12523.1
- Han, Q., Zhang, S., Liu, D., Yan, F., Wang, H., Huang, P., et al. (2020). Development of a Visible Reverse Transcription-Loop-Mediated Isothermal Amplification Assay for the Detection of Rift Valley Fever Virus. *Front Microbiol* 11:590732. doi: 10.3389/fmicb.2020.590732
- Huang, P., Wang, H., Cao, Z., Jin, H., Chi, H., Zhao, J., et al. (2018). A Rapid and Specific Assay for the Detection of MERS-CoV. *Front Microbiol* 9:1101. doi: 10.3389/fmicb.2018.01101
- Huang, W. E., Lim, B., Hsu, C. C., Xiong, D., Wu, W., Yu, Y., et al. (2020). RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2. *Microb Biotechnol* 13, 950–961. doi: 10.1111/1751-7915.13586
- Keikha, M. (2018). LAMP Method as One of the Best Candidates for Replacing with PCR Method. *Malays J Med Sci* 25, 121–123. doi: 10.21315/mjms2018.25.1.15
- Leung, K., Shum, M. H., Leung, G. M., Lam, T. T., and Wu, J. T. (2021). Early transmissibility assessment of the N501Y mutant strains of SARS-CoV-2 in the United Kingdom, October to November 2020. *Euro Surveill* 26, doi: 10.2807/1560-7917.Es.2020.26.1.2002106
- Madhi, S. A., Baillie, V., Cutland, C. L., Voysey, M., Koen, A. L., Fairlie, L., et al. (2021). Efficacy of the ChAdOx1 nCoV-19 Covid-19 Vaccine against the B.1.351 Variant. *N Engl J Med* 384, 1885–1898. doi: 10.1056/NEJMoa2102214
- Mori, Y., Nagamine, K., Tomita, N., and Notomi, T. (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem Biophys Res Commun* 289, 150–154. doi: 10.1006/bbrc.2001.5921
- Munster, V. J., Koopmans, M., van Doremalen, N., van Riel, D., and de Wit, E. (2020). A Novel Coronavirus Emerging in China - Key Questions for Impact Assessment. *N Engl J Med* 382, 692–694. doi: 10.1056/NEJMp2000929
- Nagamine, K., Hase, T., and Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol Cell Probes* 16, 223–229. doi: 10.1006/mcpr.2002.0415
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., et al. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28, E63. doi: 10.1093/nar/28.12.e63
- Oloniniyi, O. K., Kurosaki, Y., Miyamoto, H., Takada, A., and Yasuda, J. (2017). Rapid detection of all known ebolavirus species by reverse transcription-loop-mediated isothermal amplification (RT-LAMP). *J Virol Methods* 246, 8–14. doi: 10.1016/j.jviromet.2017.03.011
- Planas, D., Veyer, D., Baidaliuk, A., Staropoli, I., Guivel-Benhassine, F., Rajah, M. M., et al. (2021). Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. *Nature* 596, 276–280. doi: 10.1038/s41586-021-03777-9
- Rambaut, A., Loman, N., Pybus, O., Barclay, W., Barrett, J., Carabelli, A., et al. (2021). Preliminary genomic characterisation of an emergent SARS-CoV-2 lineage in the UK defined by a novel set of spike mutations [Online]. Available: <https://virological.org/t/preliminary-genomic-characterisation-of-an-emergent-sars-cov-2-lineage-in-the-uk-defined-by-a-novel-set-of-spike-mutations/563> [Accessed].
- Sanche, S., Lin, Y. T., Xu, C., Romero-Severson, E., Hengartner, N., and Ke, R. (2020). High Contagiousness and Rapid Spread of Severe Acute Respiratory Syndrome Coronavirus 2. *Emerg Infect Dis* 26, 1470–1477. doi: 10.3201/eid2607.200282
- Shi, J., Wen, Z., Zhong, G., Yang, H., Wang, C., Huang, B., et al. (2020). Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. *Science* 368, 1016–1020. doi: 10.1126/science.ab b7015
- Tomita, N., Mori, Y., Kanda, H., and Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc* 3, 877–882. doi: 10.1038/nprot.2008.57
- Vogels, C. B. F., Brito, A. F., Wyllie, A. L., Fauver, J. R., Ott, I. M., Kalinich, C. C., et al. (2020). Analytical sensitivity and efficiency comparisons of SARS-CoV-2 RT-qPCR primer-probe sets. *Nat Microbiol* 5, 1299–1305. doi: 10.1038/s41564-020-0761-6
- Wang, Y., Zhang, Y., Chen, J., Wang, M., Zhang, T., Luo, W., et al. (2021). Detection of SARS-CoV-2 and Its Mutated Variants via CRISPR-Cas13-Based Transcription Amplification. *Anal Chem* 93, 3393–3402. doi: 10.1021/acs.analchem.0c04303
- Yan, C., Cui, J., Huang, L., Du, B., Chen, L., Xue, G., et al. (2020). Rapid and visual detection of 2019 novel coronavirus (SARS-CoV-2) by a reverse transcription loop-mediated isothermal amplification assay. *Clin Microbiol Infect* 26, 773–779. doi: 10.1016/j.cmi.2020.04.001

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Molecular characterization of SARS-CoV-2 detected in Tokyo, Japan during five waves: Identification of the amino acid substitutions associated with transmissibility and severity

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Many variants of SARS-CoV-2 have emerged around the world. It is therefore important to understand its global viral evolution and the corresponding mutations associated with transmissibility and severity. In this study, we analyzed 112 whole genome sequences of SARS-CoV-2 collected from patients at Juntendo University Hospital in Tokyo and the genome data from entire Japan deposited in Global Initiative on Sharing Avian Influenza Data (GISAID) to examine the relationship of amino acid changes with the transmissibility and the severity of each strain/lineage. We identified 12 lineages, including B.1.1.284, B.1.1.214, R.1, AY.29, and AY.29.1, which were prevalent specifically in Japan. B.1.1.284 was most frequently detected in the second wave, but B.1.1.214 became the predominant lineage in the third wave, indicating that B.1.1.214 has a higher transmissibility than B.1.1.284. The most prevalent lineage during the fourth and fifth wave was B.1.1.7 and AY.29, respectively. In regard to the severity of identified lineages, B.1.1.214 was significantly lower than the reference lineage, B.1.1.284. Analysis of the genome sequence and other traits of each lineage/strain revealed the mutations in S, N, and NSPs that increase the transmissibility and/or severity. These mutations include S: M153T, N: P151L, NSP3: S543P, NSP5: P108S, and NSP12: A423V in B.1.1.284; S:

W152L and E484K in R.1; S: H69del, V70del, and N501Y in the Alpha strain; S: L452R, T478K, and P681R in the Delta strain. Furthermore, it is suggested that the transmissibility of B.1.1.214 could be enhanced by the mutations N: M234I, NSP14: P43L, and NSP16: R287I. To address the issue of the virus evolution, it is necessary to continuously monitor the genomes of SARS-CoV-2 and analyze the effects of mutations for developing vaccines and antiviral drugs effective against SARS-CoV-2 variants.

KEYWORDS

SARS-CoV-2, COVID-19, Japan, genome sequencing, transmissibility, severity

Introduction

In December 2019, several cases of unknown pneumonia were detected in the city of Wuhan, Hubei province, China. Deep sequencing identified the causative agent as a novel coronavirus, which was named nCoV-2019 and later renamed to SARS-CoV-2 (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020; Huang et al., 2020; Wu et al., 2020; Zhu et al., 2020).

Rapidly spreading throughout the world, the World Health Organization (WHO) officially declared the SARS-CoV-2 outbreak as a pandemic on March 11, 2020 (World Health Organization, 2020). As of March 31, 2022, there have been 485,369,784 confirmed cases and 6,138,368 deaths around the world.¹ In Japan, the first domestic case was identified on January 16, 2020, and, after five waves, a total of 1,716,928 positive cases were detected by the end of October 2021.²

The SARS-CoV-2 genome encodes 14 open reading frames: Orf1a/ab, four structural proteins (S, E, M, and N), and nine putative accessory proteins (Hoque et al., 2020; Islam et al., 2020; Rahman et al., 2020, 2021b). The Orf1a/ab is a large polyprotein and is proteolytically processed into 16 non-structural proteins (NSPs; Hoque et al., 2020). One of them, NSP14, consists of an N-terminal exonuclease domain and a C-terminal N7-MTase domain (Ogando et al., 2020). NSP14 functions as a proofreading molecule that reduces the error rate during replication. Although SARS-CoV-2 does not show as high of a mutation rate as other RNA viruses, many SARS-CoV-2 variants are emerging because of the huge amount of viral replication from the large number of infected hosts. As a result of extended human-to-human transmission, SARS-CoV-2 has obtained amino acid changes with fitness advantages. It was reported that D614G (Korber et al., 2020; Yurkovetskiy et al., 2020; Volz et al., 2021a) and N501Y (Liu et al., 2022) in S protein enhance the transmissibility of SARS-CoV-2. In addition to S protein mutations, nucleocapsid

mutations R203K and G204R were found to increase the infectivity, fitness, and pathogenicity of SARS-CoV-2 (Rahman et al., 2021a; Wu et al., 2021). The amino acid substitution P323L in NSP12 (RNA-dependent RNA polymerase) was also identified as the highly prevalent mutation, and the significant association between the presence of P323L and severe disease was reported (Flores-Alanis et al., 2021). The deletions of the viral genes such as 382-nt deletion of ORF8 (Young et al., 2020), 81-nt deletion of ORF7a (Holland et al., 2020), 30-nt deletion of spike protein (Lau et al., 2020), and 24-nt deletion of NSP1 (Islam et al., 2020) were found, which were predicted to influence the viral adaptation or attenuation by affecting the structures and functions of the proteins (Hoque et al., 2020; Islam et al., 2020).

Since the continuous replication of SARS-CoV-2 leads to the emergence and spread of new variants with higher transmissibility and varying severity, it is necessary to monitor all of the local diversity of SARS-CoV-2 variants to understand its global viral evolution and the association of mutations with transmissibility and severity.

In this study, we performed a comprehensive genomic analysis of 112 SARS-CoV-2 strains detected at Juntendo University Hospital in Tokyo and an analysis of the sequence data from entire Japan deposited in Global Initiative on Sharing Avian Influenza Data (GISAID) to understand the changing trend of SARS-CoV-2 genomes and find the correlation of amino acid changes with the transmissibility and severity of each lineage. Investigation of the mutations in viral genomes and analysis of their phenotype will be necessary for developing effective vaccines and antivirals against variants of SARS-CoV-2 (Hoque et al., 2020; Islam et al., 2020).

Materials and methods

Specimen collection and testing

Clinical samples were collected between March 1, 2020, and July 31, 2021, at Juntendo University Hospital. One hundred and twelve specimens where SARS-CoV-2 N gene were detected with

¹ <https://covid19.who.int/>

² <https://www.mhlw.go.jp/stf/covid-19/open-data.html>

less than 30 cycle threshold (Ct) values by real-time RT-PCR tests were utilized for this study following the WHO recommendation that specimens tested positive for COVID-19 with Ct value <30 are considered good materials for sequencing the whole genome of SARS-CoV-2 (Operational considerations for COVID-19 surveillance using GISRS: interim guidance, 26 March 2020; [World Health Organization, 2020](#)). Frozen-stored nasopharyngeal swab specimens in phosphate-buffered saline (PBS) and saliva samples (−80°C, single freeze–thaw) from patients with COVID-19 were used.

Whole genome sequencing of SARS-CoV-2

One hundred and twelve purified RNAs were reverse-transcribed into cDNAs using the SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, United States). The synthesized cDNAs were amplified with the Ion AmpliSeq SARS-CoV-2 Research Panel (Thermo Fisher Scientific, Waltham, MA, United States) in the Ion GeneStudio S5 System according to the manufacturer's instructions. The Ion AmpliSeq SARS-CoV-2 Research Panel consists of two primer pools targeting 237 amplicons tiled across the SARS-CoV-2 genome, with an additional five primer pairs targeting human expression controls. The SARS-CoV-2 amplicons range from 125 to 275 bp in length. Amplified samples were then sequenced using Ion 530 chips (Thermo Fisher Scientific) with eight samples per chip on the Ion S5 system. The Torrent Suite 5.14.0 platform and specific plugins were used for Next-Generation Sequencing (NGS) data analysis. All analyzed sequences showed an alignment accuracy of over 96% and a base coverage over 50×. The pangolin software was used for the assignment of SARS-CoV-2 lineages. All sequences were then submitted as FASTA files and deposited in the EpiCoV database of GISAID ([Shu and McCauley, 2017](#)). The accession numbers of these sequences were shown in [Supplementary Table 1](#). Amino acid substitutions in the sequenced viruses were analyzed with GISAID during the registration of the viral genomes, and the information was collected from the EpiCoV database. Analysis of PANGO lineage was performed based on v.3.1.15. Moreover, we analyzed the genome data deposited in GISAID (97,458 complete sequences collected from March 1, 2020 to July 31, 2021 in Japan) to compare the patterns of the prevalent lineages between the samples collected at Juntendo University Hospital and those throughout Japan.

Phylogenetic tree analysis

A total of 121 nucleotide sequences (112 sequences from Juntendo University Hospital and nine reference sequences) were aligned with the MUSCLE program. There were a total of 29,906

positions in the final dataset. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model in MEGA 11. Initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter = 0.1000)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 48.99% sites). The tree with the highest log likelihood (−47424.62) was selected for presentation.

The severity of COVID-19 patients at Juntendo University Hospital

The severity of COVID-19 was categorized into four levels according to the WHO criteria ([World Health Organization, 2021](#)). Briefly, the mild type was defined as patients with mild clinical symptoms, but no evidence of viral pneumonia or hypoxia. The moderate type was defined as patients with fever, respiratory symptoms, or other symptoms, but with no evidence of severe pneumonia, including $\text{SpO}_2 \geq 90\%$ on room air. The severe type was defined as patients with clinical signs of pneumonia and at least one of the following: shortness of breath (breathing rate $\geq 30/\text{min}$), $\text{SpO}_2 < 90\%$ on room air, or severe respiratory distress. The critical type was defined as patients with any of the following symptoms: respiratory failure requiring mechanical ventilation, shock, or a combination of other organ failures requiring ICU monitoring treatment.

Statistical analysis

We collected the information about the infected patients including the severity and the lineage data of SARS-CoV-2 determined by whole genome sequencing. To analyze the factors associated with the severity, we re-categorized the severity status into the two groups. The mild and moderate were defined as the less severe group, and the severe and critical are defined as the severe group. We constructed unadjusted and adjusted logistic regression models for the re-defined severity risk, adjusted the relevant factors (i.e., age and sex), and estimated the effect of the lineage with B.1.1.284 as the reference. Odds Ratios (ORs) and 95% CIs were estimated. A two-sided α of < 0.05 was considered statistically significant. Statistical analyses were conducted using SAS version 9.4 (SAS Institute, Cary, North Carolina).

Ethical approval

This study complied with all relevant national regulations and institutional policies and was conducted in accordance

with the tenets of the Helsinki Declaration. This study was approved by the institutional review board (IRB) at Juntendo University Hospital (IRB #20–036). The need for informed consents from individual patients was waived because all samples were de-identified in line with the Declaration of Helsinki.

Results

Analysis of SARS-CoV-2 lineages in the patients at Juntendo University Hospital in Tokyo, Japan

To determine the genetic characteristics of the SARS-CoV-2 detected at Juntendo University Hospital, we performed whole genome sequencing of clinical specimens. Throughout the five waves, a total of 970 cases were identified as positive for SARS-CoV-2 *via* RT-PCR at Juntendo University Hospital (Figure 1A). One hundred and twelve specimens with a lower Ct value were selected for sequencing analysis. We identified 12 lineages, such as B.1.1, B.1.1.284, B.1.1.214, and AY.29 (Supplementary Table 1). During the first wave, B.1.1 was most frequently detected, but the predominant lineage became B.1.1.284 in the second wave (Figure 1B). The most prevalent lineage during the third, fourth, and fifth wave was B.1.1.214, B.1.1.7 (the Alpha strain), and AY.29 (the Delta strain), respectively. In the fourth wave, R.1 was the second most frequent lineage. AY.29.1, a sub-lineage of AY.29, was also found in the fifth wave. Furthermore, we compared our data with the genome sequences of prevalent viruses in Japan that were downloaded from GISAID (97,458 sequences). We found that the patterns of the dominant lineages during five waves were similar between our data and the data of Japan (Figure 1B).

Phylogenetic analysis of SARS-CoV-2 genomes identified at Juntendo University Hospital

The SARS-CoV-2 genomes sequenced at Juntendo University Hospital formed three clusters consisting of the GISAID clades GR/GRY/O, GH, and GK (Figure 2). The GR/GRY/O cluster was composed of four subclusters, each of which included B.1.1.284, B.1.1.214, R.1, and the Alpha strain, respectively. B.1.1.284 and B.1.1.214 were the domestic lineages that circulated mainly in Japan, while R.1 was chiefly identified in the United States and Japan. The subcluster of the Alpha strain included B.1.1.7 and Q.1. The two viruses belonging to the B.1.346 lineage were situated in the GH clade, with the reference strain derived from Canada. The GK cluster contained the lineages AY.29 and AY.29.1, both of which were the Delta strain chiefly identified in Japan.

Analysis of the amino acid changes in SARS-CoV-2 genomes detected at Juntendo University Hospital

We analyzed 112 SARS-CoV-2 genomes and identified 2,209 amino acid changes (Table 1; Figure 3). Of these amino acid changes, 736 mutations were found in the S protein, which plays a vital role in SARS-CoV-2 infection. The most common change in the S region was D614G. We observed 395 amino acid changes in the N protein, where R203K and G204R were the most widespread mutations. In addition to structural proteins, such as S and N, there were 786 amino acid alterations in ORF1ab, which were cleaved into 16 nonstructural proteins (NSPs). All of the analyzed viruses possessed the amino acid substitution P323L in NSP12.

In B.1.1, which was the most frequently detected lineage during the latter period of the first wave, four amino acid substitutions were commonly identified: S: D614G, N: R203K, N: G204R, and NSP12: P323L (Table 2; Figure 3). These mutations have been maintained in many other lineages. B.1.1.284, the most predominant lineage in the former and latter period of the second wave, had S: M153T and N: P151L in the structural proteins, and NSP3: S543P, NSP5: P108S, and NSP12: A423V in the non-structural proteins in addition to the common mutations. Regarding B.1.1.214, the major lineage before and after the peak of the third wave, no amino acid substitutions were found in the spike region except for D614G. In the other region of this lineage, only N: M234I, NSP14: P43L, and NSP16: R287I were observed. The Alpha viruses, including B.1.1.7 and Q.1 (the most widespread during the former and latter period of the fourth wave), had 25 amino acid changes: 10 changes in S; four changes in N; three changes in ORF8, NSP3, and NSP6; and one change in NSP12 and NSP13. Mutations in S protein involved H69del, V70del, Y144del, N501Y, A570D, D614G, P681H, T716I, S982A, and D1118H. The second most prevalent lineage in the former period of the fourth wave was R.1, where 12 mutations were identified, including S: W152L, S: E484K, S: D614G, and S: G769V. The Delta viruses (AY.29 and AY.29.1) were heavily mutated and had 32 amino acid changes: 11 changes in Spike; four changes in N and NSP3; two changes in ORF7a, NSP4, and NSP12; and one change in M, ORF3, ORF7b, ORF8, NSP6, NSP13, and NSP14. S protein mutations were as follows: T19R, T95I, G142D, E156G, F157del, R158del, L452R, T478K, D614G, P681R, and D950N.

Severity of the patients' symptoms who were infected with the representative lineages at Juntendo University Hospital

To determine whether some lineages had different pathogenicity from the others, the severity of COVID-19 patients' symptoms in Juntendo University Hospital was evaluated. B.1.1.284 was used as the basis for comparison because B.1.1.284 was the earliest lineage that was included in more than 10 samples in our study. We found that the

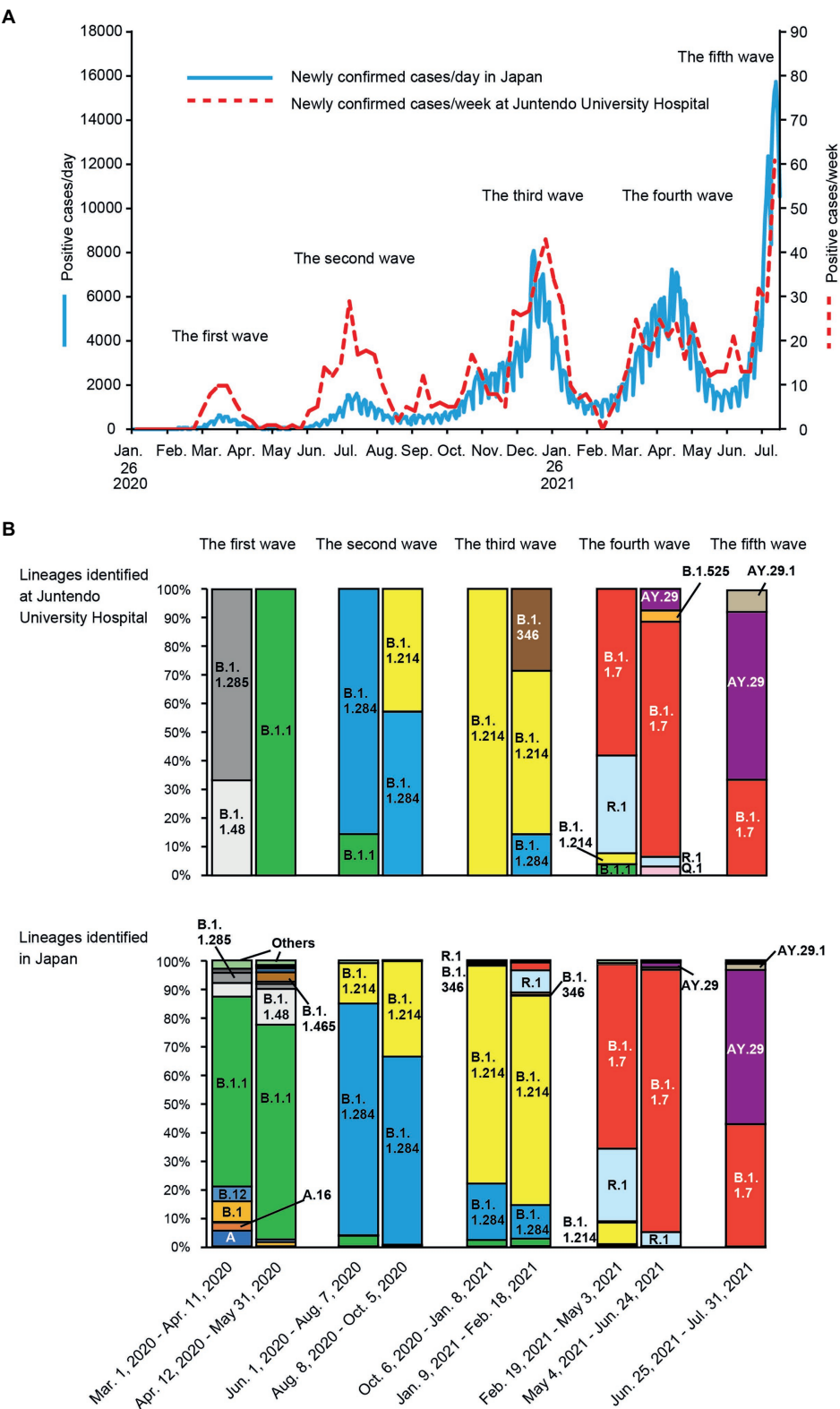


FIGURE 1
Trend of SARS-CoV-2 positive cases and lineage analysis of the viruses in each wave. **(A)** the numbers of newly confirmed cases per week in Japan and newly confirmed cases per day at Juntendo University Hospital are shown by the blue solid line and the red dotted line, respectively. **(B)** the ratios of the identified lineages at Juntendo University Hospital and in entire Japan during the designated periods are displayed.

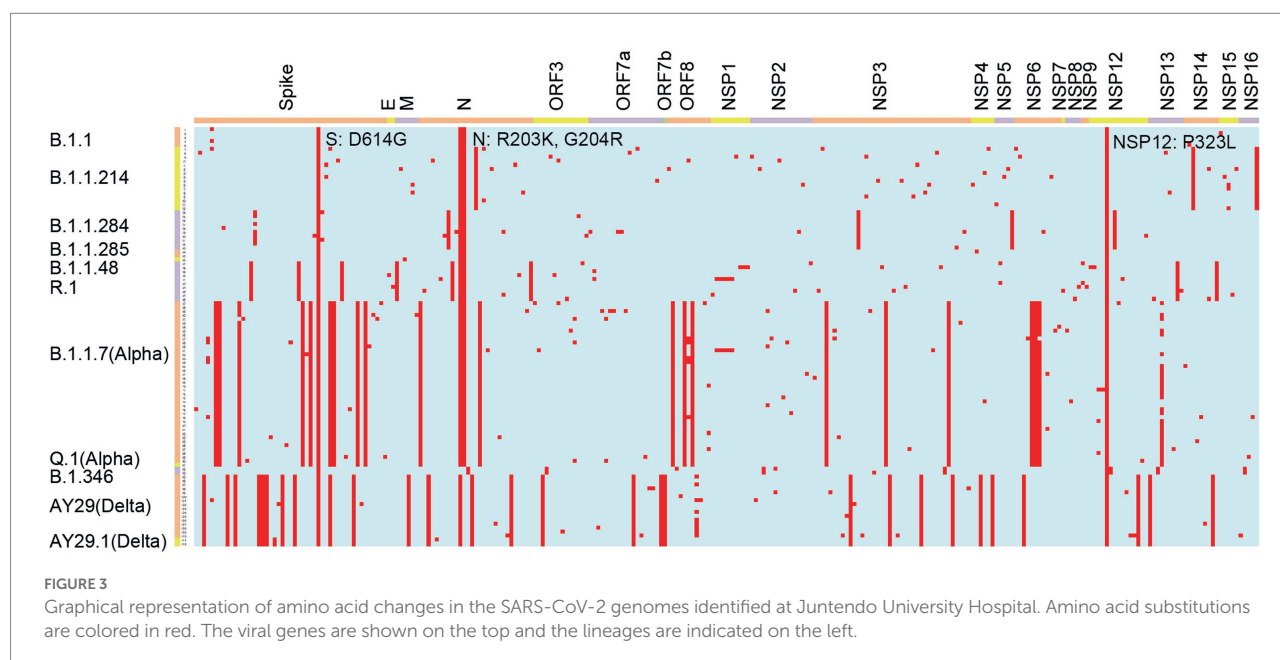


FIGURE 2

Phylogenetic tree of the SARS-CoV-2 genomes collected at Juntendo University Hospital. This tree includes 112 genomes from Juntendo University Hospital and nine reference sequences. The reference strain hCoV-19/Wuhan/WIV04/2019 is shown in red and the other references are shown in blue.

TABLE 1 The number of amino acid changes observed in SARS-CoV-2 sequenced at Juntendo University Hospital.

Genome segment	Missense mutation	In-frame deletion	Stop-gained	Total
Spike	574	162	0	736
E	2	0	0	2
M	33	0	0	33
N	395	0	0	395
ORF3	35	0	0	35
ORF7a	54	1	0	55
ORF7b	18	0	0	18
ORF8	107	0	42	149
ORF1a/ab NSP1	2	13	0	15
NSP2	20	0	0	20
NSP3	243	0	0	243
NSP4	41	0	0	41
NSP5	15	0	0	15
NSP6	26	126	0	152
NSP7	1	0	0	1
NSP8	4	0	0	4
NSP9	3	0	0	3
NSP12	149	0	0	149
NSP13	64	0	0	64
NSP14	51	0	0	51
NSP15	7	0	0	7
NSP16	21	0	0	21
Total	1,865	302	42	2,209



severity of the patients with B.1.1.214 was significantly lower than those with B.1.1.284 as shown in Tables 3, 4 (odds ratio 0.08, 95%CI 0.01–0.84, $p=0.0277$ in the univariate model; odds ratio 0.04, 95%CI 0.00–0.58, $p=0.0373$ in the multivariate model). B.1.1, R.1, Alpha, and Delta exhibited no significant difference in the severity in

comparison with B.1.1.284. Since the patients infected with the Delta strain involved vaccinated and unvaccinated individuals (11 unvaccinated; six vaccinated once; and one vaccinated twice), we also compared the severity between those with and without vaccination. There was no significant difference in the severity between vaccinated

TABLE 2 Amino acid substitutions observed in the representative lineage for each wave in comparison with hCoV-19/Wuhan/WIV04/2019.

Lineage	B.1.1	B.1.1.284	B.1.1.214	R.1	Alpha	Delta
Period	1st wave	2nd wave	3rd wave	4th wave	4th wave	5th wave
Amino acid changes	S: L54F	S: M153T	S: D614G	S: W152L	S: H69del	S: T19R
	S: D614G	S: D614G		S: E484K	S: V70del	S: T95I
				S: D614G	S: Y144del	S: G142D
				S: G769V	S: N501Y	S: E156G
					S: A570D	S: F157del
					S: D614G	S: R158del
					S: P681H	S: L452R
					S: T716I	S: T478K
					S: S982A	S: D614G
					S: D1118H	S: P681R
						S: D950N
				M: F28L		M: I82T
	N: R203K	N: P151L	N: R203K	N: S187L	N: D3L	N: D63G
	N: G204R	N: R203K	N: G204R	N: R203K	N: R203K	N: R203M
		N: G204R	N: M234I	N: G204R	N: G204R	N: G215C
				N: Q418H	N: S235F	N: D377Y
					ORF8: Q27stop	ORF3: S26L
					ORF8: R52I	ORF7a: V82A
					ORF8: Y73C	ORF7a: T120I
						ORF7b: T40I
						ORF8: P93S
	NSP12: P323L	NSP3: S543P	NSP12: P323L	NSP12: P323L	NSP3: T183I	NSP3: A488S
		NSP5: P108S	NSP14: P43L	NSP13: G439R	NSP3: A890D	NSP3: V932A
		NSP12: P323L	NSP16: R287I	NSP14: P412H	NSP3: I1412T	NSP3: P1228L
		NSP12: A423V			NSP6: S106del	NSP3: P1469S
					NSP6: G107del	NSP4: V167L
					NSP6: F108del	NSP4: T492I
					NSP12: P323L	NSP6: T77A
					NSP13: E261D	NSP12: P323L
						NSP12: G671S
						NSP13: P77L
						NSP14: A394V

TABLE 3 Severity of the patients infected with the representative lineages at Juntendo University Hospital.

Severity	The number of patients (% of total)							All lineages
	B.1.1	B.1.1.284	B.1.1.214	R.1	Alpha (B.1.1.7 + Q.1)	Delta (AY.29 + AY.29.1)	Others	
Mild	3 (50.0%)	4 (36.4%)	12 (75.0%)	3 (30.0%)	12 (26.7%)	7 (38.9%)	1 (16.7%)	42 (37.5%)
Moderate	1 (16.7%)	2 (18.2%)	3 (18.8%)	2 (20.0%)	12 (26.7%)	4 (22.2%)	3 (50.0%)	27 (24.1%)
Severe	2 (33.3%)	3 (27.3%)	1 (6.3%)	5 (50.0%)	19 (42.2%)	7 (38.9%)	2 (33.3%)	39 (34.8%)
Critical	0 (0.0%)	2 (18.2%)	0 (0.0%)	0 (0.0%)	2 (4.4%)	0 (0.0%)	0 (0.0%)	4 (3.6%)
Total	6 (100.0%)	11 (100.0%)	16 (100.0%)	10 (100.0%)	45 (100.0%)	18 (100.0%)	6 (100.0%)	112 (100.0%)

and unvaccinated Delta-infected patients in this study ($p=0.5842$ in the univariate model; $p=0.1571$ in the multivariate model).

Furthermore, we observed that age was significantly associated with the progression of symptoms (odds ratio 1.07, 95%CI 1.04–1.10,

$p<0.0001$ in the univariate model; odds ratio 1.09, 95%CI 1.05–1.13, $p<0.0001$ in the multivariate model), but sex was not (odds ratio 2.24, 95%CI 0.97–5.15, $p=0.0584$ in the univariate model; odds ratio 2.06, 95%CI 0.72–5.58, $p=0.1758$ in the multivariate model).

TABLE 4 Association between genotype and risks of severity in COVID-19 patients.

Variables	Univariate			Multivariate*		
	Odds ratio (95% CI)		p value	Odds ratio (95% CI)		p value
Age	1.07	(1.04–1.10)	< 0.0001	1.09	(1.05–1.13)	<0.0001
Sex	2.24	(0.97–5.15)	0.0584	2.25	(0.76–6.64)	0.1409
Vaccine	0.62	(0.12–3.37)	0.5842	0.18	(0.02–1.95)	0.1571
Lineage						
Alpha vs. B.1.1.284	1.05	(0.28–3.94)	0.1315	0.80	(0.13–4.98)	0.0303
B.1.1 vs. B.1.1.284	0.60	(0.08–4.76)	0.9986	0.22	(0.02–3.10)	0.7775
B.1.1.214 vs. B.1.1.284	0.08	(0.01–0.84)	0.0277	0.03	(0.00–0.56)	0.0299
Delta vs. B.1.1.284	0.76	(0.17–3.49)	0.6216	0.98	(0.11–8.88)	0.0851
R.1 vs. B.1.1.284	1.20	(0.22–6.68)	0.9986	0.07	(0.00–1.49)	0.2066
Others vs. B.1.1.284	0.60	(0.08–4.76)	0.2472	0.34	(0.03–3.75)	0.8152

*ORs and 95% CIs were estimated by logistic regression model adjusting for age and sex. The bold values mean that the difference was significant (p value was less than 0.05 and 95% CI did not cross 1).

Discussion

In this study, we analyzed 112 whole genome sequences of SARS-CoV-2 samples collected at Juntendo University Hospital as well as the genome data deposited in GISAID (97,458 complete sequences obtained in Japan) to investigate the pattern of mutations and the correlation of mutations with transmissibility and severity.

We found that the most predominant lineage of SARS-CoV-2 changed in each wave in Japan (Figure 1; Supplementary Tables 1, 2). It is likely that the major lineages in the later waves would be selected as a result of the advantageous transmissibility and/or immune escape potential (Korber et al., 2020; Mlcochova et al., 2021; Planas et al., 2021; Volz et al., 2021b; Wu et al., 2021).

Our sequence data showed that the viral protein with the largest number of mutations was S protein (Table 1). This reflects the important roles of the S protein in the transmission and survival of SARS-CoV-2. The amino acid substitutions in spike allow the virus to bind with greater strength to ACE2, fuse more efficiently with its target cell, and/or escape from neutralizing antibodies.

B.1.1.284 became the most widespread lineage in the second wave, replacing B.1.1, the dominant lineage in the first wave (Figure 1; Supplementary Tables 1, 2). B.1.1.284 would have higher transmissibility than B.1.1, and some newly acquired mutations in B.1.1.284 would be responsible for its enhanced transmissibility. It is implied that S: M153T, N: P151L, NSP3: S543P, NSP5: P108S, and/or NSP12: A423V in B.1.1.284 might confer the higher transmissibility of this lineage, allowing it to surpass B.1.1 (Table 2).

B.1.1.214 is considered to have elevated transmissibility in comparison with B.1.1.284 because B.1.1.214 exceeded B.1.1.284 in infections and became the major lineage in the third wave (Figure 1; Supplementary Tables 1, 2). However, B.1.1.214 has only D614G in the S protein and has fewer spike mutations than B.1.1.284 and B.1.1, which have lower transmissibility than B.1.1.284 (Table 2). These results suggest that the enhanced transmissibility of B.1.1.214 is due to amino acid changes in the viral protein rather than spike.

The specific mutations of B.1.1.214 outside S protein were N: M234I, NSP14: P43L, and NSP16: R287I. It is speculated that these amino acid substitutions increase the efficiency of viral RNA replication and contribute to the wider spread of SARS-CoV-2.

Regarding the severity of infected patients' symptoms, only patients with B.1.1.214 exhibited significantly lower severity than those with B.1.1.284 (Tables 3, 4). The absence of mutations other than D614G in S protein may be associated with lower severity of B.1.1.214. In other words, the acquisition of S protein mutations such as L54F in B.1.1 and M153T in B.1.1.284 may increase the severity of COVID-19.

R.1, which was the second most dominant lineage in the fourth wave, harbored E484K in the S protein. It is expected that E484K results in a stronger interaction between the S protein and ACE2 due to a charge switch and conformational changes (Nelson et al., 2021). Furthermore, previous reports showed that an E484K mutation reduces the neutralizing activity of convalescent and mRNA vaccine-elicited sera/plasma against SARS-CoV-2 (Cavanaugh et al., 2021; Hacisuleyman et al., 2021; Nonaka et al., 2021; Wang et al., 2021). In addition, a W152L mutation in the N-terminal domain potentially allows for immune escape (Chi et al., 2020).

The Alpha strains, including B.1.1.7 and Q.1, were most frequently detected in the fourth wave (Figure 1; Supplementary Tables 1, 2). These results indicate that the Alpha strains are more transmissible than the previously dominant strains, B.1.1.214 and R.1. Our finding is consistent with a previous report that showed an increased reproduction number of the Alpha strain (Davies et al., 2021a; Leung et al., 2021; Volz et al., 2021b). The spike of the Alpha strain has N501Y, which plays an important role in increasing affinity of S protein to ACE2 (Ali et al., 2021; Laffebert et al., 2021; Tian et al., 2021). Moreover, H69del and V70del were reported to increase infectivity through efficient incorporation of cleaved spike into virions (Meng et al., 2021).

Spike mutations N501Y, H69del, and V70del may increase severity and transmissibility, but Alpha variants showed no significant difference in severity from the reference lineage, B.1.1.284, in this study (Tables 3, 4). Our results were similar to a previous

paper that did not show a significant association of the Alpha strain with higher disease severity (Davies et al., 2021a). However, it was inconsistent with a report indicating an increased mortality of B.1.1.7-infected patients (Davies et al., 2021b). The reason why the Alpha variant did not show significantly higher severity than B.1.1.284 in this study may be due to the small sample size.

The Delta strain, including AY.29 and AY.29.1, replaced the Alpha strain and became the most common strain in the fifth wave (Figure 1; Supplementary Tables 1, 2). From these results, it is concluded that the Delta strain has higher transmissibility than the Alpha strain. Previous reports also showed that the Delta strain is more transmissible than the Alpha strain (Alizon et al., 2021; Campbell et al., 2021; Liu and Rocklöv, 2021; Allen et al., 2022). The Delta strains that were sequenced in our laboratory had 11 amino acid changes in the S protein, including L452R, T478K, and P681R.

The L452R is situated in the receptor binding domain (RBD) and is presumed to stabilize the complex of RBD and ACE2 (Motozono et al., 2021). The L452R mutation leads to increased infectivity of the virus. In addition to infectivity, L452R has been reported to be associated with escape from neutralizing antibodies (Deng et al., 2021). L452R caused a 3–10-fold reduction of susceptibility to about one third of vaccine and convalescent plasma samples (Ferreira et al., 2021; Greaney et al., 2021).

T478K is also located in RBD, and *in silico* analysis of spike structure has predicted that T478K may alter the electrostatic surface and increase steric hindrance of the S protein (Di Giacomo et al., 2021). It is suggested that T478K could enhance the binding affinity of RBD to ACE2.

The P681R mutation is present near the furin cleavage site and affects the efficiency of the cleavage reaction. It has been reported that P681R facilitates S protein cleavage, accelerates viral fusion and cell-to-cell infection, and enhances viral pathogenicity in hamster models (Mlcochova et al., 2021; Saito et al., 2021).

It is suspected that the Delta strain causes more severe disease than the preexisting strains due to S protein mutations. However, in the present study, the severity of the Delta strain was not significantly higher than that of B.1.1.284 (Tables 3, 4). Some studies showed that the Delta strain was associated with the higher severity (Sheikh et al., 2021; Twohig et al., 2022), while others reported that the severity of the Delta strain was not significantly elevated (Gunadi et al., 2021; Taylor et al., 2021). The reason why there is no significant difference between the Delta strain and the reference lineage in our study may be that the sample size was small, and that 44.4 percent of the patients infected with the Delta strain were vaccinated at least once while those with B.1.1.284 were not vaccinated.

In addition to the small sample size, a limitation of this research is that there is no experimental data using recombinant SARS-CoV-2 with or without specific amino acid changes to confirm the effects of the mutations.

In summary, we analyzed the sequences of 112 SARS-CoV-2 genomes detected at Juntendo University Hospital and examined the correlation of the amino acid changes with the transmissibility and the severity of each strain/lineage. It is concluded that

mutations in S, N, and NSPs increase transmissibility and/or severity. These mutations include S: M153T, N: P151L, NSP3: S543P, NSP5: P108S, and NSP12: A423V in B.1.1.284; S: W152L and E484K in R.1; S: H69del, V70del, and N501Y in the Alpha strain; S: L452R, T478K, and P681R in the Delta strain. Furthermore, it is suggested that the transmissibility of the virus could be enhanced by the mutations in proteins other than spike, such as N: M234I, NSP14: P43L, and NSP16: R287I in B.1.1.214. The evolution of the virus occurs because of mutations and natural selection of the variants. To address this issue, continuous monitoring of the mutations in the viral genomes and analysis of their effects will be required to develop vaccines and antiviral drugs effective against emerging SARS-CoV-2 variants (Hoque et al., 2020; Islam et al., 2020).

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 the institutional review board at Juntendo University Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

YT and NY conceived the study. YT, MH, YM, HM, SH, TM, KTa, and TN coordinated collection and processing the clinical samples. KTs, YH, MW, and SM processed samples. KTs and YH performed RNA isolation and RT-PCR. KTs, YH, MW, SM, and YT performed NGS experiments. KTs, YH, YT, and NY performed sequencing data analysis. NY performed phylogenetic analysis. KTs, YH, YT, NY, and SN contributed to data analysis. YT, SN, and NY prepared and wrote 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.

References

- Ali, F., Kasry, A., and Amin, M. (2021). The new SARS-CoV-2 strain shows a stronger binding affinity to ACE2 due to N501Y mutant. *Med. Drug Discov.* 10:100086. doi: 10.1016/j.medidd.2021.100086
- Alizon, S., Haim-Boukobza, S., Foulongne, V., Verdurme, L., Trombert-Paolantoni, S., Lecorche, E., et al. (2021). Rapid spread of the SARS-CoV-2 Delta variant in some French regions, June 2021. *Euro Surveill.* 26:2100573. doi: 10.2807/1560-7917.ES.2021.26.28.2100573
- Allen, H., Vusirikala, A., Flannagan, J., Twohig, K. A., Zaidi, A., Chudasama, D., et al. (2022). Household transmission of COVID-19 cases associated with SARS-CoV-2 Delta variant (B.1.617.2): national case-control study. *Lancet Reg. Health Eur.* 12:100252. doi: 10.1016/j.lanepe.2021.100252
- Campbell, F., Archer, B., Laurenson-Schafer, H., Jinnai, Y., Konings, F., Batra, N., et al. (2021). Increased transmissibility and global spread of SARS-CoV-2 variants of concern as at June 2021. *Euro Surveill.* 26:2100509. doi: 10.2807/1560-7917.ES.2021.26.24.2100509
- Cavanaugh, A. M., Fortier, S., Lewis, P., Arora, V., Johnson, M., George, K., et al. (2021). COVID-19 outbreak associated with a SARS-CoV-2 R.1 lineage variant in a skilled nursing facility after vaccination program—Kentucky, March 2021. *MMWR Morb. Mortal. Wkly Rep.* 70, 639–643. doi: 10.15585/mmwr.mm7017e2
- Chi, X., Yan, R., Zhang, J., Zhang, G., Zhang, Y., Hao, M., et al. (2020). A neutralizing human antibody binds to the N-terminal domain of the spike protein of SARS-CoV-2. *Science* 369, 650–655. doi: 10.1126/science.abc6952
- Coronaviridae Study Group of the International Committee on Taxonomy of Viruses (2020). The species severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat. Microbiol.* 5, 536–544. doi: 10.1038/s41564-020-0695-z
- Davies, N. G., Abbott, S., Barnard, R. C., Jarvis, C. I., Kucharski, A. J., Munday, J. D., et al. (2021a). Estimated transmissibility and impact of SARS-CoV-2 lineage B.1.1.7 in England. *Science* 372:eabg3055. doi: 10.1126/science.abg3055
- Davies, N. G., Jarvis, C. I., CMMID COVID-19 Working Group, Edmunds, W. J., Jewell, N. P., Diaz-Ordaz, K., et al. (2021b). Increased mortality in community-tested cases of SARS-CoV-2 lineage B.1.1.7. *Nature* 593, 270–274. doi: 10.1038/s41586-021-03426-1
- Deng, X., Garcia-Knight, M. A., Khalid, M. M., Servellita, V., Wang, C., Morris, M. K., et al. (2021). Transmission, infectivity, and neutralization of a spike L452R SARS-CoV-2 variant. *Cell* 184, 3426–3437.e8. doi: 10.1016/j.cell.2021.04.025
- Di Giacomo, S., Mercatelli, D., Rakhimov, A., and Giorgi, F. M. (2021). Preliminary report on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike mutation T478K. *J. Med. Virol.* 93, 5638–5643. doi: 10.1002/jmv.27062
- Ferreira, I., Kemp, S. A., Dattir, R., Saito, A., Meng, B., Rakshit, P., et al. (2021). SARS-CoV-2 B.1.617 mutations L452R and E484Q are not synergistic for antibody evasion. *J. Infect. Dis.* 224, 989–994. doi: 10.1093/infdis/jiab368
- Flores-Alanis, A., Cruz-Rangel, A., Rodriguez-Gomez, F., Gonzalez, J., Torres-Guerrero, C. A., Delgado, G., et al. (2021). Molecular epidemiology surveillance of SARS-CoV-2: mutations and genetic diversity one year after emerging. *Pathogens* 10:184. doi: 10.3390/pathogens10020184
- Greaney, A. J., Loes, A. N., Gentles, L. E., Crawford, K. H. D., Starr, T. N., Malone, K. D., et al. (2021). Antibodies elicited by mRNA-1273 vaccination bind more broadly to the receptor binding domain than do those from SARS-CoV-2 infection. *Sci. Transl. Med.* 13:eabi9915. doi: 10.1126/scitranslmed.abi9915
- Gunadi, , Hakim, M. S., Wibawa, H., Marcellus, , Setiawaty, V., Slamet, , et al. (2021). Is the infection of the SARS-CoV-2 delta variant associated with the outcomes of COVID-19 patients? *Front. Med.* 8:780611. doi: 10.3389/fmed.2021.780611
- Hacisuleyman, E., Hale, C., Saito, Y., Blachere, N. E., Bergh, M., Conlon, E. G., et al. (2021). Vaccine breakthrough infections with SARS-CoV-2 variants. *N. Engl. J. Med.* 384, 2212–2218. doi: 10.1056/NEJMoa2105000
- Holland, L. A., Kaelin, E. A., Maqsood, R., Estifanos, B., Wu, L. I., Varsani, A., et al. (2020). An 81-nucleotide deletion in SARS-CoV-2 ORF7a identified from sentinel surveillance in Arizona (January to March 2020). *J. Virol.* 94, e00711–e00720. doi: 10.1128/JVI.00711-20
- Hoque, M. N., Chaudhury, A., Akanda, M. A. M., Hossain, M. A., and Islam, M. T. (2020). Genomic diversity and evolution, diagnosis, prevention, and therapeutics of the pandemic COVID-19 disease. *PeerJ* 8:e9689. doi: 10.7717/peerj.9689
- Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 395, 497–506. doi: 10.1016/S0140-6736(20)30183-5
- Islam, M. R., Hoque, M. N., Rahman, M. S., Alam, A., Akther, M., Puspo, J. A., et al. (2020). Genome-wide analysis of SARS-CoV-2 virus strains circulating worldwide implicates heterogeneity. *Sci. Rep.* 10, 14004. doi: 10.1038/s41598-020-70812-6
- Korber, B., Fischer, W. M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., et al. (2020). Tracking changes in SARS-CoV-2 spike: evidence that D614G increases infectivity of the COVID-19 virus. *Cell* 182, 812–827.e19. doi: 10.1016/j.cell.2020.06.043
- Laffey, C., de Koning, K., Kanaar, R., and Lebbink, J. H. G. (2021). Experimental evidence for enhanced receptor binding by rapidly spreading SARS-CoV-2 variants. *J. Mol. Biol.* 433:167058. doi: 10.1016/j.jmb.2021.167058
- Lau, S. Y., Wang, P., Mok, B. W., Zhang, A. J., Chu, H., Lee, A. C., et al. (2020). Attenuated SARS-CoV-2 variants with deletions at the S1/S2 junction. *Emerg. Microb. Infect.* 9, 837–842. doi: 10.1080/22221751.2020.1756700
- Leung, K., Shum, M. H., Leung, G. M., Lam, T. T., and Wu, J. T. (2021). Early transmissibility assessment of the N501Y mutant strains of SARS-CoV-2 in the United Kingdom, October to November 2020. *Euro Surveill.* 26:2002106. doi: 10.2807/1560-7917.ES.2020.26.1.2002106
- Liu, Y., Liu, J., Plante, K. S., Plante, J. A., Xie, X., Zhang, X., et al. (2022). The N501Y spike substitution enhances SARS-CoV-2 infection and transmission. *Nature* 602, 294–299. doi: 10.1038/s41586-021-04245-0
- Liu, Y., and Rocklöv, J. (2021). The reproductive number of the Delta variant of SARS-CoV-2 is far higher compared to the ancestral SARS-CoV-2 virus. *J. Travel Med.* 28:taab124. doi: 10.1093/jtm/taab124
- Meng, B., Kemp, S. A., Papa, G., Dattir, R., Ferreira, I., Marelli, S., et al. (2021). Recurrent emergence of SARS-CoV-2 spike deletion H69/V70 and its role in the alpha variant B.1.1.7. *Cell Rep.* 35:109292. doi: 10.1016/j.celrep.2021.109292
- Mlcochova, P., Kemp, S. A., Dhar, M. S., Papa, G., Meng, B., Ferreira, I., et al. (2021). SARS-CoV-2 B.1.617.2 Delta variant replication and immune evasion. *Nature* 599, 114–119. doi: 10.1038/s41586-021-03944-y
- Motozono, C., Toyoda, M., Zahradnik, J., Saito, A., Nasser, H., Tan, T. S., et al. (2021). SARS-CoV-2 spike L452R variant evades cellular immunity and increases infectivity. *Cell Host Microbe* 29, 1124–1136.e11. doi: 10.1016/j.chom.2021.06.006

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Supplementary material

The Supplementary materials for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.912061/full#supplementary-material>

- Nelson, G., Buzko, O., Spilman, P., Niazi, K., Rabizadeh, S., and Soon-Shiong, P. (2021). Molecular dynamic simulation reveals E484K mutation enhances spike RBD-ACE2 affinity and the combination of E484K, K417N and N501Y mutations (501Y.V2 variant) induces conformational change greater than N501Y mutant alone, potentially resulting in an escape mutant. *bioRxiv* [Preprint]. doi: 10.1101/2021.01.13.426558
- Nonaka, C. K. V., Franco, M. M., Graf, T., de Lorenzo Barcia, C. A., de Avila Mendonca, R. N., de Sousa, K. A. F., et al. (2021). Genomic evidence of SARS-CoV-2 reinfection involving E484K spike mutation, Brazil. *Emerg. Infect. Dis.* 27, 1522–1524. doi: 10.3201/eid2705.210191
- Ogando, N. S., Zevenhoven-Dobbe, J. C., van der Meer, Y., Bredenbeek, P. J., Posthuma, C. C., and Snijder, E. J. (2020). The enzymatic activity of the nsp14 exoribonuclease is critical for replication of MERS-CoV and SARS-CoV-2. *J. Virol.* 94, e01246–e01320. doi: 10.1128/JVI.01246-20
- Planas, D., Veyer, D., Baidaliuk, A., Staropoli, I., Guivel-Benhassine, F., Rajah, M. M., et al. (2021). Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. *Nature* 596, 276–280. doi: 10.1038/s41586-021-03777-9
- Rahman, M. S., Hoque, M. N., Islam, M. R., Akter, S., Rubayet Ul Alam, A. S. M., Siddique, M. A., et al. (2020). Epitope-based chimeric peptide vaccine design against S, M and E proteins of SARS-CoV-2, the etiologic agent of COVID-19 pandemic: an in silico approach. *PeerJ* 8:e9572. doi: 10.7717/peerj.9572
- Rahman, M. S., Islam, M. R., Alam, A., Islam, I., Hoque, M. N., Akter, S., et al. (2021a). Evolutionary dynamics of SARS-CoV-2 nucleocapsid protein and its consequences. *J. Med. Virol.* 93, 2177–2195. doi: 10.1002/jmv.26626
- Rahman, M. S., Islam, M. R., Hoque, M. N., Alam, A., Akther, M., Puspo, J. A., et al. (2021b). Comprehensive annotations of the mutational spectra of SARS-CoV-2 spike protein: a fast and accurate pipeline. *Transbound. Emerg. Dis.* 68, 1625–1638. doi: 10.1111/tbed.13834
- Saito, A., Irie, T., Suzuki, R., Maemura, T., Nasser, H., Uriu, K., et al. (2021). Enhanced fusogenicity and pathogenicity of SARS-CoV-2 Delta P681R mutation. *Nature* 602, 300–306. doi: 10.1038/s41586-021-04266-9
- Sheikh, A., McMenamin, J., Taylor, B., and Robertson, C. Public Health Scotland and the EAVE II Collaborators (2021). SARS-CoV-2 Delta VOC in Scotland: demographics, risk of hospital admission, and vaccine effectiveness. *Lancet* 397, 2461–2462. doi: 10.1016/S0140-6736(21)01358-1
- Shu, Y., and McCauley, J. (2017). GISAID: Global initiative on sharing all influenza data - from vision to reality. *Euro Surveill* 22:30494. doi: 10.2807/1560-7917.ES.2017.22.13.30494
- Taylor, C. A., Patel, K., Pham, H., Whitaker, M., Anglin, O., Kambhampati, A. K., et al. (2021). Severity of disease among adults hospitalized with laboratory-confirmed COVID-19 before and during the period of SARS-CoV-2 B.1.617.2 (Delta) predominance—COVID-NET, 14 states, January–august 2021. *MMWR Morb. Mortal. Wkly Rep.* 70, 1513–1519. doi: 10.15585/mmwr.mm7043e1
- Tian, F., Tong, B., Sun, L., Shi, S., Zheng, B., Wang, Z., et al. (2021). N501Y mutation of spike protein in SARS-CoV-2 strengthens its binding to receptor ACE2. *elife* 10. doi: 10.7554/eLife.69091
- Twohig, K. A., Nyberg, T., Zaidi, A., Thelwall, S., Sinnathamby, M. A., Aliabadi, S., et al. (2022). Hospital admission and emergency care attendance risk for SARS-CoV-2 delta (B.1.617.2) compared with alpha (B.1.1.7) variants of concern: a cohort study. *Lancet Infect. Dis.* 22, 35–42. doi: 10.1016/S1473-3099(21)00475-8
- Volz, E., Hill, V., McCrone, J. T., Price, A., Jorgensen, D., O'Toole, A., et al. (2021a). Evaluating the effects of SARS-CoV-2 spike mutation D614G on transmissibility and pathogenicity. *Cell* 184, 64–75. doi: 10.1016/j.cell.2020.11.020
- Volz, E., Mishra, S., Chand, M., Barrett, J. C., Johnson, R., Geidelberg, L., et al. (2021b). Assessing transmissibility of SARS-CoV-2 lineage B.1.1.7 in England. *Nature* 593, 266–269. doi: 10.1038/s41586-021-03470-x
- Wang, P., Nair, M. S., Liu, L., Iketani, S., Luo, Y., Guo, Y., et al. (2021). Antibody resistance of SARS-CoV-2 variants B.1.351 and B.1.1.7. *Nature* 593, 130–135. doi: 10.1038/s41586-021-03398-2
- World Health Organization (2020). WHO Director-General's Opening Remarks at the Media Briefing on COVID-19. Available at: https://apps.who.int/iris/bitstream/handle/10665/331589/WHO-2019-nCoV-Leveraging_GISRS-2020.1-eng.pdf
- World Health Organization (2021). Clinical Management of COVID-19.
- Wu, H., Xing, N., Meng, K., Fu, B., Xue, W., Dong, P., et al. (2021). Nucleocapsid mutations R203K/G204R increase the infectivity, fitness, and virulence of SARS-CoV-2. *Cell Host Microbe* 29, 1788–1801. doi: 10.1016/j.chom.2021.11.005
- Wu, F., Zhao, S., Yu, B., Chen, Y. M., Wang, W., Song, Z. G., et al. (2020). A new coronavirus associated with human respiratory disease in China. *Nature* 579, 265–269. doi: 10.1038/s41586-020-2008-3
- Young, B. E., Fong, S. W., Chan, Y. H., Mak, T. M., Ang, L. W., Anderson, D. E., et al. (2020). Effects of a major deletion in the SARS-CoV-2 genome on the severity of infection and the inflammatory response: an observational cohort study. *Lancet* 396, 603–611. doi: 10.1016/S0140-6736(20)31757-8
- Yurkovetskiy, L., Wang, X., Pascal, K. E., Tomkins-Tinch, C., Nyalile, T. P., Wang, Y., et al. (2020). Structural and functional analysis of the D614G SARS-CoV-2 spike protein variant. *Cell* 183, 739–751.e8. doi: 10.1016/j.cell.2020.09.032
- Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733. doi: 10.1056/NEJMoa2001017



Comparison of the Performance of 24 Severe Acute Respiratory Syndrome Coronavirus 2 Antibody Assays in the Diagnosis of Coronavirus Disease 2019 Patients

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Background: The accurate detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the key to control Coronavirus Disease-2019 (COVID-19). The performance of different antibody detection methods for diagnosis of COVID-19 is inconclusive.

Methods: Between 16 February and 28 February 2020, 384 confirmed COVID-19 patients and 142 healthy controls were recruited. 24 different serological tests, including 4 enzyme-linked immunosorbent assays (EIAs), 10 chemiluminescent immunoassays (CLIAs), and 10 lateral flow immunoassays (LFIAs), were simultaneously performed.

Results: The sensitivities of anti-SARS-CoV-2 IgG and IgM antibodies with different reagents ranged from 75 to 95.83% and 46.09 to 92.45%, respectively. The specificities of both anti-SARS-CoV-2 IgG and IgM were relatively high and comparable among different reagents, ranged from 88.03 to 100%. The area under the curves (AUCs) of different tests ranged from 0.733 to 0.984, and the AUCs of EIAs or CLIAs were significantly higher than those of LFIAs. The sensitivities of both IgG and IgM gradually increased with increase of onset time. After 3–4 weeks, the sensitivities of anti-SARS-CoV-2 IgG were maintained at a certain level but the sensitivities of IgM were gradually decreased. Six COVID-19 patients who displayed negative anti-SARS-CoV-2 results were associated with the factors such as older age, having underlying diseases, and using immunosuppressant.

Conclusion: Besides the purpose of assessing the impact of the SARS-CoV-2 pandemic in the population, SARS-CoV-2 antibody assays may have an adjunct role in the diagnosis and exclusion of COVID-19, especially by using high-throughput technologies (EIAs or CLIAs).

Keywords: COVID-19, SARS-CoV-2, serological tests, lateral flow immunoassay, enzyme-linked immunosorbent assay, chemiluminescent immunoassay

INTRODUCTION

Coronavirus disease 2019 (COVID-19), the emerging infectious disease caused by a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the greatest threat to public health worldwide in recent 2 years (Phelan et al., 2020; The, 2020; Zhu et al., 2020). Globally, as of February 11, 2022, there have been 404 million confirmed cases of COVID-19, including 5.7 million deaths, reported to the World Health Organization. To this day, COVID-19 pandemic is still the most critical problem in the global health agenda.

The rapid and accurate diagnosis of COVID-19 is the key to control the epidemic of this disease. The diagnosis of COVID-19 is mainly based on epidemiology, clinical symptoms, radiology, and laboratory pathogen detection. The clinical symptoms of COVID-19 include many typical respiratory manifestations (fever, cough, chest pain, or shortness of breath) and other manifestations (fever, muscle ache, fatigue, diarrhea, or headache) (Baj et al., 2020; Brendish et al., 2020; Pan et al., 2020), which are similar to that of influenza (Wang et al., 2014). The asymptomatic COVID-19 patients were also reported previously, increasing the difficulty of diagnosis based solely on clinical features (Rothe et al., 2020; Ralli et al., 2021; Temkin and Healthcare Worker COVID-19 Surveillance Working Group, 2021). The characteristics of radiology of COVID-19 are also unspecific and the diagnosis of which based on radiology has variation among different radiologists (Chung et al., 2020; Kuo et al., 2021). The positive detection of SARS-CoV-2 virus nucleic acid by using real-time reverse transcription polymerase chain reaction (RT-PCR) is the most important diagnostic tool for COVID-19 (Islam and Iqbal, 2020; Pascarella et al., 2020; Zowawi et al., 2021). However, nucleic acid testing has some limitations, such as requiring certified laboratories, experienced technicians and expensive equipment, long turnaround time, and the existence of false negative results (Liu et al., 2020; Sule and Oluwayelu, 2020).

Serological tests are readily available in clinical laboratories in most hospitals and are easier to carry out than molecular tests (Lisboa Bastos et al., 2020; Xiao S.Y. et al., 2020). Besides the purpose of assessing the impact of the SARS-CoV-2 pandemic in the population and evaluating antibody titers from previous exposures to SARS-CoV-2 or from vaccine treatment, serological tests were also recommended to be used for diagnosing or excluding suspected cases in the guideline of diagnosis and treatment for SARS-CoV-2 pneumonia made by Chinese National Health Commission. SARS-CoV-2-specific IgM antibody level peaks at week 3 and then declines, whereas IgG antibodies to spike protein can persist long-term, even beyond 1 year after infection (Gudbjartsson et al., 2020; Hou et al., 2020, 2021; Xiao A.T. et al., 2020). Importantly, although there were many studies focused on the role of SARS-CoV-2-specific antibodies in the diagnosis, prognosis and management of COVID-19 (Lisboa Bastos et al., 2020; Ong et al., 2021), there was rare study to evaluated the performance of different antibody detection methods in clinical practice. Given there

are many commercially available anti-SARS-CoV-2 IgM and IgG detection kits developed, it is necessary to verify the accuracy of serological tests in the diagnosis of COVID-19 in clinical practice.

In the current study, we compared the performance of 24 different serological tests, which were classified as enzyme-linked immunosorbent assay (ELISA or EIA), chemiluminescent immunoassay (CLIA), and point-of-care testing (POCT) technology such as lateral flow immunoassay (LFIA), in the diagnosis of COVID-19 patients. We also analyzed the sensitivity of these methods in patients at different time after disease onset. This study is useful for developing the standards for antibody testing and further understanding the appearance and persistence of SARS-CoV-2 antibodies in COVID-19 patients.

MATERIALS AND METHODS

Patients

Between 16 February and 28 February 2020, a total number of 384 COVID-19 patients and 142 healthy controls were continuously recruited from Tongji Hospital (the largest hospital in central region of China), Wuhan, China. The diagnosis of COVID-19 was according to the guideline of diagnosis and treatment for SARS-CoV-2 pneumonia made by Chinese National Health Commission. The confirmed COVID-19 patients were defined as having positive SARS-CoV-2 real-time RT-PCR results in clinical samples, together with typical clinical symptoms (fever, cough, or shortness of breath) and radiological characteristics (unilateral pneumonia, bilateral pneumonia, or ground-glass opacity). The healthy controls were defined as individuals without signs or symptoms of active disease by clinical interview and physical examination, and with negative SARS-CoV-2 real-time RT-PCR results. Five milliliter of venous blood from each participant were collected into a test tube for serum separation, which was then stored at -80°C until use. The serum was thawed and mixed before measurement. The demographic and clinical information, and laboratory results were collected from electronic medical records. This study was approved by the ethical committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (TJ-C20200128).

Severe Acute Respiratory Syndrome Coronavirus 2-Specific Antibody Detection

ELISAs

Four ELISA kits were obtained from Livzon [Anti-SARS-CoV-2 ELISA Kit (IgG), Anti-SARS-CoV-2 ELISA Kit (IgM), Livzon Pharmaceutical Group Inc., Zhuhai, China] (Livzon-EIA-IgG, Livzon-EIA-IgM) or WANTAI [Anti-SARS-CoV-2 ELISA Kit (Ab total), Anti-SARS-CoV-2 ELISA Kit (IgM), WANTAI BioPharm Group Inc., Beijing, China] (WANTAI-EIA-Ab total, WANTAI-EIA-IgM) respectively, and performed according to the manufacturers' instructions. Briefly, -80°C stored serum

samples were thawed and mixed before use. After that, the serum samples and reagents were added to 96-well microtiter plates pre-coated with SARS-CoV-2-specific antigens and detected by automated analyzers. Signal to cutoff value (S/CO) ≥ 1 is reactive (positive), and $S/CO < 1$ is non-reactive (negative).

Chemiluminescent Immunoassays

Similarly, -80°C stored serum samples were thawed and mixed before use. Ten anti-SARS-CoV-2 CLIA kits were obtained from InnoDx (InnoDx biotechnology Co., Ltd., Xiamen, China) (InnoDx-CLIA-Ab total, InnoDx-CLIA-IgM), Beier (Beier bioengineering Co., Ltd., Beijing, China) (Beier-CLIA-IgG, Beier-CLIA-IgM), YHLO (YHLO Biotech Co., Ltd., Shenzhen, China) (YHLO-CLIA-IgG, YHLO-CLIA-IgM), Orienter (Orienter Biotechnology Co., Ltd. Chengdu, China) (Orienter-CLIA-IgG, Orienter-CLIA-IgM), and Maccura (Maccura Biotechnology Co., Ltd., Chengdu, China) (Maccura-CLIA-IgG, Maccura-CLIA-IgM), respectively, and performed by automated chemiluminescence analyzers (Caris200, InnoDx; VI-180, Beier; iFlash 3000-C, YHLO; LA2000, Orienter; i 3000, Maccura). For InnoDx, Orienter and Maccura, $S/CO \geq 1$ is reactive (positive), and $S/CO < 1$ is non-reactive (negative). For YHLO, the results ≥ 10 AU/ml is reactive (positive), and the results < 10 AU/ml is non-reactive (negative). The cutoff value is 5 RU/ml for Beier.

Lateral Flow Immunoassays

Ten POCT anti-SARS-CoV-2 LFIA kits were obtained from Livzon (Livzon-LFIA-IgG, Livzon-LFIA-IgM), WANTAI (WANTAI-LFIA-IgG, WANTAI-LFIA-IgM), Beier (Beier-LFIA-IgG, Beier-LFIA-IgM), HEALGEN (Orient Gene Biotech Co., Ltd., Huzhou, China) (HEALGEN-LFIA-IgG, HEALGEN-LFIA-IgM), and Innovita (Innovita Biological Technology Co., Ltd., Beijing, China) (Innovita-LFIA-IgG, Innovita-LFIA-IgM), respectively, and performed according to the manufacturers' instructions. Briefly, -80°C stored serum samples were thawed and mixed before use. After that, the serum samples were diluted and added to colloidal gold immunochromatographic strip. The results were finally read by the eyes.

For the antibody detection reagents, YHLO-CLIA-IgG, YHLO-CLIA-IgM, HEALGEN-LFIA-IgG, and HEALGEN-LFIA-IgM have received CE certification in Europe, and InnoDx-CLIA-Ab total, Innovita-LFIA-IgG, and Innovita-LFIA-IgM have been approved by the China National Medical Product Administration.

Statistical Analysis

Data analyses were performed with SPSS 21.0 (SPSS, Inc.) or GraphPad Prism 6.0.1 (GraphPad). Unless otherwise specified, the data are expressed as mean $\pm$ standard deviation (SD). Continuous variables were compared with Mann-Whitney U -test. Receiver-operator characteristic (ROC) curve analysis was used to compare the performance of different anti-SARS-CoV-2 assays for diagnosis of COVID-19. The area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), together with 95%

confidence interval (CI), were identified. Statistical significance was determined as $p < 0.05$.

RESULTS

Participant Characteristics

A total of 384 COVID-19 patients (males, 197; females, 187) were enrolled in this study. The median age was 65 years (range 5–91 years). The median time from onset of symptoms to antibody detection was 21 days (range 3–78 days). A total of 142 healthy individuals (males, 80; females, 62) who tested negative for SARS-CoV-2 were enrolled as control subjects. The median age of healthy controls was 42 years (range 2–90 years).

The Performance of 24 Severe Acute Respiratory Syndrome Coronavirus 2 Antibody Assays for Coronavirus Disease-2019 Diagnosis

The sensitivity and specificity of these 24 antibody reagents are shown in **Table 1**. The sensitivities of anti-SARS-CoV-2 IgG or total antibodies with different reagents ranged from 75 to 95.83%. The sensitivities of anti-SARS-CoV-2 IgM ranged from 46.09 to 92.45%. The specificities of both anti-SARS-CoV-2 IgG and IgM with different reagents were relatively high, ranged from 88.03 to 100%. The specificities of IgG and IgM in most reagents were comparable. Our data showed that most commercially available anti-SARS-CoV-2 detection kits, especially the high-throughput technologies (EIAs or CLIAs), have relatively high sensitivity and specificity for COVID-19 diagnosis.

ROC analysis for each assay was determined. As shown in **Figure 1**, the AUCs of these 24 assays ranged from 0.733 to 0.984. The AUCs of EIAs or CLIAs, no matter in IgG or IgM, were significantly higher than those of LFIAs (**Figure 2**). The AUCs of these 24 assays ranged in a descending order from: Orienter-CLIA-IgG > Maccura-CLIA-IgG > InnoDx-CLIA-Ab total > WANTAI-EIA-Ab total > Livzon-EIA-IgG > YHLO-CLIA-IgG > Beier-CLIA-IgG > Innovita-LFIA-IgG > HEALGEN-LFIA-IgG > Livzon-LFIA-IgG > Beier-LFIA-IgG > WANTAI-LFIA-IgG for IgG or total antibody detection; and Orienter-CLIA-IgM > Maccura-CLIA-IgM > WANTAI-EIA-IgM > InnoDx-CLIA-IgM > Beier-CLIA-IgM > YHLO-CLIA-IgM > Livzon-EIA-IgM > Beier-LFIA-IgM > HEALGEN-LFIA-IgM > Innovita-LFIA-IgM > WANTAI-LFIA-IgM > Livzon-LFIA-IgM for IgM detection.

The sensitivities of anti-SARS-CoV-2 IgM were significantly lower than those of anti-SARS-CoV-2 IgG or total antibodies ($p < 0.01$), but the specificities between anti-SARS-CoV-2 IgG and IgM had no difference ($p = 0.568$) (**Figure 3**). The sensitivities of both IgG and IgM were gradually increased with increase of onset time, and reached the peak after about 3–4 weeks. After that, the sensitivities of anti-SARS-CoV-2 IgG were maintained at a certain level but the sensitivities of anti-SARS-CoV-2 IgM were gradually decreased (**Figure 4**).

TABLE 1 | The sensitivity and specificity of 24 SARS-CoV-2 antibody assays.

	Sensitivity (%)	Positive/Total	Specificity (%)	Negative/Total	Positive predictive value (%)	Negative predictive value (%)
Livzon-EIA-IgG	92.19	354/384	96.48	137/142	98.61	82.04
WANTAI-EIA-Ab total	95.83	368/384	97.18	138/142	98.92	89.61
InnoDx-CLIA-Ab total	93.49	359/384	99.30	141/142	99.72	84.94
Beier-CLIA-IgG	75.00	288/384	99.30	141/142	99.65	59.49
YHLO-CLIA-IgG	95.05	365/384	88.03	125/142	95.55	86.81
Orienter-CLIA-IgG	94.27	362/384	97.18	138/142	98.91	86.25
Maccura-CLIA-IgG	92.19	354/384	100	142/142	100	82.56
Livzon-LFIA-IgG	92.71	356/384	99.30	141/142	99.72	83.43
WANTAI-LFIA-IgG	83.33	320/384	95.77	136/142	98.16	68.00
Beier-LFIA-IgG	81.34	231/284*	100	142/142	100	72.82
HEALGEN-LFIA-IgG	91.93	353/384	100	142/142	100	82.08
INNOVITA-LFIA-IgG	92.97	357/384	99.30	141/142	99.73	84.43
Livzon-EIA-IgM	47.14	181/384	99.30	141/142	99.45	40.99
WANTAI-EIA-IgM	85.68	329/384	97.89	139/142	99.10	71.65
InnoDx-CLIA-IgM	89.58	344/384	99.30	141/142	99.71	77.90
Beier-CLIA-IgM	64.32	247/384	97.18	138/142	98.41	50.18
YHLO-CLIA-IgM	84.11	323/384	90.85	129/142	96.13	67.89
Orienter-CLIA-IgM	90.36	347/384	97.89	139/142	99.14	78.98
Maccura-CLIA-IgM	92.45	355/384	100	142/142	100	83.04
Livzon-LFIA-IgM	46.09	177/384	100	142/142	100	40.69
WANTAI-LFIA-IgM	57.81	222/384	99.30	141/142	99.55	46.53
Beier-LFIA-IgM	71.48	203/284*	100	142/142	100	63.68
HEALGEN-LFIA-IgM	69.79	268/384	100	142/142	100	55.04
INNOVITA-LFIA-IgM	67.71	260/384	97.18	138/142	98.48	52.67

*Due to a shortage of reagents or samples, only 284 cases of COVID-19 were detected by Beier-LFIA-IgG/IgM.

False-Negative Results of Severe Acute Respiratory Syndrome Coronavirus 2 Antibody Assays

There were 6 COVID-19 patients who had negative anti-SARS-CoV-2 results by any detection kits. We observed that days from onset to antibody detection were between 3 and 12 days in these patients. Furthermore, five of six patients had underlying diseases such as hypertension, diabetes, and coronary heart disease. One patient with systemic lupus erythematosus was undergoing immunosuppressant treatment (rituximab). The demographic and clinical characteristics of these patients are shown in **Table 2**. These data suggested that the false negative results of anti-SARS-CoV-2 results may be caused by the variability in the time from onset of illness to detection or the immunosuppression status in COVID-19 patients.

DISCUSSION

The early diagnosis and isolation of COVID-19 patients are the key to control the outbreak of the disease. Given the false-negative results of SARS-CoV-2 RT-PCR is common in clinical samples, especially in patients with increased time since symptom onset or with oropharyngeal samples rather than nasopharyngeal samples, it is unsuitable for use of the method to exclude COVID-19 (Arevalo-Rodriguez et al., 2020; Wikramaratna et al., 2020). With the outbreak of COVID-19, many SARS-CoV-2

antibody detection methods based on different methodologies such as ELISA, CLIA and LFIA have been developed. The current view emphasizes that SARS-CoV-2 antibodies serve as an complement to RT-PCR in the diagnosis of acute infection (Sidiq et al., 2020). However, the performance of these antibody detection methods for the diagnosis of COVID-19 patients is inconclusive. In this study, we compared the performance of almost all current commercially available assays for anti-SARS-CoV-2 detection in China. Our data showed that the performance of high-throughput technologies including EIAs and CLIAs was superior to POCT. Moreover, most EIAs and CLIAs had high sensitivity and specificity and comparable diagnostic accuracy, which confirmed that SARS-CoV-2 antibody detection may have an adjunct role in the diagnosis of COVID-19.

Regarding using serological tests for COVID-19 diagnosis, there were two main aspects that should be considered. First, the test should have enough sensitivity and specificity to facilitate COVID-19 diagnosis. Second, technical efficiency and bio-safety also counted. Our data showed that most anti-SARS-CoV-2 IgG tests and many anti-SARS-CoV-2 IgM tests such as Orienter-CLIA-IgM and Maccura-CLIA-IgM, achieved over 90% sensitivity in the diagnosis of COVID-19, which was in accordance with previous study (Li et al., 2020). Thus, SARS-CoV-2 antibody detection was of important value in early diagnosis of COVID-19, especially in patients suspected as SARS-CoV-2 infection but with negative RT-PCR results. On the other hand, both anti-SARS-CoV-2 IgG and IgM detection had high

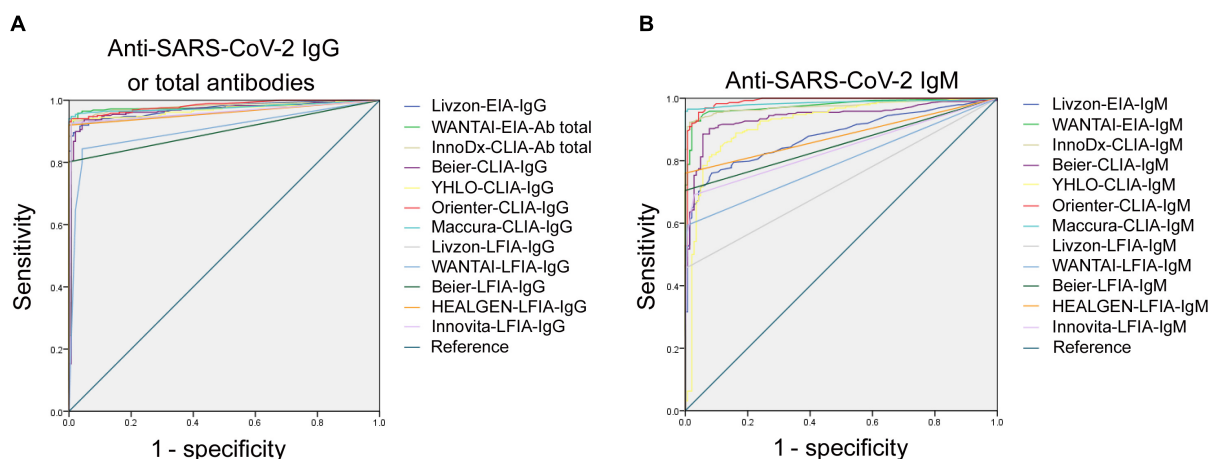
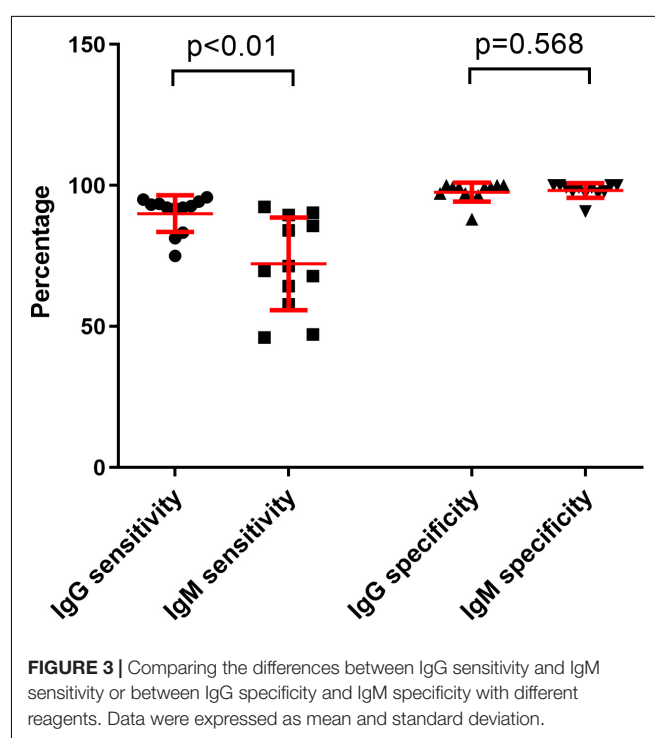
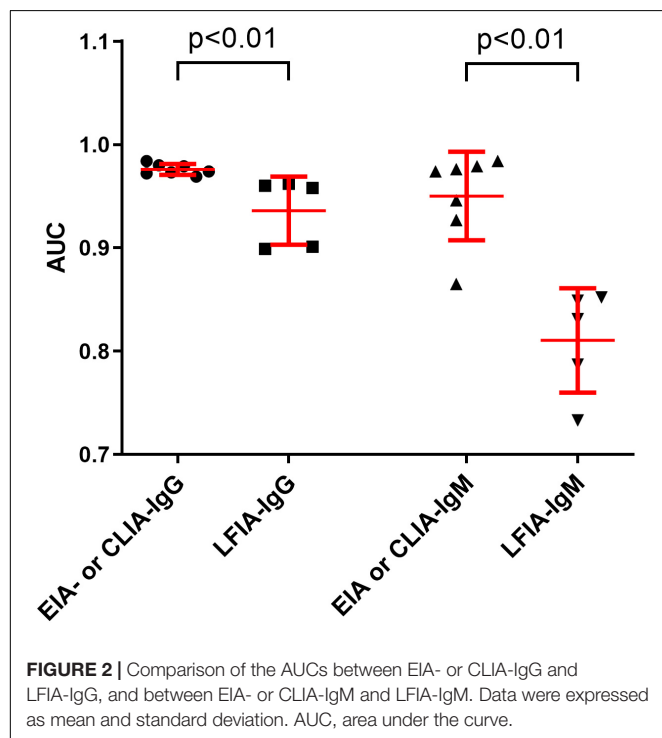


FIGURE 1 | ROC analysis of 24 SARS-CoV-2 antibody assays. **(A)** ROC analysis of anti-SARS-CoV-2 IgG or total antibodies with different reagents. AUCs were 0.973 (95% CI, 0.961–0.985), 0.974 (95% CI, 0.957–0.990), 0.979 (95% CI, 0.968–0.990), 0.969 (95% CI, 0.953–0.986), 0.972 (95% CI, 0.959–0.985), 0.984 (95% CI, 0.976–0.993), 0.980 (95% CI, 0.968–0.991), 0.958 (95% CI, 0.941–0.976), 0.899 (95% CI, 0.871–0.927), 0.901 (95% CI, 0.872–0.930), 0.960 (95% CI, 0.943–0.979), 0.962 (95% CI, 0.945–0.979), and 0.968 (95% CI, 0.954–0.983) for Livzon-EIA-IgG, WANTAI-EIA-Ab total, InnoDx-CLIA-Ab total, Beier-CLIA-IgG, YHLO-CLIA-IgG, Orienter-CLIA-IgG, Maccura-CLIA-IgG, Livzon-LFIA-IgG, WANTAI-LFIA-IgG, Beier-LFIA-IgG, HEALGEN-LFIA-IgG, and Innovita-LFIA-IgG, respectively. **(B)** ROC analysis of anti-SARS-CoV-2 IgM with different reagents. AUCs were 0.865 (95% CI, 0.835–0.896), 0.976 (95% CI, 0.964–0.988), 0.974 (95% CI, 0.962–0.987), 0.946 (95% CI, 0.926–0.966), 0.927 (95% CI, 0.900–0.955), 0.984 (95% CI, 0.973–0.995), 0.979 (95% CI, 0.966–0.991), 0.733 (95% CI, 0.692–0.774), 0.787 (95% CI, 0.750–0.824), 0.852 (95% CI, 0.817–0.887), 0.849 (95% CI, 0.818–0.880), and 0.831 (95% CI, 0.797–0.864) for Livzon-EIA-IgM, WANTAI-EIA-IgM, InnoDx-CLIA-IgM, Beier-CLIA-IgM, YHLO-CLIA-IgM, Orienter-CLIA-IgM, Maccura-CLIA-IgM, Livzon-LFIA-IgM, WANTAI-LFIA-IgM, Beier-LFIA-IgM, HEALGEN-LFIA-IgM, and Innovita-LFIA-IgM, respectively. CI, confidence interval.

specificity (mostly higher than 95%) for diagnosis of COVID-19. Hereafter, these data suggest that SARS-CoV-2 antibody detection plays an important role in the diagnosis and exclusion of COVID-19 patients.

Except for Beier, the sensitivities of all other anti-SARS-CoV-2 IgG detection methods were relatively high. Nevertheless, the sensitivities of anti-SARS-CoV-2 IgM detection methods varied greatly, and the sensitivities of WANTAI-EIA-IgM,



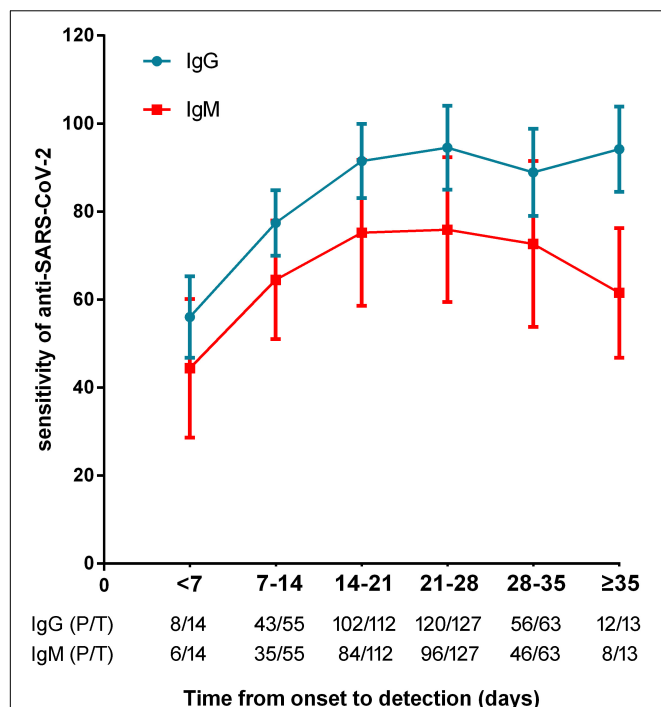


FIGURE 4 | The sensitivities of anti-SARS-CoV-2 antibodies in patients with different timepoints (calculated from onset of symptoms to antibody detection). P/T, positive number/total number.

InnoDx-CLIA-IgM, YHLO-CLIA-IgM, Orienter-CLIA-IgM and Maccura-CLIA-IgM were higher than those of others. Overall, the sensitivities of anti-SARS-CoV-2 IgG in different methods were higher than IgM, but the specificity of both anti-SARS-CoV-2 IgG and IgM had no difference among these methods. Generally, antigen-specific IgM can be early detected after pathogen infection and then rapidly decreases in several weeks. In contrast, IgG usually appears later but maintains at a certain level for a long time. Consistent with this notion, it was reported that IgM could be detected in peripheral blood of COVID-19 patients after 3–7 days and that IgG could be detected after 7–8 days (Zhou et al., 2020). It is worthy to note that, the median time from onset to antibody detection was 21 days in the present study, and we speculated that our enrolled patients were in the middle stage of infection or recovery period. This could be used to explain the low sensitivity of anti-SARS-CoV-2 IgM in these patients. Our findings indicated that the COVID-19 patients with decreased level of IgM but with maintained level of IgG may be in the status of recovery. These data suggest that SARS-CoV-2 antibody detection could also play an important role in the treatment monitoring and prognosis of COVID-19.

Due to the highly contagious nature of the disease, even asymptomatic carriers could spread SARS-CoV-2 virus, which made the control of COVID-19 outbreak more difficult (Rothe et al., 2020; Zou et al., 2020). Given that the sensitivities of SARS-CoV-2 antibody assays were high, these assays had great value in screening asymptomatic SARS-CoV-2 carriers. However, we still observed 6 COVID-19 patients with false-negative results of

TABLE 2 | The demographic and clinical characteristics of 6 COVID-19 patients with negative anti-SARS-CoV-2 results in all 24 assays.

No.	Sex	Age (years)	Time from onset to detection (days)	Severity	Underlying disease
19	Female	75	12	Severe	Hypertension, coronary heart disease
162	Male	34	5	Mild	None
191	Female	22	7	Extremely severe	Systemic lupus erythematosus, using immunosuppressant (rituximab)
208	Female	27	3	Mild	Hyperthyreosis, pregnancy
219	Female	66	7	Severe	Hypertension, diabetes, coronary heart disease, endometrial cancer (after surgery)
236	Male	57	3	Severe	Hypertension, diabetes, lung cancer (after surgery)

all antibody detection methods (Table 2). The reasons could be as follows. First, low concentration of antibodies could lead to false negative results. As shown in Table 2, the days from onset to antibody detection of five patients were within 7 days, while IgM and IgG levels may be below the detection limit during this period. Second, the heterogeneity of immune response to SARS-CoV-2 in different individuals may cause delayed antibody production in some individuals. Third, the patients with underlying conditions may be one of the important reasons contributing to false-negative results of antibody detection. As shown in Table 2, one patient (No. 19) was a seventy-five-year-old female who might have impaired immunity because of older age (Chandra, 2002). Another three patients (No. 191, No. 219, No. 236) had immunocompromised conditions such as diabetes, lung cancer, and undergoing immunosuppressant treatment, which could affect the producing of antibodies in these patients. In addition, previous study has shown that the possibility of false-negative results of SARS-CoV-2 antibody assays should be considered if the sample was pre-inactivated by heating, which suggests that heat inactivation prior to immunoanalysis is not recommended (Hu et al., 2020). On the other hand, the false-positive results of SARS-CoV-2 antibody assays maybe due to cross-reactivity with anti-HBV, anti-influenza, and rheumatoid factor (Tre-Hardy et al., 2020).

Some limitations of the study should be mentioned. First, since Tongji Hospital was one of designated hospitals for transfer of patients with COVID-19 from other hospitals, the enrolled patients in this study had a relatively prolonged time from onset of symptoms to admission. This is the reason why the median time from onset to antibody detection was 21 days, which could affect the results of antibody detection. Second, we did not continuously monitor the producing of antibodies in the same patients, and further study is needed.

Taken together, the present study confirms that SARS-CoV-2 antibody assays have good performance in the diagnosis and exclusion of COVID-19 patients, especially by using high-throughput technologies (EIAs or CLIAs), which suggests that antibody detection of SARS-CoV-2 may play an important role in the control of COVID-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

This study was approved by the ethical committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (TJ-C20200128). Written informed consent for participation was not required for this study

REFERENCES

- Arevalo-Rodriguez, I., Buitrago-Garcia, D., Simancas-Racines, D., Zambrano-Achig, P., Del Campo, R., Ciapponi, A., et al. (2020). False-negative results of initial RT-PCR assays for COVID-19: a systematic review. *PLoS One* 15:e0242958. doi: 10.1371/journal.pone.0242958
- Baj, J., Karakula-Juchnowicz, H., Teresinski, G., Buszewicz, G., Ciesielka, M., Sitarz, E., et al. (2020). COVID-19: Specific and Non-Specific Clinical Manifestations and Symptoms: The Current State of Knowledge. *J. Clin. Med.* 9:1753. doi: 10.3390/jcm9061753
- Brendish, N. J., Poole, S., Naidu, V. V., Mansbridge, C. T., Norton, N., Borca, F., et al. (2020). Clinical characteristics, symptoms and outcomes of 1054 adults presenting to hospital with suspected COVID-19: a comparison of patients with and without SARS-CoV-2 infection. *J. Infect.* 81, 937–943. doi: 10.1016/j.jinf.2020.09.033
- Chandra, R. K. (2002). Nutrition and the immune system from birth to old age. *Eur. J. Clin. Nutr.* 56, S73–S76. doi: 10.1038/sj.ejcn.1601492
- Chung, M., Bernheim, A., Mei, X., Zhang, N., Huang, M., Zeng, X., et al. (2020). CT Imaging Features of 2019 Novel Coronavirus (2019-nCoV). *Radiology* 295, 202–207. doi: 10.1148/radiol.20200230
- Gudbjartsson, D. F., Norddahl, G. L., Melsted, P., Gunnarsdottir, K., Holm, H., Eythorsson, E., et al. (2020). Humoral Immune Response to SARS-CoV-2 in Iceland. *N. Engl. J. Med.* 383, 1724–1734. doi: 10.1056/NEJMoa2026116
- Hou, H., Wang, T., Zhang, B., Luo, Y., Mao, L., Wang, F., et al. (2020). Detection of IgM and IgG antibodies in patients with coronavirus disease 2019. *Clin. Transl. Immunol.* 9:e01136. doi: 10.1002/cti2.1136
- Hou, H., Zhang, Y., Tang, G., Luo, Y., Liu, W., Cheng, C., et al. (2021). Immunologic memory to SARS-CoV-2 in convalescent COVID-19 patients at 1 year postinfection. *J. Allergy Clin. Immunol.* 148, 1481–1492.e2. doi: 10.1016/j.jaci.2021.09.008
- Hu, X., An, T., Situ, B., Hu, Y., Ou, Z., Li, Q., et al. (2020). Heat inactivation of serum interferes with the immunoanalysis of antibodies to SARS-CoV-2. *J. Clin. Lab. Anal.* 34:e23411. doi: 10.1002/jcla.23411
- Islam, K. U., and Iqbal, J. (2020). An Update on Molecular Diagnostics for COVID-19. *Front. Cell. Infect. Microbiol.* 10:560616. doi: 10.3389/fcimb.2020.560616
- Kuo, B. J., Lai, Y. K., Tan, M. L. M., and Goh, X. C. (2021). Utility of Screening Chest Radiographs in Patients with Asymptomatic or Minimally Symptomatic COVID-19 in Singapore. *Radiology* 298, E131–E140. doi: 10.1148/radiol.2020203496
- Li, Z., Yi, Y., Luo, X., Xiong, N., Liu, Y., Li, S., et al. (2020). Development and clinical application of a rapid IgM-IgG combined antibody test for SARS-CoV-2 infection diagnosis. *J. Med. Virol.* 92, 1518–1524. doi: 10.1002/jmv.25727
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AUTHOR CONTRIBUTIONS

SW and FW wrote the first draft of the manuscript and involved in data curation and project administration. HL managed reagents. HH and TW researched the literature and conceived the study. WW and MZ collected the specimen. SW and BY involved in experiment operation. MH, ZS, and FW involved in protocol development, gaining ethical approval, patient recruitment, and data analysis. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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- Lisboa Bastos, M., Tavaziva, G., Abidi, S. K., Campbell, J. R., Haraoui, L. P., Johnston, J. C., et al. (2020). Diagnostic accuracy of serological tests for covid-19: systematic review and meta-analysis. *BMJ* 370:m2516. doi: 10.1136/bmj.m2516
- Liu, R., Han, H., Liu, F., Lv, Z., Wu, K., Liu, Y., et al. (2020). Positive rate of RT-PCR detection of SARS-CoV-2 infection in 4880 cases from one hospital in Wuhan, China, from Jan to Feb 2020. *Clin. Chim. Acta* 505, 172–175. doi: 10.1016/j.cca.2020.03.009
- Ong, D. S. Y., Fragkou, P. C., Schweitzer, V. A., Chemaly, R. F., Moschopoulos, C. D., Skevaki, C., et al. (2021). How to interpret and use COVID-19 serology and immunology tests. *Clin. Microbiol. Infect.* 27, 981–986. doi: 10.1016/j.cmi.2021.05.001
- Pan, L., Mu, M., Yang, P., Sun, Y., Wang, R., Yan, J., et al. (2020). Clinical Characteristics of COVID-19 Patients With Digestive Symptoms in Hubei, China: A Descriptive, Cross-Sectional, Multicenter Study. *Am. J. Gastroenterol.* 115, 766–773. doi: 10.14309/ajg.0000000000000620
- Pascarella, G., Strumia, A., Piliego, C., Bruno, F., Del Buono, R., Costa, F., et al. (2020). COVID-19 diagnosis and management: a comprehensive review. *J. Intern. Med.* 288, 192–206. doi: 10.1111/joim.13091
- Phelan, A. L., Katz, R., and Gostin, L. O. (2020). The Novel Coronavirus Originating in Wuhan, China: Challenges for Global Health Governance. *JAMA* 323, 709–710. doi: 10.1001/jama.2020.1097
- Ralli, M., Morrone, A., Arcangeli, A., and Ercoli, L. (2021). Asymptomatic patients as a source of transmission of COVID-19 in homeless shelters. *Int. J. Infect. Dis.* 103, 243–245. doi: 10.1016/j.ijid.2020.12.031
- Rothe, C., Schunk, M., Sothmann, P., Bretzel, G., Froeschl, G., Wallrauch, C., et al. (2020). Transmission of 2019-nCoV Infection from an Asymptomatic Contact in Germany. *N. Engl. J. Med.* 382, 970–971. doi: 10.1056/NEJMc2001468
- Sidiq, Z., Hanif, M., Dwivedi, K. K., and Chopra, K. K. (2020). Benefits and limitations of serological assays in COVID-19 infection. *Indian J. Tuberc.* 67, S163–S166. doi: 10.1016/j.ijtb.2020.07.034
- Sule, W. F., and Oluwayelu, D. O. (2020). Real-time RT-PCR for COVID-19 diagnosis: challenges and prospects. *Pan. Afr. Med. J.* 35:121. doi: 10.11604/pamj.supp.2020.35.24258
- Temkin, E., and Healthcare Worker COVID-19 Surveillance Working Group (2021). Extremely low prevalence of asymptomatic COVID-19 among healthcare workers caring for COVID-19 patients in Israeli hospitals: a cross-sectional study. *Clin. Microbiol. Infect.* 27:130.e1–130.e4.
- The, L. (2020). Emerging understandings of 2019-nCoV. *Lancet* 395:311. doi: 10.1016/S0140-6736(20)30186-0
- Tre-Hardy, M., Wilmet, A., Beukinga, I., Dogne, J. M., Douxfils, J., and Blairon, L. (2020). Validation of a chemiluminescent assay for specific SARS-CoV-2 antibody. *Clin. Chem. Lab. Med.* 58, 1357–1364. doi: 10.1515/cclm-2020-0594

- Wang, C., Yu, H., Horby, P. W., Cao, B., Wu, P., Yang, S., et al. (2014). Comparison of patients hospitalized with influenza A subtypes H7N9, H5N1, and 2009 pandemic H1N1. *Clin. Infect. Dis.* 58, 1095–1103. doi: 10.1093/cid/ciu053
- Wikramaratna, P. S., Paton, R. S., Ghafari, M., and Lourenco, J. (2020). Estimating the false-negative test probability of SARS-CoV-2 by RT-PCR. *Euro Surveill.* 25:2000568. doi: 10.2807/1560-7917.ES.2020.25.50.2000568
- Xiao, A. T., Gao, C., and Zhang, S. (2020). Profile of specific antibodies to SARS-CoV-2: the first report. *J. Infect.* 81, 147–178. doi: 10.1016/j.jinf.2020.03.012
- Xiao, S. Y., Wu, Y., and Liu, H. (2020). Evolving status of the 2019 novel coronavirus infection: proposal of conventional serologic assays for disease diagnosis and infection monitoring. *J. Med. Virol.* 92, 464–467. doi: 10.1002/jmv.25702
- Zhou, P., Yang, X. L., Wang, X. G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579, 270–273. doi: 10.1038/s41586-020-2012-7
- Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733. doi: 10.1056/NEJMoa2001017
- Zou, L., Ruan, F., Huang, M., Liang, L., Huang, H., Hong, Z., et al. (2020). SARS-CoV-2 Viral Load in Upper Respiratory Specimens of Infected Patients. *N. Engl. J. Med.* 382, 1177–1179. doi: 10.1056/NEJMc2001737
- Zowawi, H. M., Alenazi, T. H., AlOmaim, W. S., Wazzan, A., Alsufayan, A., Hasanain, R. A., et al. (2021). Portable RT-PCR System: a Rapid and Scalable Diagnostic Tool for COVID-19 Testing. *J. Clin. Microbiol.* 59:e03004–20. doi: 10.1128/JCM.03004-20

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Receptor-binding domain-anchored peptides block binding of severe acute respiratory syndrome coronavirus 2 spike proteins with cell surface angiotensin-converting enzyme 2

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Background: The COVID-19 pandemic has killed over 6 million people worldwide. Despite the accumulation of knowledge about the causative pathogen severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the pathogenesis of this disease, cures remain to be discovered. We searched for certain peptides that might interfere with spike protein (S protein)-angiotensin-converting enzyme 2 (ACE2) interactions.

Methods: Phage display (PhD)-12 peptide library was screened against recombinant spike trimer (S-trimer) or receptor-binding domain (S-RBD) proteins. The resulting enriched peptide sequences were obtained, and their potential binding sites on S-trimer and S-RBD 3D structure models were searched. Synthetic peptides corresponding to these and other reference sequences were tested for their efficacy in blocking the binding of S-trimer protein onto recombinant ACE2 proteins or ACE2-overexpressing cells.

Results: After three rounds of phage selections, two peptide sequences (C2, DHAQRYGAGHSG; C6, HWKAVNWLKPWT) were enriched by S-RBD, but only

C2 was present in S-trimer selected phages. When the 3D structures of static monomeric S-RBD (6M17) and S-trimer (6ZGE, 6ZGG, 7CAI, and 7CAK, each with different status of S-RBDs in the three monomer S proteins) were scanned for potential binding sites of C2 and C6 peptides, C6 opt to bind the saddle of S-RBD in both 6M17 and erected S-RBD in S-trimers, but C2 failed to cluster there in the S-trimers. In the competitive S-trimer-ACE2-binding experiments, synthetic C2 and C6 peptides inhibited S-trimer binding onto 293T-ACE2hR cells at high concentrations (50 μ M) but not at lower concentrations (10 μ M and below), neither for the settings of S-trimer binding onto recombinant ACE2 proteins.

Conclusion: Using PhD methodology, two peptides were generated bearing potentials to interfere with S protein-ACE2 interaction, which might be further exploited to produce peptidomimetics that block the attachment of SARS-CoV-2 virus onto host cells, hence diminishing the pathogenesis of COVID-19.

KEYWORDS

SARS-CoV-2, spike protein, ACE2, RBD domain, phage display

Introduction

The COVID-19 pandemic had caused 6.35 million death worldwide as of 8 July 2022 and still poses a serious challenge in some nations. Worse was that over two more folds of “excess deaths” might have occurred due to indirect consequences of the pandemic, such as changes in “social, economic, and behavioral responses to the pandemic, including strict lockdowns” (Collaborators, 2022). While vaccinations and natural infections build herd immunity that helps to protect people from infection or prevent pandemic recurrence (Lipsitch and Dean, 2020; Mobarak et al., 2022), cures are still lacking for the infected individuals in most areas. Among the scientific efforts, various therapeutics have been tried, such as cells (Sang et al., 2021), engineered antibodies (Matthay and Luetkemeyer, 2021), natural products (Bhattacharya and Paul, 2021), synthetical biologicals (Robson, 2020), and small molecules (Tiwari et al., 2020). Intended targets included viral structural proteins (Sheward et al., 2022), host products [e.g., interleukin 6 (IL-6)] (Murthy and Lee, 2021), viral replication process (Schafer et al., 2022), or host-virus interactions (Gordon et al., 2020). The strategies aiming at the first step of virus-host interactions sound most attractive. The viral spike (S) protein trimers (S-trimers) are thought to be the main molecules mediating the affinity of exogenous severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus for angiotensin-converting enzyme 2 (ACE2) or other less-attended molecules, such as TMPRSS2 on host cells (Hoffmann et al., 2020). Since the structures of both S and ACE2 proteins are known, computation or computer-based methods are thought to be high for novel drug discovery (Cao et al., 2020; Jawad et al., 2021).

However, though a few candidates had been proposed in these *in silico* studies, only part of them had been proven effective in functional experimental studies, highlighting the demand for more robust strategies that mimic the actual virus-host interactions more faithfully.

Phage display (PhD) methodology, as exemplified in other infectious diseases (Huang et al., 2012; Alfaleh et al., 2020; Roth et al., 2021), met this end and has been tried in the context of SARS-CoV-2 or COVID-19. In detail, PhD has been successful in producing antibodies for neutralizing or detection (Noy-Porat et al., 2020; Bertoglio et al., 2021) in identifying COVID-19-induced antibodies to the virus (Zhao et al., 2021) or in searching for viral epitopes responsible for virus escaping immune responses (Garrett et al., 2021). Based on our previous experience using PhD in studies of the host-pathogen interactions (Zhao et al., 2012; Shen et al., 2017), we performed PhD screening to search for peptides that would bind the receptor-binding domain (RBD) domain of SARS-CoV-2 S protein (S-RBD). Theoretically, if such peptides could bind the site(s) critical for S-RBD interaction with its receptors (e.g., ACE2 or other molecules), they should interfere with S-trimer-ACE2 interactions. Furthermore, such an S-protein Entrapped Affinity Ligand (SEAL) peptide should be able to block the binding of the viruses with their target cells. Here, we report that two SEAL peptides were obtained *via* phage displaying against S-RBD and S-trimer proteins, and preliminary functional studies demonstrated weak blocking effects at high concentrations. Encouragingly, while this project was ongoing, three groups reported their results obtained by protocols mainly relying on PhD (Petrenko et al., 2022; Sevenich et al., 2022; Yang et al., 2022).

The promises and limitations of these studies were also discussed.

Materials and methods

Phage display screening against spike receptor-binding domain or spike trimer proteins and confirmation of affinity of promising phages

Recombinant SARS-CoV-2 S-trimer proteins were from the commercial resource (Cat# DRA49, MW 136.6 kDa; Novoprotein Company, Suzhou, China), and recombinant S-RBD products corresponding to aa319-541 of YP_009724390.1, MW 30.7 kDa (Lan et al., 2020) was a generous gift from Li (Tsinghua University, Beijing, China). PhD-12 Peptide PhD Library Kit (New England BioLabs, Beverly, MA, United States) was used for PhD screening against these two proteins. Briefly, S-trimer proteins were immobilized overnight at 4°C on the enzyme-linked immunosorbent assay (ELISA) plates at 100 µg/ml in 0.1 M NaHCO₃, pH 8.6. The plates were then blocked with 0.5% bovine serum albumin (BSA) in 0.1 M NaHCO₃ buffer (containing 0.02% NaN₃) for 1 h. After six washes with tris base-buffered saline solution (TBST buffer containing 0.01% Tween-20), 2×10^{11} phages in TBST buffer were added for 45 min at room temperature. After ten washes, bound phages were recovered, amplified in *Escherichia coli*, harvested into TBS buffer (containing 0.02% NaN₃), quantified with a plaque-forming assay, and used the product for the second round display. After two or three screening rounds, bound phages were harvested into elution buffer (0.2 M Glycine-HCl, 1 mg/ml BSA, pH 2.2) and neutralized with 1 M tris base-HCl buffer, pH 9.1. After dilution, the phage mix was applied onto bacterial plates to obtain blue plaques. Thirty (after the second round) or 25 (after the third round) isolated plaques were randomly picked for phage DNA sequencing using the primers in the kit. The resulting 12-amino acids peptides translated from phage DNA inserts were analyzed, and the most promising sequence was used for subsequent studies.

Enzyme-linked immunoassay (ELISA) was used to confirm the affinity of the resulting monoclonal phages for targeted proteins. ELISA plates (Corning, NY, United States) were coated with 10 µg/ml S-trimer or S-RBD proteins. With the starting original library phages (O virions) as control, all selected interest phages were amplified, titrated, and added to the plates at different concentrations (2.5×10^9 , 1×10^{10} , 4×10^{10} , 1.6×10^{11} , and 6.4×10^{11} phage virions in 100 µl) for 1 h at room temperature. The plates were washed six times with TBST washing buffer and then incubated with diluted horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal antibody

(GE Healthcare, Piscataway, NJ, United States) for 1 h. After six washes, 3,3', 5,5-tetramethylbenzidine (TMB) solution (Beyotime Biotechnology, Shanghai, China) was added to the plate, and after 10 min of development, the reaction was stopped by adding 2 M H₂SO₄ solution. Optical absorbance was measured at 450 nm in a microplate reader (Synergy H1, BioTek Instruments, Inc., Winooski, VT, United States).

PEP-SiteFinder modeling of candidate spike-protein entrapped affinity ligand peptides docking onto monomeric receptor-binding domain or spike trimer proteins

The surface of RBD or S-trimer proteins was scanned using the PEP-SiteFinder (Saladin et al., 2014). The 3D models of RBD to locate the potential docking site(s) of interest peptide(s) on S proteins (Yan et al., 2020) or S-trimers (Lv et al., 2020; Wrobel et al., 2020) were retrieved from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB). The top 50 poses of each peptide in each protein model were checked to identify the most likely binding site(s). Cn3D (Wang et al., 2000) was also utilized for viewing these protein structures.

Measurement of the effect of synthetic peptides on spike trimer-angiotensin-converting enzyme 2 binding

293T-ACE2hR cells, a cell line consistently expressing human ACE2 (hACE2) on the cell surface (Supplementary Figure 1; Zhang et al., 2021), or recombinant ACE2 proteins (Cat#10108-H02H, Novoprotein) were utilized to test the potential effect of interest peptides on S-trimer-ACE2 binding. In brief, two possible SEAL peptides derived from the above analysis (C2 and C6) and two reference peptides [spike-binding peptide 1 (SBP1) and spike-binding peptide 1 (SBP2)] (Zhang et al., 2020) were ordered from Biotech Bioscience and Technology (Shanghai, China) and dissolved in PBS. Their sequences were as follows: C2, DHAQRYGAGHSG; C6, HWKAVNWLKPWT; SBP1, IEEQAKTFLDKFNHEAEDLFYQSK; and SBP2, TFLDKFNHEAED. 293T-ACE2hR cells were grown in 96-well plates until confluent in the first measurement setting. S-trimer at 2 nM was mixed with equal volume (25 µl) of peptides at different concentrations (0, 0.16, 0.8, 4, 20, and 100 µM) and kept at room temperature for 1 h. After removing the culture medium from the cells, the mixture was added (50 µl/well) and kept at room temperature for 1 h. Unbound peptides and proteins were removed, and the cells were washed three times

with PBS. HRP-conjugated Anti-6X His tag[®] antibody (diluted at 1:10,000; Abcam, Cambridge, MA, United States) was added to each well for 1 h at room temperature. After three washes with PBS, TMB Solution (Beyotime Biotechnology) was added to the plate, and the plate was read at OD370 nm in a Multiskan Go Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States).

Then, 293T-ACE2hR cells were substituted by recombinant ACE2 proteins and coated onto ELISA plates in the other measurement setting. In brief, recombinant ACE2 proteins (Novoprotein) were immobilized overnight at room temperature on ELISA plates at a 5 µg/ml concentration in 0.1 M NaHCO₃, pH 9.6. The plates were then blocked with 10% fetal calf serum for 2 h and washed with tris-buffered saline solution containing 0.1% Tween-20 [phosphate-buffered saline (PBST) buffer]. Then a premixture of S-trimer proteins (final concentration 0.01 µM) with peptides (C2, C6, SBP1, and SBP2) of different concentrations (0.5, 1.65, and 5 µM) was added to each well (50 µl/well). The following procedures were described above for the 293T-ACE2hR cells setting.

Results and discussion

Obtainment of two promising spike-protein entrapped affinity ligand peptide sequences displayed against spike-receptor-binding domain

Phage display has been widely used in identifying interacting partners of target molecules that were included in previous projects of this team (Zhao et al., 2012; Wang et al., 2019). In the current study, we applied PhD on S-trimers or S-RBD proteins, aiming to obtain peptides supposedly able to “seal” the potent binding site on their surface. After three rounds of panning PhD against recombinant S-RBD proteins, two phages with peptide sequences DHAQRYGAGHSG (C2) and HWKAVNWLKPWT (C6) were enriched in the elutes, each of them accounting for 10 clones in all 25 sequenced clones. Interestingly, C2 and C6 accounted for 7 and 6 clones in the elute after the second panning in all 30 sequenced clones. Therefore, we did not attempt more rounds of panning. When S-trimer proteins were used for panning, only the C2 sequence dominated the elutes, accounting for 20 of 30 clones after the second panning and 16 of 25 phages after the third panning, respectively. Next, using the starting library phages (O virions) as control, the ELISA assay demonstrated dose-dependent binding of monoclonal C2 and C6 phages to immobilized S-RBD proteins and C2 phages for S-trimer protein (Figure 1). An accurate comparison between affinity of C2 and C6 phages for the same target (e.g., S-RBD proteins)

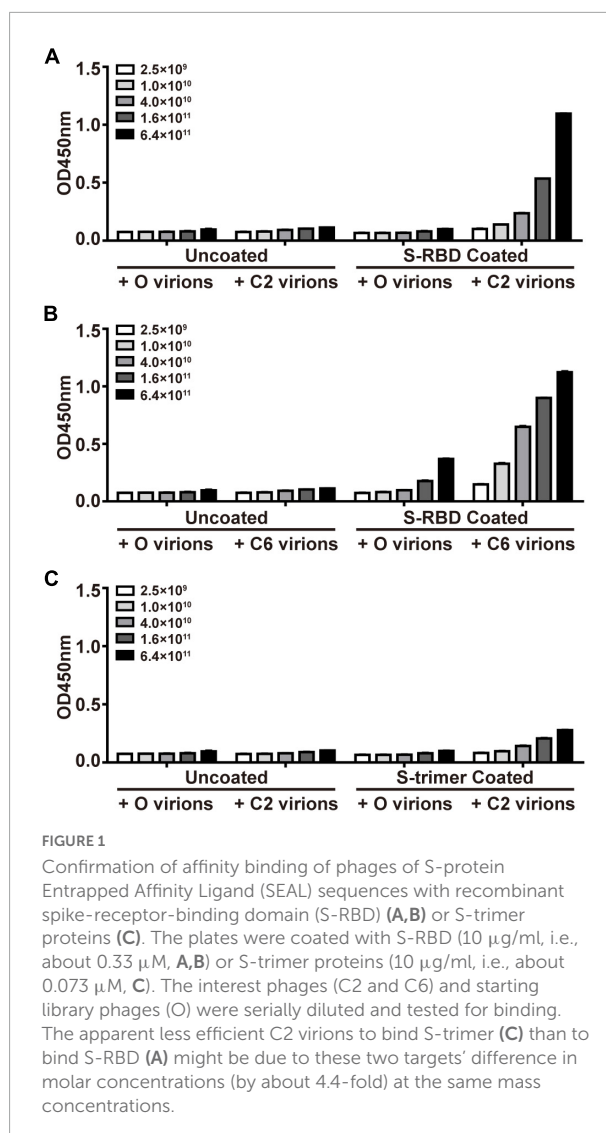


FIGURE 1

Confirmation of affinity binding of phages of S-protein Entrapped Affinity Ligand (SEAL) sequences with recombinant spike-receptor-binding domain (S-RBD) (A,B) or S-trimer proteins (C). The plates were coated with S-RBD (10 µg/ml, i.e., about 0.33 µM, A,B) or S-trimer proteins (10 µg/ml, i.e., about 0.073 µM, C). The interest phages (C2 and C6) and starting library phages (O virions) were serially diluted and tested for binding. The apparent less efficient C2 virions to bind S-trimer (C) than to bind S-RBD (A) might be due to these two targets' difference in molar concentrations (by about 4.4-fold) at the same mass concentrations.

was not attempted, or between affinities of C2 phages for different target proteins (i.e., S-RBD or S-trimer). Measurement of affinity of C6 phages for S-trimer was not attempted either.

Three-dimensional modeling of peptides binding onto spike receptor-binding domain or spike trimer

Previous crystal structural studies suggested that the resting S-trimers on the virus surface took a “closed” figuration and, upon contacting ACE2 (or other receptors) on host cells, went through the opening process and exposed the RBD (Wrobel et al., 2020). Surely, this opening process would also alter the configuration of the whole molecule. When the

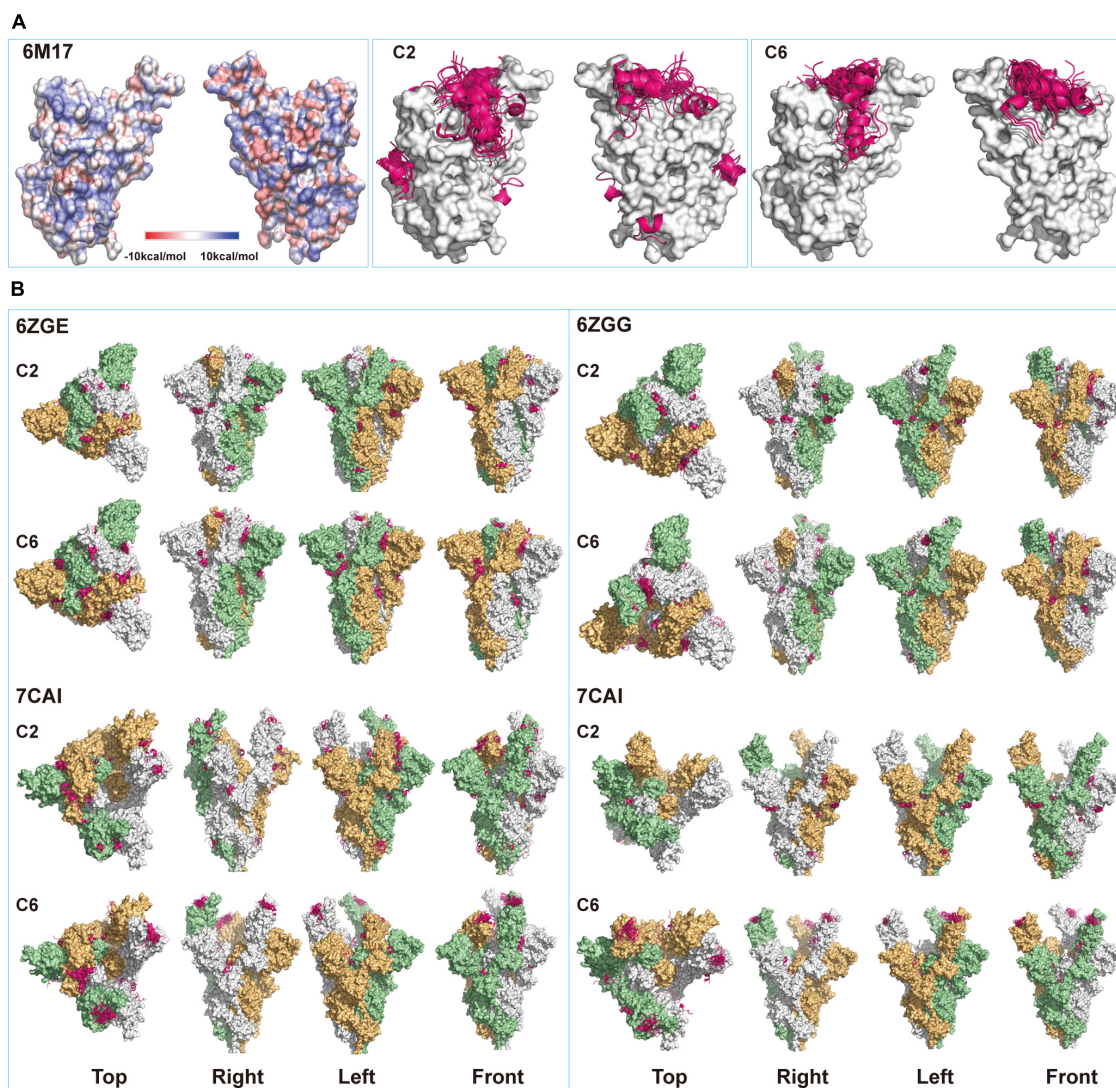


FIGURE 2

Structural modeling of peptides-spike interactions. **(A)** Comparing binding patterns for C2 and C6 peptides (pink belts) onto spike-receptor-binding domain (S-RBD). Shown are the fifty poses with the highest of each binding exclusively onto the saddle of RBD (6M17, open), some poses of C2 peptide bind onto other sites on the surface of RBD. **(B)** Four configurations corresponding to the different status of RBDs in the S-trimers (6ZGE, 6ZGG, 7CAI, and 7CAK for 0, 1, 2, and 3 RBDs open, respectively) were compared for their potential bindings with the two peptides. Each configuration was given a view from the top, right, left, and front sides.

static model of single S-RBD protein (6M17) was used for predicting peptide binding sites, it was found that all fifty C6 peptide poses were docked onto the saddle of S-RBD, while a fraction of C2 peptide poses were docked onto other sites that were supposedly not to directly affect S-RBD's receptor binding functions (Figure 2A). When S-trimer was used for modeling, binding sites for C2 poses were even more dispersed, and few of them would dock onto S-RBD saddlebacks, independent of S-trimers' configuration (Figure 2B). For example, in the all-closing status of the S-trimer (6ZGE), 11 of 50 C2 poses were located in the spaces among the three S-RBDs, 6 between neighboring S-RBDs, and 8 in the middle

between S-RBD and N-terminal domain (NTD). In the all-open configuration (7CAK), only 6 poses were related to S-RBD and not any poses were docked onto the saddle of open S-RBDs.

On the contrary, once a single S-RBD was erected (6ZGG), several C6 poses were docked onto its saddle. When one or two more S-RBDs were erected (7CAI and 7CAK, respectively), almost all C6 poses were on their saddlebacks. We assumed that in an actual environment that contained both virus and host cells, if the C2 or C6 peptides were present when S-trimers were in the closed configuration just like in resting virus, their binding onto the surface of S-trimers might facilitate or hinder

the opening or erection of S-RBD, which deserved in-depth investigation in future. However, once the S-trimer opening process was initiated, C6 peptides should be able to bind the saddle of S-RBDs. Since it has been well documented that the saddle section was critical for RBD functions, such as binding with ACE2 (Lan et al., 2020; Wang et al., 2020) and being immunogenic (Greaney et al., 2021), we proposed that C6 peptides might interfere with the interaction of S-trimer with ACE2 by SEALing the S-RBD saddle(s). The differential docking sites' prediction observed for C2 and C6 might partially explain that C2 virions were enriched by both S-RBD and S-trimer proteins, while the C6 virions were enriched by S-RBD proteins only (Figure 1).

Synthetic C2 and C6 peptides blocked the binding of spike-trimers onto 293T-ACE2hR cells

When the above hypothesis was tested on recombinant ACE2 proteins coated on a solid surface, no blocking effect was observed for any tested peptides even at a 500:1 (5 μ M vs. 0.01 μ M, peptide vs. S-trimer) ratio (Figure 3A). These peptides' ineffectiveness in blocking S-trimer binding with ACE2 at equivalent concentrations was also observed when 293T-ACE2hR cells were used as the source of S-trimer targets in living cells. When 50,000-fold overdose of C2 or C6 peptides was present, namely, 50 μ M peptides vs. 0.001 μ M S-trimer proteins, a blocking effect was observed for C2 and C6, but still not for SBP1 or SBP2 peptides (Figure 3B).

Failure of 500-fold overdose of C2 or C6 peptides to block S-trimers binding with their receptors was disappointing, and we suggested two possible explanations for this failure, especially for C6. First, the occupation of the saddle by C6 did not cause enough stereotype blockade as expected, which might be due to the small size of this 12-aa peptide, especially when most poses lay on the saddle "seat" in a vertical-crossing orientation

rather than paralleling along the saddle axis (Figure 2A). It has been demonstrated that the "candle" and "pommel" contributed more than the "seat" (Supplementary Figure 2) to the overall affinity and configuration fitting between S-RBD and ACE2 (Lan et al., 2020; Wang et al., 2020). Second, the affinity of these peptides for S-RBD might be too low to constitute effective competitors when natural and intact receptors (i.e., ACE2 molecules) were present, which might be the case for the SBP1 and SBP2 peptides. SBP1 was from the N-terminal sequence of hACE2 (e.g., α -helix 1) that was supposed to contact S-RBD if it intact ACE2 (Supplementary Figure 2; Zhang et al., 2020). Though those investigators demonstrated the association of SBP1 peptides with S-RBD proteins at the level of micromolar scales using bio-layer interferometry, SBP1 peptides neither associated with cell surface S-RBD proteins nor did they outcompete ACE2 binding onto S-RBD proteins (Zhang et al., 2020). More rigorous studies should verify such explanations and determine the associated factors between C6 and C2 peptides for RBD or S-trimers.

Another issue deserving discussion was why C2 and C6 behaved similarly in the competition assay (Figure 3) though they manifested different enrichment patterns in PhD panning (Figure 1), as well as different binding properties in the 3D modeling assay (Figure 2). It was known that configuration changes of S-trimers when virus encountered host components were critical for higher affinity interactions between S-trimers and ACE2 molecules. It was understandable that such interactions would be unique in many aspects and depend on the dynamic minutiae of all parties, and we would arbitrarily assume that the factors causing differential binding patterns of C2 or C6 virions onto S-RBD/S-trimers in PhD assays did not contribute enough interference to the actual interactions between S-trimers and ACE2 in the competition context, which are reflected in Figure 3.

Lastly, as a useful tool for studying molecular interactions at both biophysical and functional levels, PhD has been successful in identifying peptides that might be directly utilized to block

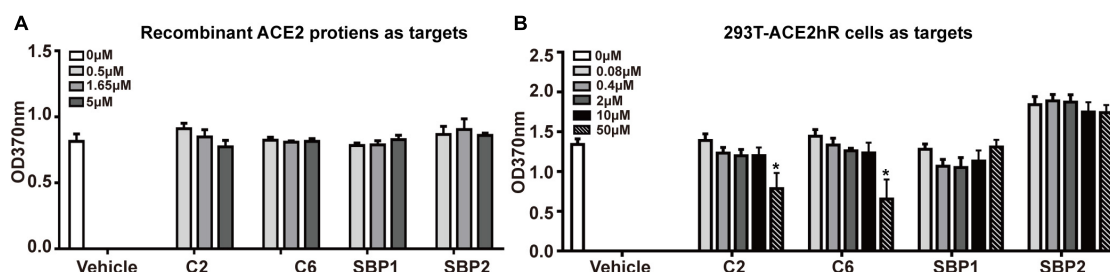


FIGURE 3

The potential blocking effect of peptides on the binding of S-trimer onto cellular or soluble angiotensin-converting enzyme 2 (ACE2). (A) The enzyme-linked immunoassay (ELISA) was applied to assess the blocking ability of synthetic peptides C2 and C6 at binding between ACE2 molecules and S-trimer protein, and the peptide SBP1 was used as positive control and peptide spike-binding peptide 2 (SBP2) as the negative control. (B) ELISA was applied to assess the blocking ability of synthetic peptides C2 and C6 at binding between 293T-ACE2hR cells and S-trimer protein. The peptide SBP1 was used as a positive and peptide SBP2 as the negative control. * $p < 0.05$, vs. vehicle, all by Student's t -test.

pathogen invasions in this lab (Zhao et al., 2012) or others (Hall et al., 2009; Wei et al., 2020). This study and several other efforts (Anand et al., 2021; Sokullu et al., 2021; Ballmann et al., 2022; Labriola et al., 2022; Petrenko et al., 2022; Sevenich et al., 2022; Yang et al., 2022) demonstrated that PhD was a plausible method for generating possible therapeutics to treat COVID-19 as well. For example, Petrenko used phage-displayed spike S1 protein mimotopes to search for “all” cellular receptors, including authentic and alternative ones. Interestingly, FGFR3 was identified as an alternative receptor to S proteins (Petrenko et al., 2022). Since FGFR3 manifested a distribution pattern different from that of ACE2, Petrenko’s work expanded the area of SARS-CoV-2 targets and might lead to the discovery of novel pathogenesis of SARS-CoV-2. Like us, Sevenich performed three rounds of screening on S-RBD proteins using a 16-aa phage library combined with high throughput sequencing. The five final sequences they obtained manifested variable affinity for S-RBD proteins in confirmation assays, with Kd from 1.3 to 89.4 μ M (Sevenich et al., 2022). Others also demonstrated the stringent dependence of efficacy of intended therapeutics on their molecular compositions and configuration (Raghuvamsi et al., 2021). With the help of computation of RBD-ACE2 interactions, the Baker’s team generated mini-proteins of 56–64 residues with inhibitory concentrations of 24–35 nM for RBD-ACE2 binding (Cao et al., 2020). On the contrary, also based on analysis of motifs or amino acids involved in RBD-ACE2 interaction, Chitsike proposed six peptides (20–29 aa) mimicking S-RBD fragment or hACE2 fragment but found that their IC50 for inhibiting RBD-ACE2 binding in experiments varied from 27 to 363 μ M (Chitsike et al., 2021). For us, the direction of our future study would be to use the current C6 SEAL peptides as the core sequences and to develop them into larger molecules (e.g., mini-protein) or other types of peptides (e.g., circular peptides) that would have a better chance to block RBD-ACE2 binding (Pomplun, 2020).

Conclusion

For the great endanger of the COVID-19 pandemic to the human, any strategies or approaches that might lead to the discovery of therapeutics or cures deserve a try (Singh et al., 2021; Vivekanandhan et al., 2021). We utilized PhD to generate two 12-aa peptides with the potentials to inhibit S-protein bindings onto cellular ACE2. Structural modeling revealed that one (C6) might take effect by binding onto the S-RBD-ACE2 interaction face. More efforts should be made to improve the binding affinity of the peptides for S proteins, such as by modifying or transforming them into other types of molecules to block S protein-ACE2 adherence more efficiently. Ultimately, such peptides or their derivatives might be developed into therapeutics that block virus-host attachment and hinder disease onset.

Data availability statement

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

Author contributions

YQW conceived and designed the study. TW, JX, YLW, and WZ performed the experiments. YX and QY supplied reagents. BW performed the 3D structural modeling. TW, JX, and YQW analyzed the data and prepared the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.910343/full#supplementary-material>

References

- Alfaleh, M. A., Alsaab, H. O., Mahmoud, A. B., Alkayyal, A. A., Jones, M. L., Mahler, S. M., et al. (2020). Phage display derived monoclonal antibodies: From bench to bedside. *Front. Immunol.* 11:1986. doi: 10.3389/fimmu.2020.01986
- Anand, T., Virmani, N., Bera, B. C., Vaid, R. K., Vashisth, M., Bardajaty, P., et al. (2021). Phage display technique as a tool for diagnosis and antibody selection for coronaviruses. *Curr. Microbiol.* 78, 1124–1134. doi: 10.1007/s00284-021-02398-9
- Ballmann, R., Hotop, S. K., Bertoglio, F., Steinke, S., Heine, P. A., Chaudhry, M. Z., et al. (2022). ORFeome phage display reveals a major immunogenic epitope on the s2 subdomain of SARS-CoV-2 spike protein. *Viruses* 14:1326. doi: 10.3390/v14061326
- Bertoglio, F., Meier, D., Langreder, N., Steinke, S., Rand, U., Simonelli, L., et al. (2021). SARS-CoV-2 neutralizing human recombinant antibodies selected from pre-pandemic healthy donors binding at RBD-ACE2 interface. *Nat. Commun.* 12:1577. doi: 10.1038/s41467-021-21609-2
- Bhattacharya, S., and Paul, S. M. N. (2021). Efficacy of phytochemicals as immunomodulators in managing COVID-19: A comprehensive view. *Virusdisease* 32, 435–445. doi: 10.1007/s13337-021-00706-2
- Cao, L., Goresnik, I., Coventry, B., Case, J. B., Miller, L., Kozodoy, L., et al. (2020). De novo design of picomolar SARS-CoV-2 miniprotein inhibitors. *Science* 370, 426–431. doi: 10.1126/science.abd9909
- Chitsike, L., Krstenansky, J., and Duerksen-Hughes, P. J. (2021). ACE2 : S1 RBD interaction-targeted peptides and small molecules as potential COVID-19 therapeutics. *Adv. Pharmacol. Pharm. Sci.* 2021:1828792. doi: 10.1155/2021/1828792
- Collaborators, C.-E. M. (2022). Estimating excess mortality due to the COVID-19 pandemic: A systematic analysis of COVID-19-related mortality, 2020–21. *Lancet* 399, 1513–1536. doi: 10.1016/S0140-6736(21)02796-3
- Garrett, M. E., Galloway, J., Chu, H. Y., Itell, H. L., Stoddard, C. I., Wolf, C. R., et al. (2021). High-resolution profiling of pathways of escape for SARS-CoV-2 spike-binding antibodies. *Cell* 184, 2927–2938.e11. doi: 10.1016/j.cell.2021.04.045
- Gordon, D. E., Jang, G. M., Bouhaddou, M., Xu, J., Obernier, K., White, K. M., et al. (2020). A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* 583, 459–468. doi: 10.1038/s41586-020-2286-9
- Greaney, A. J., Starr, T. N., Gilchuk, P., Zost, S. J., Binshtein, E., Loes, A. N., et al. (2021). Complete mapping of mutations to the SARS-CoV-2 Spike receptor-binding domain that escape antibody recognition. *Cell Host Microbe* 29, 44–57.e9. doi: 10.1016/j.chom.2020.11.007
- Hall, P. R., Hjelle, B., Njus, H., Ye, C., Bondu-Hawkins, V., Brown, D. C., et al. (2009). Phage display selection of cyclic peptides that inhibit Andes virus infection. *J. Virol.* 83, 8965–8969. doi: 10.1128/JVI.00606-09
- Hoffmann, M., Kleine-Weber, H., Schroeder, S., Kruger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 181, 271–280.e8. doi: 10.1016/j.cell.2020.02.052
- Huang, J. X., Bishop-Hurley, S. L., and Cooper, M. A. (2012). Development of anti-infectives using phage display: Biological agents against bacteria, viruses, and parasites. *Antimicrob. Agents Chemother.* 56, 4569–4582. doi: 10.1128/AAC.00567-12
- Jawad, B., Adhikari, P., Podgornik, R., and Ching, W. Y. (2021). Key interacting residues between RBD of SARS-CoV-2 and ACE2 receptor: Combination of molecular dynamics simulation and density functional calculation. *J. Chem. Inf. Model.* 61, 4425–4441. doi: 10.1021/acs.jcim.1c00560
- Labriola, J. M., Miersch, S., Chen, G., Chen, C., Pavlenko, A., Saberianfar, R., et al. (2022). Peptide-Antibody Fusions Engineered by Phage Display Exhibit an Ultrapotent and Broad Neutralization of SARS-CoV-2 Variants. *ACS Chem. Biol.* 17, 1978–1988. doi: 10.1021/acscchembio.2c00411
- Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., et al. (2020). Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature* 581, 215–220. doi: 10.1038/s41586-020-2180-5
- Lipsitch, M., and Dean, N. E. (2020). Understanding COVID-19 vaccine efficacy. *Science* 370, 763–765. doi: 10.1126/science.abe5938
- Ly, Z., Deng, Y. Q., Ye, Q., Cao, L., Sun, C. Y., Fan, C., et al. (2020). Structural basis for neutralization of SARS-CoV-2 and SARS-CoV by a potent therapeutic antibody. *Science* 369, 1505–1509. doi: 10.1126/science.abc5881
- Matthay, M. A., and Luetkemeyer, A. F. (2021). IL-6 receptor antagonist therapy for patients hospitalized for covid-19: Who, when, and how? *JAMA* 326, 483–485. doi: 10.1001/jama.2021.11121
- Mobarak, A. M., Miguel, E., Abaluck, J., Ahuja, A., Alsan, M., Banerjee, A., et al. (2022). End COVID-19 in low- and middle-income countries. *Science* 375, 1105–1110. doi: 10.1126/science.abo4089
- Murthy, S., and Lee, T. C. (2021). IL-6 blockade for COVID-19: A global scientific call to arms. *Lancet Respir. Med.* 9, 438–440. doi: 10.1016/S2213-2600(21)00127-2
- Noy-Porat, T., Makdasi, E., Alcalay, R., Mechaly, A., Levy, Y., Bercovich-Kinori, A., et al. (2020). A panel of human neutralizing mAbs targeting SARS-CoV-2 spike at multiple epitopes. *Nat. Commun.* 11:4303. doi: 10.1038/s41467-020-18159-4
- Petrenko, V. A., Gillespie, J. W., De Plano, L. M., and Shokhen, M. A. (2022). Phage-displayed mimotopes of SARS-CoV-2 spike protein targeted to authentic and alternative cellular receptors. *Viruses* 14:384. doi: 10.3390/v14020384
- Pomplun, S. (2020). Targeting the SARS-CoV-2-spike protein: From antibodies to miniproteins and peptides. *RSC Med. Chem.* 12, 197–202. doi: 10.1039/d0md00385a
- Raghuvamsi, P. V., Tulsian, N. K., Samsudin, F., Qian, X., Purushothaman, K., Yue, G., et al. (2021). SARS-CoV-2 S protein:ACE2 interaction reveals novel allosteric targets. *Elife* 10:e63646. doi: 10.7554/eLife.63646
- Robson, B. (2020). Computers and viral diseases. Preliminary bioinformatics studies on the design of a synthetic vaccine and a preventative peptidomimetic antagonist against the SARS-CoV-2 (2019-nCoV, COVID-19) coronavirus. *Comput. Biol. Med.* 119:103670. doi: 10.1016/j.combiomed.2020.103670
- Roth, K. D. R., Wenzel, E. V., Ruschig, M., Steinke, S., Langreder, N., Heine, P. A., et al. (2021). Developing recombinant antibodies by phage display against infectious diseases and toxins for diagnostics and therapy. *Front. Cell. Infect. Microbiol.* 11:697876. doi: 10.3389/fcimb.2021.697876
- Saladin, A., Rey, J., Thevenet, P., Zacharias, M., Moroy, G., and Tuffery, P. (2014). PEP-SiteFinder: A tool for the blind identification of peptide binding sites on protein surfaces. *Nucleic Acids Res.* 42:W221–W226. doi: 10.1093/nar/gku404
- Sang, L., Guo, X., Shi, J., Hou, S., Fan, H., and Lv, Q. (2021). Characteristics and developments in mesenchymal stem cell therapy for COVID-19: An update. *Stem Cells Int.* 2021:5593584. doi: 10.1155/2021/5593584
- Schafer, A., Martinez, D. R., Won, J. J., Meganck, R. M., Moreira, F. R., Brown, A. J., et al. (2022). Therapeutic treatment with an oral prodrug of the remdesivir parental nucleoside is protective against SARS-CoV-2 pathogenesis in mice. *Sci. Transl. Med.* 14:eabm3410. doi: 10.1126/scitranslmed.abm3410
- Sevenich, M., Thul, E., Lakomek, N. A., Klunemann, T., Schubert, M., Bertoglio, F., et al. (2022). Phage display-derived compounds displace hACE2 from Its complex with SARS-CoV-2 spike protein. *Biomedicines* 10:441. doi: 10.3390/biomedicines10020441
- Shen, Y., Chen, L., Wang, M., Lin, D., Liang, Z., Song, P., et al. (2017). Flagellar hooks and hook protein FlgE participate in host microbe interactions at immunological level. *Sci. Rep.* 7:1433. doi: 10.1038/s41598-017-01619-1
- Sheward, D. J., Kim, C., Ehling, R. A., Pankow, A., Castro Dopico, X., Dyrda, R., et al. (2022). Neutralisation sensitivity of the SARS-CoV-2 omicron (B.1.1.529) variant: A cross-sectional study. *Lancet Infect. Dis.* 22, 813–820. doi: 10.1016/S1473-3099(22)00129-3
- Singh, I. K., Kumari, P., Mittal, P., Kumar, A., Singal, B., Hasan, G. M., et al. (2021). Emerging Therapeutic Approaches to COVID-19. *Curr. Pharm. Des.* 27, 3370–3388. doi: 10.2174/1381612827666210125160703
- Sokullu, E., Gauthier, M. S., and Coulombe, B. (2021). Discovery of Antivirals Using Phage Display. *Viruses* 13:1120. doi: 10.3390/v13061120
- Tiwari, V., Beer, J. C., Sankaranarayanan, N. V., Swanson-Mungerson, M., and Desai, U. R. (2020). Discovering small-molecule therapeutics against SARS-CoV-2. *Drug Discov. Today* 25, 1535–1544. doi: 10.1016/j.drudis.2020.06.017
- Vivekanandhan, K., Shanmugam, P., Barabadi, H., Arumugam, V., Daniel Raj, Daniel Paul Raj, D., et al. (2021). Emerging therapeutic approaches to combat COVID-19: Present status and future perspectives. *Front. Mol. Biosci.* 8:604447. doi: 10.3389/fmolb.2021.604447
- Wang, Q., Zhang, Y., Wu, L., Niu, S., Song, C., Zhang, Z., et al. (2020). Structural and functional basis of SARS-CoV-2 entry by using human ACE2. *Cell* 181, 894–904.e9. doi: 10.1016/j.cell.2020.03.045
- Wang, T., Shen, Y., Li, Y., Wang, B., Wang, B., Wu, D., et al. (2019). Ectopic ATP synthase beta subunit proteins on human leukemia cell surface interact with platelets by binding glycoprotein IIb. *Haematologica* 104:e364–e368. doi: 10.3324/haematol.2019.216390
- Wang, Y., Geer, L. Y., Chappey, C., Kans, J. A., and Bryant, S. H. (2000). Cn3D: Sequence and structure views for Entrez. *Trends Biochem. Sci.* 25, 300–302. doi: 10.1016/S0968-0004(00)01561-9
- Wei, J., Hameed, M., Wang, X., Zhang, J., Guo, S., Anwar, M. N., et al. (2020). Antiviral activity of phage display-selected peptides against Japanese encephalitis virus infection in vitro and in vivo. *Antiviral Res.* 174:104673. doi: 10.1016/j.antiviral.2019.104673

Wrobel, A. G., Benton, D. J., Xu, P., Roustan, C., Martin, S. R., Rosenthal, P. B., et al. (2020). SARS-CoV-2 and bat RaTG13 spike glycoprotein structures inform on virus evolution and furin-cleavage effects. *Nat. Struct. Mol. Biol.* 27, 763–767.

Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., and Zhou, Q. (2020). Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. *Science* 367, 1444–1448. doi: 10.1126/science.abb2762

Yang, F., Liu, L., Neuenschwander, P. F., Idell, S., Vankayalapati, R., Jain, K. G., et al. (2022). Phage display-derived peptide for the specific binding of SARS-CoV-2. *ACS Omega* 7, 3203–3211. doi: 10.1021/acsomega.1c04873

Zhang, G., Pomplun, S., Loftis, A. R., Tan, X., Loas, A., and Pentelute, B. L. (2020). Investigation of ACE2 N-terminal fragments binding to SARS-CoV-2 Spike RBD. *bioRxiv* [Preprint]. doi: 10.1101/2020.03.19.999318

Zhang, Y., Wang, S., Wu, Y., Hou, W., Yuan, L., Shen, C., et al. (2021). Virus-Free and live-cell visualizing SARS-CoV-2 cell entry for studies of neutralizing antibodies and compound inhibitors. *Small Methods* 5:2001031. doi: 10.1002/smt.202001031

Zhao, G., Li, S., Zhao, W., He, K., Xi, H., Li, W., et al. (2012). Phage display against corneal epithelial cells produced bioactive peptides that inhibit *Aspergillus* adhesion to the corneas. *PLoS One* 7:e33578. doi: 10.1371/journal.pone.0033578

Zhao, S., Zhang, H., Yang, X., Zhang, H., Chen, Y., Zhan, Y., et al. (2021). Identification of potent human neutralizing antibodies against SARS-CoV-2 implications for development of therapeutics and prophylactics. *Nat. Commun.* 12:4887. doi: 10.1038/s41467-021-25153-x



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Systematic benchmarking of nanopore Q20+ kit in SARS-CoV-2 whole genome sequencing

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Whole genome sequencing provides rapid insight into key information about the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), such as virus typing and key mutation site, and this information is important for precise prevention, control and tracing of coronavirus disease 2019 (COVID-19) outbreak in conjunction with the epidemiological information of the case. Nanopore sequencing is widely used around the world for its short sample-to-result time, simple experimental operation and long sequencing reads. However, because nanopore sequencing is a relatively new sequencing technology, many researchers still have doubts about its accuracy. The combination of the newly launched nanopore sequencing Q20+ kit (LSK112) and flow cell R10.4 is a qualitative improvement over the accuracy of the previous kits. In this study, we firstly used LSK112 kit with flow cell R10.4 to sequence the SARS-CoV-2 whole genome, and summarized the sequencing results of the combination of LSK112 kit and flow cell R10.4 for the 1200bp amplicons of SARS-CoV-2. We found that the proportion of sequences with an accuracy of more than 99% reached 30.1%, and the average sequence accuracy reached 98.34%, while the results of the original combination of LSK109 kit and flow cell R9.4.1 were 0.61% and 96.52%, respectively. The mutation site analysis showed that it was completely consistent with the final consensus sequence of next generation sequencing (NGS). The results showed that the combination of LSK112 kit and flow cell R10.4 allowed rapid whole-genome sequencing of SARS-CoV-2 without the need for verification of NGS.

KEYWORDS

nanopore sequencing, Q20+ kit, SARS-CoV-2, coronavirus, accuracy

Introduction

Coronavirus disease 2019 (COVID-19), which occurs at the end of 2019, is a very serious infectious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and poses a huge public health challenge to the world (Wu et al., 2020). SARS-CoV-2 is an enveloped virus with a positive-sense, single-stranded RNA genome of ~30 kb. The COVID-19 epidemic is currently occurring in almost every country in the world, with over 520 million cases of infection and over 6.25 million deaths as of the end of May 2022. Because of the highly transmissible nature of SARS-CoV-2 and the easy mutation nature of single-stranded RNA viruses, SARS-CoV-2 is constantly mutating and undergoing immune escape (Garcia-Beltran et al., 2021; Harvey et al., 2021).

Currently, the world health organization (WHO) has defined five specific Variants of Concern (VOCs¹), in particular B.1.617.2 (Delta) and B.1.1.529 (Omicron). Delta was the key strain that caused the early COVID-19 epidemic, with the D614G mutation contributing to the rapid spread of SARS-CoV-2 (Korber et al., 2020; Jackson et al., 2021; Plante et al., 2021). Omicron has been responsible for the rapid re-transmission of COVID-19 epidemic since 2021, and the K417N mutation caused the immune escape of Omicron strain against SARS-CoV-2 vaccine (Cao et al., 2021, 2022; Li et al., 2021). In fact, more than 90% of the sites of SARS-CoV-2 genome have been mutated. According to the PANGOLIN SARS-CoV-2 typing system,² hundreds of SARS-CoV-2 genotypes have appeared, and only whole genome sequencing can detect all genotypes at once.

Nanopore sequencing is a technology with many advantages such as simplicity, real-time rapid sequencing, and long reads. It has been used to sequence pathogens in several previous outbreaks, such as Ebola, Zika, and Lassa virus (Hoenen, 2016; Quick et al., 2017; Kafetzopoulou et al., 2019). The earliest ARTIC sequencing protocol for sequencing SARS-CoV-2 was also derived from the nanopore sequencing protocol of the Zika virus (Quick et al., 2017). At present, nanopore sequencing is widely used for the whole genome sequencing of SARS-CoV-2. A large number of SARS-CoV-2 sequences in databases such as Global Initiative of Sharing All Influenza Data (GISAID) and National Center for Biotechnology Information (NCBI) are sequenced by nanopore sequencing. In addition, the nanopore-based direct RNA sequencing is also used to study the subgenomic structure and RNA modification of SARS-CoV-2, providing scientists with the complete transcriptome structure of SARS-CoV-2 (Davidson et al., 2020; Kim et al., 2020; Chang et al., 2021; Wang et al., 2021; Ugolini et al., 2022).

Although nanopore sequencing has excellent performance in SARS-CoV-2 sequencing, with a sensitivity and specificity of more than 99% based on a sequencing depth greater than 60x, compared with the next generation sequencing (NGS) technologies represented by Illumina (Bull et al., 2020). There are still scientists who are concerned about the accuracy of nanopore sequencing and still perform NGS to verify the nanopore sequencing results when studying the transmission relationship between cases. Recently, Oxford Nanopore Technologies (ONT) launched Q20+ kit (LSK112), which claimed to produce duplex data (~Q30) and achieve simplex accuracies of over 99%, enhanced high-precision consensus sequence as well as mutation identification, when combined with the latest flow cell R10.4. In this study, we firstly utilized Q20+ kit in combination with flow cell R10.4 for whole-genome sequencing of SARS-CoV-2, and we compared the sequencing results with the results of NGS and the combination of the previous nanopore sequencing kit LSK109 and flow cell R9.4.1 to observe whether Q20+ kit showed significant improvement in the accuracy of SARS-CoV-2 whole-genome sequencing. We found that the SARS-CoV-2 consensus sequences of the combination of Q20+ kit and flow cell R10.4 were completely consistent with the sequences generated by the NGS, with a very significant improvement in single-molecule accuracy, particularly for the homopolymer region where nanopore sequencing was most likely to be incorrect in the past. Comparing with the old kit LSK109 with R9.4, the new Q20+ kit (LSK112) with flow cell R10.4 improved the average sequence accuracy in sequencing SARS-CoV-2 96.25% to 98.34% and the proportion of sequences with an accuracy of more than 99 to 30.1% from 0.61%, which greatly reduced the background noise that may interfere with variants calling.

Materials and methods

Sample preparation

A total of 15 samples were selected in this study, all of which have been sequenced by NGS, and all of them were provided by the Institutes of Pathogenic Microbiology of Jiangsu Provincial Center for Disease Control and Prevention. The sample information is shown in Table 1. According to the operating instruction of the automatic nucleic acid extractor, RNA was extracted by using accompanying nucleic acid extraction kits. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) of RNA was performed using COVID-19 Coronavirus Real Time PCR Kit (bioPerfectus technologies and Daan Gene, China) in CFX Connect Real-Time PCR Detection System (96-Well 0.2 mL Block) (Bio-Rad, American).

1 <https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/>

2 https://cov-lineages.org/lineage_list.html

TABLE 1 The information of 15 samples.

Strain number	VOCs	Ct value(ORF1ab/N)	Sample type	Source	NGS instrument
20216080-9	Delta	18.8/15.6	Throat Swab	Nanjing	Illumina miseq
20216110-27	Delta	21/24	Throat Swab	Yangzhou	Ion Torrent Genexus
20216085-26	Delta	26/24.5	Throat Swab	Yangzhou	MGISEQ-2000
20216080-10	Delta	23.1/20	Throat Swab	Nanjing	Illumina miseq
20216097-3	Delta	22/21	Throat Swab	Yangzhou	Illumina miseq
20216097-25	Delta	25/23	Throat Swab	Yangzhou	Illumina miseq
20216085-11	Delta	24/25	Throat Swab	Yangzhou	MGISEQ-2000
20216085-30	Delta	25/25	Throat Swab	Yangzhou	MGISEQ-2000, Ion S5 XL
20216085-31	Delta	27/26	Throat Swab	Yangzhou	MGISEQ-2000
2022030-11	Omicron	17/20	Throat Swab	Suzhou	Illumina NextSeq 2000
2022030-8	Omicron	22/23	Throat Swab	Suzhou	Illumina NextSeq 2000
2022030-7	Omicron	18/20	Throat Swab	Suzhou	Illumina NextSeq 2000
2022071-1	Omicron	20/21	Throat Swab	Nantong	MGI DNBSEQ-E5
2022071-2	Omicron	15/16	Throat Swab	Nantong	MGI DNBSEQ-E5
2022071-3	Omicron	14/16	Throat Swab	Nantong	MGI DNBSEQ-E5

Reverse-transcriptase polymerase chain reaction

Short Fragment (400bp) Target Capture Kit and Long Fragment (1200bp) Target Capture Kit for SARS-CoV-2 Whole Genome (Baiyi Technology Co., Ltd., China, BK-WCoV024TS and BK-WCoV024ITS) were selected to reverse transcribe the extracted RNA and amplify the SARS-CoV-2 whole genome. The top three samples in [Table 1](#) were amplified using the short fragment target capture kit and the other samples were amplified using the long fragment target capture kit. RNA was reverse transcribed into cDNA with reverse transcriptase and random primers, and the cDNA was amplified by multiple polymerase chain reaction (Multiple PCR) using primer pool 1 and primer pool 2 provided in the kit, respectively. The conditions of Multiple PCR: 98°C for 30s followed by 25 cycles of 98°C for 15s, 65°C for 5min, and 72°C for 2min. The Multiplex PCR products were purified by AMPure XP beads (Beckman coulter, United States) and then quantified using a Qubit 2.0 Fluorometer and Qubit dsDNA BR Assay kit (Thermo Fisher Scientific, Q32850).

Next generation sequencing

Illumina sequencing was performed using Nextera XT DNA Library Preparation Kit (Illumina, FC-131-1096) for library building and sequencing on Miseq or NextSeq 2000 (300 cycles for 150bp paired end read type). BGI sequencing was performed using ATOplex RNA Library Prep Set for library construction and sequencing on MGISEQ-2000, and using DNBelab-D4RS Digital Sample Preparation System and accompanying kits for library building and sequencing on

DNBSEQ-E5. Both sequencers of Applied Biosystems were automatic operating systems, using matching kits and materials for library building and sequencing.

Nanopore sequencing

Libraries were built according to the manufacturer's protocols of Sequencing Ligation Kit (ONT, SQK-LSK109 or SQK-LSK112) and Native Barcoding Kit (ONT, EXP-NBD104) or Native Barcoding Kit 24 (ONT, SQK-NBD112.24), respectively. After quantitative dilution, the libraries were loaded onto flow cell R9.4.1 (ONT, FLO-MIN106D) and flow cell R10.4 (ONT, FLO-MIN112), respectively, and were sequenced on GridION X5. The run was terminated after achieving sufficient sequencing data and the flow cell was washed using flow cell Wash Kit (ONT, EXP-WSH004), allowing it to be reused in subsequent sequencings.

Data analysis

The fast5 electrical signal files were obtained from the nanopore sequencing down-machine data, and then the fast5 data were converted to standard fastq files using Guppy (v 6.0.1³) to study the effect of different base-calling strategies on the accuracy of the nanopore sequencing data. We used three modes from the configuration file – config in guppy: dna_r9.4.1_450bps_fast.cfg, dna_r9.4.1_450bps_hac.cfg and dna_r9.4.1_450bps_sup.cfg, corresponding to the conversion

³ https://community.nanoporetech.com/docs/prepare/library_prep_protocols/Guppy-protocol/v/gpb_2003_v1_revai_14dec2018

modes: fast, hac and sup mode, respectively. The average Q value for each reads was counted using Seqkit tool (v.2.2.0⁴) (Shen et al., 2016) and the accuracy density curves were plotted based on the obtained Q values using the ggplot2 package in R language (v 4.1.3⁵). When analyzing the homopolymer accuracy of the SARS-CoV-2, we used Seqkit tool to obtain all homopolymer positions and corresponding sequences on the reference genome Wuhan-Hu-1, and then used Seqkit tool to count the number of different homopolymers matched to the sample data, using the ggplot2 package for line plotting.

The data analysis process was carried out using BAIYI MicroGeno Platform (v 4.0⁶, Hangzhou Baiyi Technology Co., Ltd.). The raw data were first quality controlled using NanoPlot (v.1.30.0⁷) (Coster et al., 2018) and then the low quality and sequences less than 200bp were filtered using Filtlong (v.0.2.0⁸) based on the quality control results. The filtered clean data were compared with the reference genome Wuhan-Hu-1. When processing the NGS data, we used BWA (v 0.7.17⁹) (Li, 2018) for comparison and minimap2 (v 2.22¹⁰) (Li, 2018) when processing the nanopore data. Mutation site detection was performed using freebayes (v 1.1.2¹¹), with reference assembly of the SARS-CoV-2 whole genome sequence using bcftools (v 1.12¹²) (Danecek et al., 2021). We calculated the Shannon entropy of variant sites in nanopore sequencing and NGS to analyze the accuracy of sequenced sites (formula of Shannon entropy: $H(x) = -\sum_x P(x) \log_2[P(x)]$), using the ggplot2 package for line plotting.

Results

The basic sequencing data

Fifteen samples were selected for SARS-CoV-2 whole genome sequencing by NGS and nanopore sequencing, including 9 Delta samples and 6 Omicron samples. Short Fragment (400bp) Target Capture Kit and Long Fragment (1200bp) Target Capture Kit for SARS-CoV-2 Whole Genome were selected to reverse transcribe the extracted RNA and amplify the SARS-CoV-2 whole genome. Among nine Delta samples, three samples were amplified by the 400bp capture kit, six samples were amplified by the 1200bp capture kit. Six Omicron samples were amplified by the 1200bp capture kit. Then, fifteen samples were sequenced by NGS, method A

(using LSK112 kit with flow cell R10.4) and method B (using LSK109 kit with flow cell R9.4.1) respectively (the details of amplification and sequencing protocol are given in the methods and materials). The time from a sample to sequencing result was 21–29 h for NGS and 7–8 h for nanopore sequencing. Statistical analysis of the sequencing results showed that the sequencing depth of each sequencing method was greater than 230, and the whole genome sequences of 15 samples were basically obtained, with most of sequences coverage above 99% (Table 2). The amount of the sequencing data is showed in Table 3.

Analysis of accuracy

The effect of different base calling strategies on the accuracy of nanopore sequencing

Considering that nanopore sequencing is a technology based on electrical signal sequencing, different base calling strategies can be chosen during the conversion of electrical signal fast5 data into fastq data. Guppy, providing three base calling strategies (fast, hac, and sup modes), was utilized to analyze the effect of different data conversion modes on sequence accuracy. The density distribution of sequence accuracy showed that the sup mode had higher accuracy for both method A and method B (Figures 1A,B). The fast and hac modes were not suitable for analyzing Q20 data, with the fast mode being more obvious (Figure 1B). Therefore, we consistently chose the sequence accuracy in the sup mode to evaluate both nanopore sequencing methods. It could be found that the sequence accuracy of method A was significantly better than that of method B for both the 400bp capture kit and the 1200bp capture kit. This illustrated that regardless of the length of the sequenced fragments, Q20+ kit had a great improvement in sequence accuracy, reaching an accuracy of 99% (Figures 1C,D).

The effect of different amplicon lengths on the accuracy of nanopore sequencing

In method B, the average sequencing fragment lengths obtained by using the 400bp capture kit and the 1200bp capture kit were around 376bp and 1058bp, respectively, with no significant difference in accuracy. In contrast, in method A, the average reads accuracy of the 1200bp amplicon improved significantly compared to that of the 400bp amplicon, from 96.5 to 97.5%, and the average proportion of data above Q20 rose from 23 to 28.8% (Table 4). This led us to further consider whether different amplicon lengths had an effect on nanopore sequencing accuracy? As could be seen in the single-base accuracy analysis, the average single-base Quality value (Q value) of the 400bp amplicon was indeed lower than that of the 1200bp amplicon (Supplementary Figure 1). Interestingly, we also found that in method A, the single-base Q values for the first 20–30 bp was very low (Figure 2A), possibly due to an unstable electrical signal generated when the DNA fragment just passed through the nanopore. However, the first 20–30 bp

⁴ <https://github.com/shenwei356/seqkit>

⁵ <https://www.r-project.org/>

⁶ <http://www.baiyi-tech.cn/>

⁷ <https://github.com/wdecoster/NanoPlot>

⁸ <https://github.com/rrwick/Filtlong>

⁹ <https://github.com/lh3/bwa>

¹⁰ <https://github.com/lh3/minimap2>

¹¹ <https://github.com/freebayes/freebayes>

¹² <https://github.com/samtools/bcftools>

TABLE 2 Information on whole genome sequencing data from 15 samples.

Strain number	Sequencing depth			Coverage			Average fragment length	
	NGS	LSK109 +R9.4.1	LSK112 +R10.4	NGS	LSK109 +R9.4.1	LSK112 +R10.4	LSK109 +R9.4.1	LSK112 +R10.4
20216080-9	2765	2397	1868	98.90%	99.79%	99.08%	376.155	412.708
20216110-27	16635	1159	545	99.77%	99.78%	99.37%	805.838	881.555
20216085-26	61587	230	434	99.90%	99.61%	99.62%	692.554	801.429
20216085-11	29596	1311	3016	99.90%	99.54%	99.60%	1058.65	1095.62
20216085-30	25627	1669	4137	99.90%	99.53%	99.60%	1064.24	1096.96
20216085-31	39297	1412	3689	99.90%	99.60%	99.61%	1059.76	1091.5
20216080-10	887	3161	873	97.78%	99.60%	98.93%	895.279	518.09
20216097-3	4661	1050	3288	99.69%	99.60%	99.60%	965.401	984.236
20216097-25	1818	2426	3274	99.54%	99.60%	99.60%	992.775	1052.37
2022030-11	19799	1737	5016	99.32%	97.90%	99.28%	1085.94	1111.35
2022030-8	19209	1299	3905	99.34%	96.26%	98.19%	1037.88	1063.16
2022030-7	28698	1615	4823	86.77%	98.29%	98.33%	1083.78	1103.44
2022071-1	3109	7889	3068	99.19%	99.17%	98.60%	1062.55	1082.7
2022071-2	6051	3986	3554	99.54%	98.07%	98.07%	1102.29	1113.51
2022071-3	6231	4565	3899	99.31%	98.61%	98.35%	1084.41	1102.2

was the adapter sequence, not the true amplified fragment sequence. With this in mind, we further performed statistics on the accuracy after cutting the adapter sequence and found that was 98.27% (Figure 2B).

The effect of duplex data on the accuracy of nanopore sequencing

Currently, for DNA sequencing, ONT only supports the 1D method, but LSK112 kit is supported by the 2D method. Compared to method B, some sequences in method A are double stranded through the nanopore. In the sequences with positive and negative strand through the nanopores, we used Guppy (guppy_basecaller_duplex) with duplex tools (v 0.2.9¹³) for method A to analyze the extracted duplex data. The statistical analysis revealed an average Q value of 26.1 for the duplex data, corresponding to an accuracy of 99.75453%, and duplex data accounted 3.33% of the sequencing data of method A (Figure 3), which was relatively in line with the 1-10% range given by ONT. The results showed that duplex data was particularly effective in improving the accuracy of nanopore sequencing.

Analysis of single nucleotide polymorphism and insertion-deletion

Taking the SARS-CoV-2 genome Wuhan-Hu-1 (GenBank accession number: MN908947.3) as the reference genome, we analyzed the mutation site for each sample. It could be found

that method B had a significant increase of mutation sites in the fast mode, 30.97% of which were caused by homopolymer variation, and generated 2.92% false positive site heterozygosity in addition (Supplementary Table 1). This also confirmed that the fast mode was not suitable for accurate variants calling, meanwhile the fast mode can run faster than sup mode with lower hardware requirement, which may illustrate why some scientists still have doubt about the accuracy of nanopore sequencing technology even with the rapid development of

TABLE 3 The amount of the sequencing data.

Sample	Q20		R9.4	
	Fast	Hac	Fast	Hac
20216080-9	130 Mb	134.9 Mb	104.3 Mb	108.9 Mb
20216110-27	94.8 Mb	100.3 Mb	243.3 Mb	250.6 Mb
20216085-26	105.6 Mb	114.6 Mb	78.1 Mb	80.1 Mb
20216085-11	107.8 Mb	113.8 Mb	48.8 Mb	50 Mb
20216085-30	142.9 Mb	154 Mb	61.3 Mb	62.9 Mb
20216085-31	134.1 Mb	140.3 Mb	52.9 Mb	54.3 Mb
20216080-10	105.8 Mb	111.9 Mb	122 Mb	126.5 Mb
20216097-3	124.8 Mb	128.7 Mb	39.6 Mb	40.9 Mb
20216097-25	119.8 Mb	124.3 Mb	89.4 Mb	92.6 Mb
2022030-11	169.3 Mb	180 Mb	60.7 Mb	62.1 Mb
2022030-8	135.2 Mb	144.4 Mb	47.2 Mb	48.2 Mb
2022030-7	162.7 Mb	172.3 Mb	56.9 Mb	58.2 Mb
2022071-1	106.1 Mb	112 Mb	280.2 Mb	287.6 Mb
2022071-2	120.9 Mb	128.4 Mb	140.9 Mb	144.9 Mb
2022071-3	134.2 Mb	141.9 Mb	161.9 Mb	166.1 Mb

¹³ <https://github.com/nanoporetech/duplex-tools/>

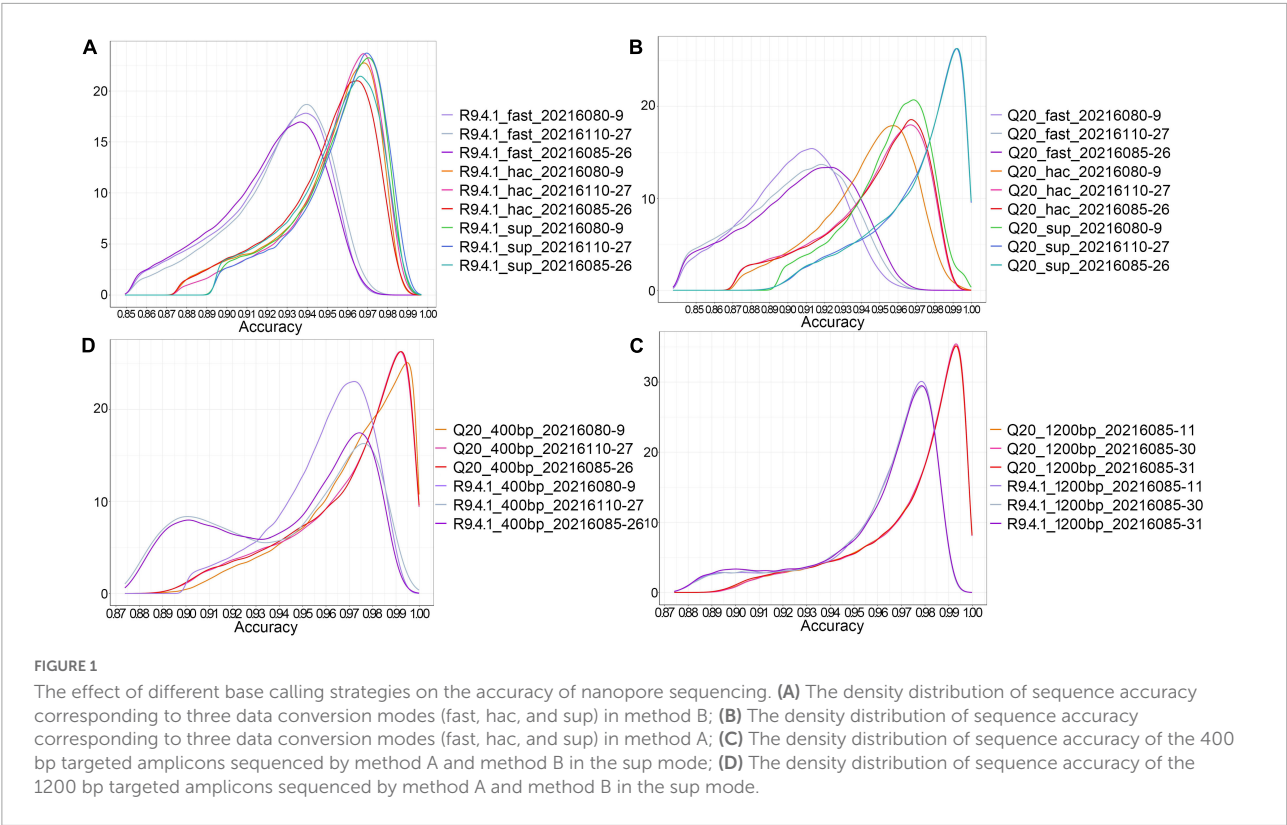


TABLE 4 The accuracy of whole genome sequencing of 15 samples.

Strain number	Targeted capture fragment length	Sequence accuracy			De-adapter sequence accuracy		Q20+ data	
		NGS	LSK109 +R9.4.1	LSK112 +R10.4	LSK109 +R9.4.1	LSK112 +R10.4	LSK109 +R9.4.1	LSK112 +R10.4
20216080-9	400bp	99.98%	95.32%	96.02%	96.37%	98.14%	1.14%	23.76%
20216110-27	400bp	99.68%	95.32%	96.61%	95.73%	98.00%	0.57%	23.76%
20216085-26	400bp	99.75%	95.43%	96.53%	96.11%	98.00%	1.43%	23.51%
20216085-11	1200bp	99.70%	96.28%	97.25%	96.69%	98.26%	0.84%	29.16%
20216085-30	1200bp	99.68%	96.20%	97.25%	96.69%	98.30%	0.86%	29.35%
20216085-31	1200bp	99.72%	96.20%	97.31%	96.69%	98.26%	0.88%	29.04%
20216080-10	1200bp	99.98%	95.43%	97.37%	96.20%	98.30%	0.47%	27.08%
20216097-3	1200bp	99.98%	95.53%	96.45%	96.20%	98.71%	0.27%	38.12%
20216097-25	1200bp	99.97%	95.53%	97.66%	96.20%	98.59%	0.31%	33.56%
2022030-11	1200bp	99.94%	96.37%	97.60%	96.76%	98.30%	0.67%	29.60%
2022030-8	1200bp	99.94%	96.37%	97.30%	96.70%	98.20%	0.86%	28.60%
2022030-7	1200bp	99.94%	96.37%	97.30%	96.70%	98.30%	0.78%	30.10%
2022071-1	1200bp	99.99%	96.02%	97.30%	96.40%	98.20%	0.48%	28.50%
2022071-2	1200bp	99.99%	96.02%	97.30%	96.30%	98.20%	0.44%	28.80%
2022071-3	1200bp	99.99%	96.02%	97.30%	96.40%	98.20%	0.46%	29.20%

accuracy in nanopore sequencing. In the sup mode, method A and method B were completely consistent with the NGS in mutation detection with the consistent site coverage. Intriguingly, in method B, we analyzed eight consecutive T-base position (genomic position 11094) in the sup mode, and found that 7 out of 15 samples were identified to be heterozygous with a deletion of one T base which proportion was greater than 50%, and the other 8 samples had low heterozygosity

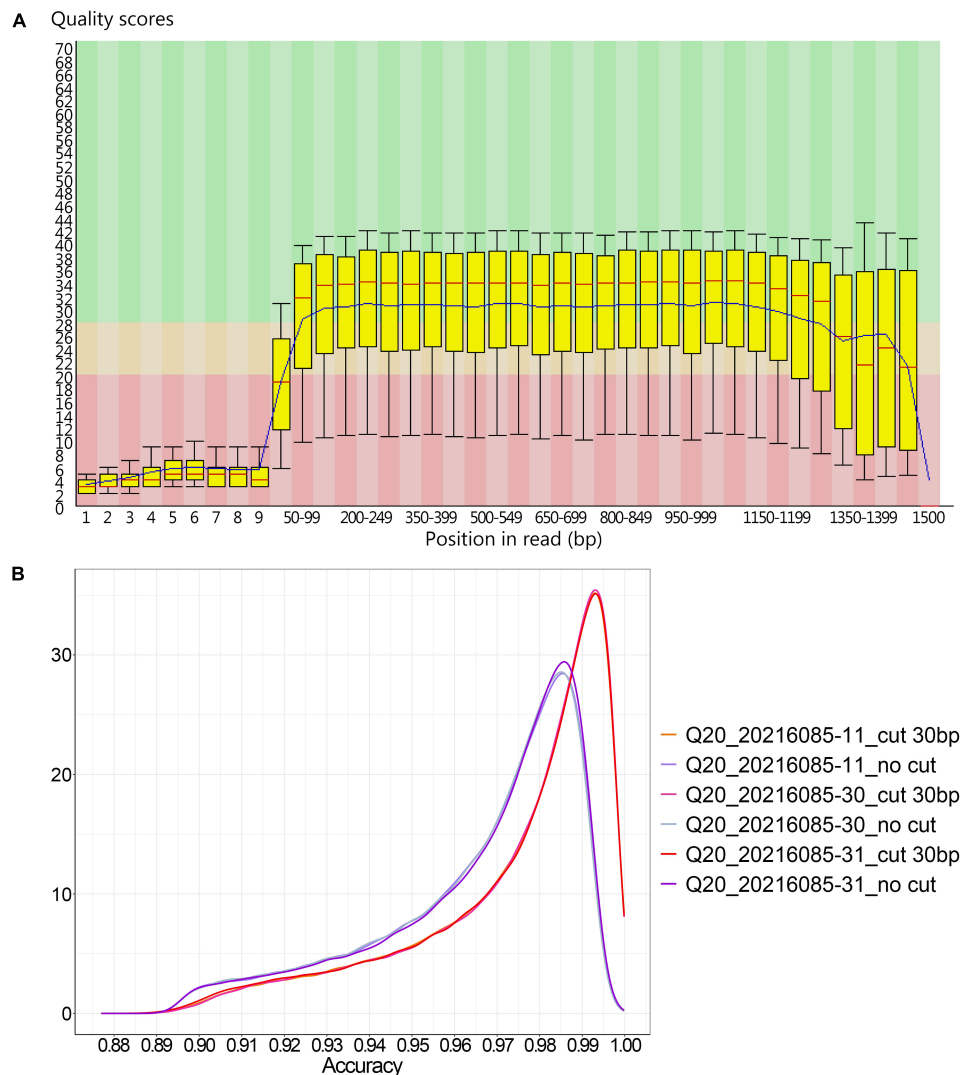


FIGURE 2

The accuracy of 1200 bp amplicons sequenced by method A. (A) The single-base Q value corresponding to the base position in the 1200 bp amplified product from method A; (B) The density distribution of sequence accuracy of the 1200 bp amplified product with and without the 30 bp adapter sequence cut in method A.

deletion variation. This false positive situation was well resolved by method A in the sup mode, with none of the 15 samples generating false positive at this position.

Analysis of homopolymer

We conducted a genome-wide scan of the SARS-CoV-2 whole genome, which had multiple regions of homopolymer, including a T-base homopolymerized region with a length up to 8, in addition to the 3' UTR. In method B, the percentage of homopolymer identification accuracy gradually decreased as the length of homopolymer increased (Figure 4A). This limited the application of this sequencing method to whole-genome sequencing of SARS-CoV-2, as it could easily cause

frame shift mutation. Method A showed high recognition accuracy for homopolymer, and still had excellent recognition accuracy for a T-base homopolymer region with a length of 8 (Figure 4B). Moreover, the recognition accuracy of homopolymer was significantly negatively correlated with the length of homopolymer, and had no significant correlation with the four base types.

Analysis of data quantity

We analyzed the data quantity generated by flow cell R10.4 and flow cell R9.4.1 over time and could see that flow cell R10.4 generated approximately 230 Mb data at 120 min, which is a significant difference compared to the

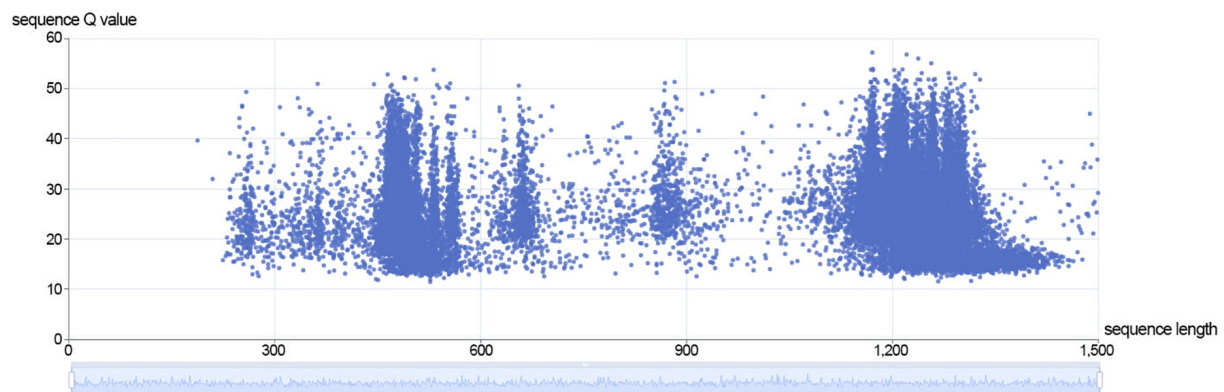


FIGURE 3
The distribution of sequence length and sequence Q value of duplex data in method A.

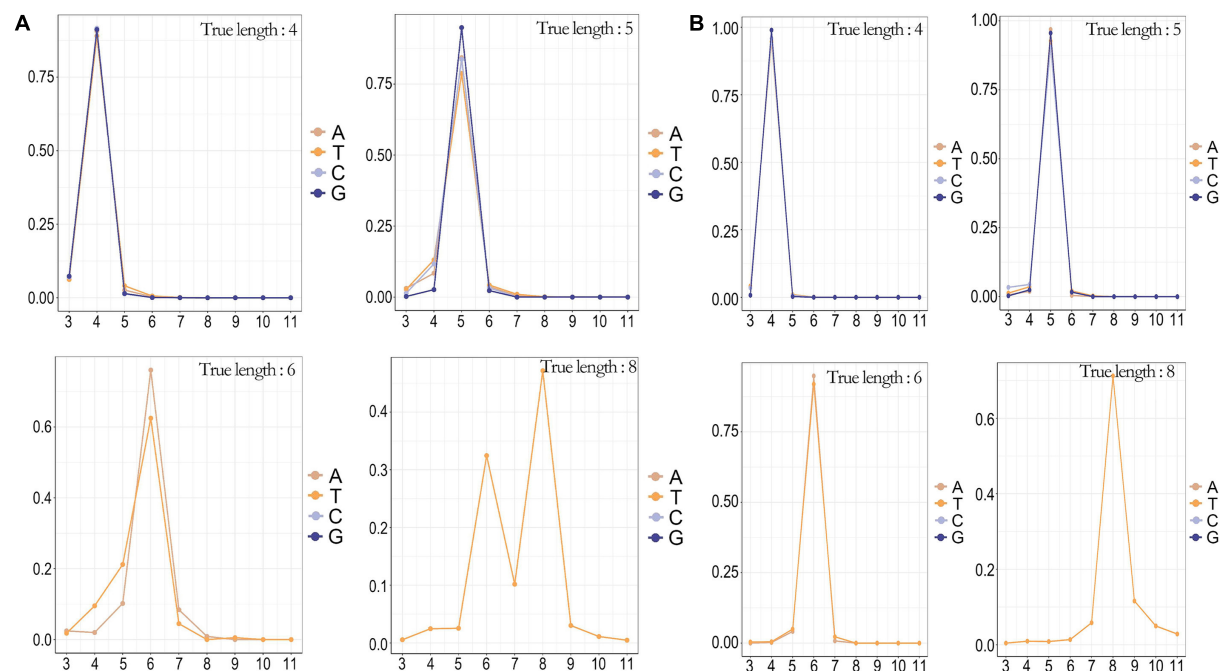


FIGURE 4
The counter density statistic for the detection of different homopolymers on the whole genome of SARS-CoV-2. (A) The counter density plot of method B in the sup mode for the detection of homopolymer with lengths of 4, 5, 6, and 8; (B) The counter density plot of method A in the sup mode for the detection of homopolymer with lengths of 4, 5, 6, and 8.

625.4 Mb data generated by flow cell R9.4.1 (Figure 5). It was a significant positive correlation with the speed through the nanopore of sequences on both flow cells. The sequencing speed of flow cell R9.4.1 is 400~450bp per second, while the sequencing speed of flow cell R10.4 is reduced to 200bp per second. As could be seen from the above analysis, method A significantly improved sequencing accuracy at the sacrifice of its data output. However, during the whole genome sequencing of SARS-CoV-2, the data output was often excessive, so the combination of LSK112 kit and flow cell R10.4 could

still meet the needs of the whole genome sequencing of SARS-CoV-2.

Discussion

Whole-genome sequencing is the best way to detect SARS-CoV-2 due to its rapidly mutating nature. On account of the advantages of rapid, simple and low-cost sequencing nature, nanopore sequencing technologies is widely used

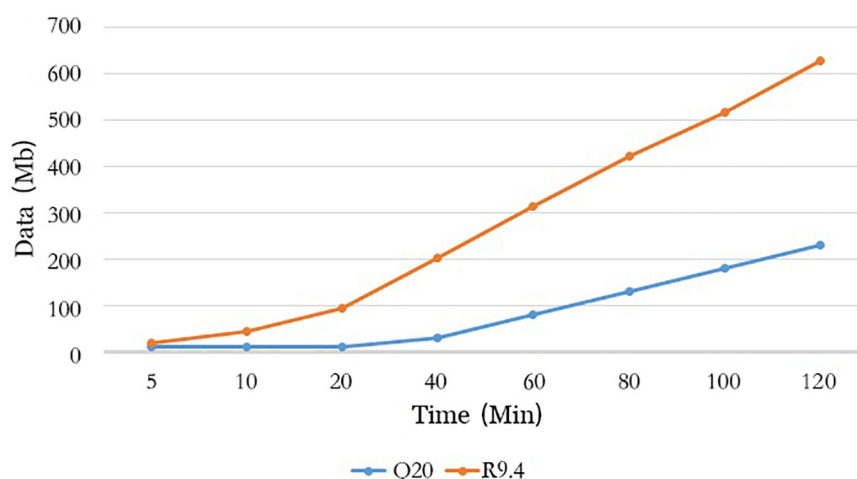


FIGURE 5

Comparison of method A with method B for data output over time.

to obtain the whole genome sequence of viruses, such as Ebola, Zika, and Lassa viruses (Hoenen, 2016; Quick et al., 2017; Kafetzopoulou et al., 2019). Actually, how to make the accuracy of nanopore sequencing technology comparable to NGS or even sanger sequencing is still the most important issue to the users. Excitingly, the emergency of nanopore Q20+ kit (LSK112 kit with flow cell R10.4) may help us to sequence the SARS-CoV-2 genome without verification from NGS or sanger sequencing, and its sequencing accuracy has been verified in bacterial, fungal, human and plants (Sereika et al., 2021; Keraite et al., 2022; Sanderson et al., 2022). This study is the first benchmark test of nanopore Q20+ sequencing in SARS-CoV-2 and viruses. Excitingly, LSK114 kit with flow cell R10.4.1 released in London Calling 2022 not only maintains the accuracy of 99%, but also improves the sequencing yield to the same level or even more as LSK109 kit with flow cell R9.4.1.

Regardless of method A or method B, there were significant differences in accuracy among three base calling modes, with the sequence accuracy decreasing significantly in the fast mode, especially in the homopolymer region. Highest accuracy was achieved by two sequencing methods in the sup mode, with some sample sequences in method A reaching an accuracy of over 99%. Method A was more accurate than method B regardless of the size of the targeted capture fragment. And the longer the fragment, the more accurate it was. Method A had duplex data with an average Q value of 26.1 and an accuracy of 99.75453%, although the percentage of duplex data was small. With the development of nanopore sequencing technology and the increasing proportion of duplex data, nanopore

sequencing is expected to achieve even higher accuracy. It could be observed that LSK112 kit did improve sequencing accuracy compared to LSK109 kit and was more suitable for sequencing long amplicons. The sequencing quality of sequences that initially enter the nanopore is poor, due to the unstable speed of the initial sequence through the nanopore. The overall sequence accuracy is greatly affected when the length of amplicon was short. It could be seen from the results that the accuracy was significantly improved after removing the adapter sequence. Therefore, we need to filter the adapter sequence and short fragment in order to achieve better analysis results in the data processing part.

In the mutation detection, it was evident that method B had a recognition error in the homopolymer region, which led to the eventual problem of frame shift mutation. This problem is even more noticeable on ONT MK1C platform and this weak point was eliminated on ONT GridION platform supporting the sup base calling mode with a huge boost of read-time computing power. Q20+ kit maintained high recognition accuracy in the homopolymer regions of the lengths of 4, 5, 6 and 8, which clearly showed that the Q20+ kit solved the homopolymer accuracy problem well. We compared the consensus sequences sequenced by method A with the consensus sequences from NGS, and the sequences were identical. The homopolymer region has been a high-incidence region with accuracy problems in the previous sequencing kits of nanopore sequencing technology. However, the continuous upgrades of sequencing kits, flow cell and algorithm are solving the shortcoming, especially Q20+ kits, such as LSK112 kit and LSK114 kit, have improved the ability of detecting homopolymers up to length of 10~12. A recent study reported sequencing bacteria genome

with LSK112 kit and flow cell R10.4 has allowed high accuracy in homopolymers regions of length up to 9 (Sereika et al., 2021). It means that LSK112 kit and flow cell R10.4 allow the accurate detection of the largest 8-base homopolymer in SARS-CoV-2 genome.

In conclusion, Q20+ kit was found to be more accurate than previous nanopore sequencing kits, especially for sequencing long amplicons. The improvement in accuracy derived from the increased 5 to 10% of duplex data, and the relatively reduced sequencing speed that resulted in increased homopolymer identification accuracy. However, to ensure high accuracy, the base calling strategy required selecting the sup mode.

At present, Nanopore sequencing is increasingly used for the whole genome sequencing of SARS-CoV-2 due to its advantages of simple, fast and real-time sequencing. The improved accuracy brought by Q20+ kit can play a more accurate and positive role in the prevention and control of epidemics and traceability analysis of SARS-CoV-2.

Data availability statement

The data presented in this study have been submitted to the National Genomics Data Center (<https://ngdc.cnecb.ac.cn/>) with submission number: CRA007743. The generated consensus sequences were submitted with accession numbers: GWHBJYX01000000–GWHBKAG01000000.

Author contributions

JL and XX: methodology establishment, data sorting and analysis, visualization, and writing – original draft. ZM: methodology establishment, data sorting, and writing – review and editing. LW: data sorting and analysis. KZ, XZ, QQ, and YG: resource and writing – review and editing. LM: conceptualization, project administration, supervision, and writing – review and editing. LC: conceptualization, funding acquisition, project administration, validation, supervision, and

writing – review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors XX, LW, and LM were employed by Hangzhou Baiyi Technology Co., Ltd.

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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Supplementary material

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References

- Bull, R. A., Adikari, T. N., Ferguson, J. M., Hammond, J. M., Stevanovski, I., Beukers, A. G., et al. (2020). Analytical validity of nanopore sequencing for rapid SARS-CoV-2 genome analysis. *Nat. Commun.* 11, 6272. doi: 10.1038/s41467-020-20075-6
- Cao, Y., Wang, J., Jian, F., Xiao, T., Song, W., Yisimayi, A., et al. (2022). Omicron escapes the majority of existing SARS-CoV-2 neutralizing antibodies. *Nature* 602, 657–663. doi: 10.1038/s41586-021-04385-3
- Cao, Y., Yisimayi, A., Bai, Y., Huang, W., Li, X., Zhang, Z., et al. (2021). Humoral immune response to circulating SARS-CoV-2 variants elicited by inactivated and RBD-subunit vaccines. *Cell Res.* 31, 732–741. doi: 10.1038/s41422-021-00514-9
- Chang, J. J., Rawlinson, D., Pitt, M. E., Taiaroa, G., Gleeson, J., Zhou, C., et al. (2021). Transcriptional and epi-transcriptional dynamics of SARS-CoV-2 during cellular infection. *Cell Rep.* 35:109108. doi: 10.1016/j.celrep.2021.109108
- Coster, W. D., D'Her, T. S., Schultz, D. T., Cruts, M., and Broeckhoven, C. V. (2018). NanoPack: Visualizing and processing long-read sequencing data. *Bioinformatics* 34, 2666–2669. doi: 10.1093/bioinformatics/bty149
- Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., et al. (2021). Twelve years of SAMtools and BCFtools. *Gigascience* 10:giab008. doi: 10.1093/gigascience/giab008
- Davidson, A. D., Williamson, M. K., Lewis, S., Shoemark, D., Carroll, M. W., Heesom, K. J., et al. (2020). Characterisation of the transcriptome and proteome

- of SARS-CoV-2 reveals a cell passage induced in-frame deletion of the furin-like cleavage site from the spike glycoprotein. *Genome Med.* 12:68. doi: 10.1186/s13073-020-00763-0
- Garcia-Beltran, W. F., Lam, E. C., St Denis, K., Nitido, A. D., Garcia, Z. H., Hauser, B. M., et al. (2021). Multiple SARS-CoV-2 variants escape neutralization by vaccine-induced humoral immunity. *Cell* 184, 2372–2383. doi: 10.1016/j.cell.2021.03.013
- Harvey, W. T., Carabelli, A. M., Jackson, B., Gupta, R. K., Thomson, E. C., Harrison, E. M., et al. (2021). SARS-CoV-2 variants, spike mutations and immune escape. *Nat. Rev. Microbiol.* 19, 409–424. doi: 10.1038/s41579-021-00573-0
- Hoenen, T. (2016). Sequencing of ebola virus genomes using nanopore technology. *Bio Protoc.* 6:e1998. doi: 10.21769/BioProtoc.1998
- Jackson, C. B., Zhang, L., Farzan, M., and Choe, H. (2021). Functional importance of the D614G mutation in the SARS-CoV-2 spike protein. *Biochem. Biophys. Res. Commun.* 538, 108–115. doi: 10.1016/j.bbrc.2020.11.026
- Kafetzopoulou, L. E., Pullan, S. T., Lemey, P., Suchard, M. A., Ehichioya, D. U., Pahlmann, M., et al. (2019). Metagenomic sequencing at the epicenter of the nigerian 2018 lassa fever outbreak. *Science* 363, 74–77. doi: 10.1126/science.aau9343
- Keraite, I., Becker, P., Canevazzi, D., Frias-López, M. C., Dabad, M., Tonda-Hernandez, R., et al. (2022). Novel method for multiplexed full-length single-molecule sequencing of the human mitochondrial genome. *bioRxiv* [Preprint]. 479581. doi: 10.1101/2022.02.08.479581
- Kim, D., Lee, J. Y., Yang, J. S., Kim, J. W., Kim, V. N., and Chang, H. (2020). The architecture of SARS-CoV-2 transcriptome. *Cell* 181, 914.e10–921.e10.
- Korber, B., Fischer, W. M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., et al. (2020). Tracking changes in SARS-CoV-2 spike: Evidence that D614G increases infectivity of the COVID-19 virus. *Cell* 182, 812–827. doi: 10.1016/j.cell.2020.06.043
- Li, H. (2018). Minimap2: Pairwise alignment for nucleotide sequences. *Bioinformatics* 34, 3094–3100. doi: 10.1093/bioinformatics/bty191
- Li, Q., Nie, J., Wu, J., Zhang, L., Ding, R., Wang, H., et al. (2021). SARS-CoV-2 501Y.V2 variants lack higher infectivity but do have immune escape. *Cell* 184, 2362–2371. doi: 10.1016/j.cell.2021.02.042
- Plante, J. A., Liu, Y., Liu, J., Xia, H., Johnson, B. A., Lokugamage, K. G., et al. (2021). Spike mutation D614G alters SARS-CoV-2 fitness. *Nature* 592, 116–121. doi: 10.1038/s41586-020-2895-3
- Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., Gangavarapu, K., et al. (2017). Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nat. Protoc.* 12, 1261–1276. doi: 10.1038/nprot.2017.066
- Sanderson, N., Kapel, N., Rodger, G., Webster, H., Lipworth, S., Street, T., et al. (2022). Comparison of R9.4.1/Kit10 and R10/Kit12 Oxford Nanopore flowcells and chemistries in bacterial genome reconstruction. *bioRxiv* [Preprint]. 490057. doi: 10.1101/2022.04.29.490057
- Sereika, M., Kirkegaard, R. H., Karst, S. M., Michaelsen, T. Y., Sørensen, E. A., Wollenberg, R. D., et al. (2021). Oxford Nanopore R10.4 long-read sequencing enables near-perfect bacterial genomes from pure cultures and metagenomes without short-read or reference polishing. *Nat. Methods* 19, 823–826. doi: 10.1038/s41592-022-01539-7
- Shen, W., Le, S., Li, Y., and Hu, F. (2016). SeqKit: A cross-platform and ultrafast toolkit for FASTA/Q file manipulation. *PLoS One* 11:e163962. doi: 10.1371/journal.pone.0163962
- Ugolini, C., Mulroney, L., Leger, A., Castelli, M., Criscuolo, E., Williamson, M. K., et al. (2022). Nanopore recappable sequencing maps SARS-CoV-2 5' capping sites and provides new insights into the structure of sgRNAs. *Nucleic Acids Res.* 50, 3475–3489. doi: 10.1093/nar/gkac144
- Wang, D., Jiang, A., Feng, J., Li, G., Guo, D., Sajid, M., et al. (2021). The SARS-CoV-2 subgenome landscape and its novel regulatory features. *Mol. Cell* 81, 2135–2147. doi: 10.1016/j.molcel.2021.02.036
- Wu, F., Zhao, S., Yu, B., Chen, Y. M., Wang, W., Song, Z. G., et al. (2020). A new coronavirus associated with human respiratory disease in China. *Nature* 579, 265–269. doi: 10.1038/s41586-020-2008-3



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Corrigendum: Systematic benchmarking of nanopore Q20+ kit in SARS-CoV-2 whole genome sequencing

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The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Unspecific reactivity must be excluded in COVID-19 epidemiological analyses or virus tracing based on serologic testing: Analysis of 46,777 post-pandemic samples and 1,114 pre-pandemic samples

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Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease 2019 (COVID-19). Serologic testing is complementary to nucleic acid screening to identify SARS-CoV-2. This study aimed to evaluate unspecific reactivity in SARS-CoV-2 serologic tests.

Materials and methods: Total anti-SARS-CoV-2 antibodies from 46,777 subjects who were screened for SARS-CoV-2 were retrospectively studied to evaluate the incidence and characteristics of the unspecific reactivity. A total of 1,114 pre-pandemic samples were also analysed to compare unspecific reactivity.

Results: The incidence of unspecific reactivity in anti-SARS-CoV-2 total antibody testing was 0.361% in 46,777 post-pandemic samples, similar to the incidence of 0.359% (4/1,114) in 1,114 pre-pandemic samples ($p = 0.990$). Subjects ≥ 19 years old had a 2.753-fold [95% confidence interval (CI), 1.130–6.706] higher probability of unspecific reactivity than subjects < 19 years old ($p = 0.026$). There was no significant difference between the sexes. The unspecific reactivity was associated with 14 categories within the disease spectrum, with three tops being the skin and subcutaneous tissue diseases (0.93%), respiratory system diseases (0.78%) and neoplasms diseases (0.76%).

The percentage of patients with a titer ≥ 13.87 cut-off index (COI) in the unspecific reactivity was 7.69%.

Conclusion: Our results suggest a unspecific reactivity incidence rate of 0.361% involving 14 categories on the disease spectrum. Unspecific reactivity needs to be excluded when performing serologic antibody testing in COVID-19 epidemiological analyses or virus tracing.

KEYWORDS

SARS-CoV-2, serologic testing, unspecific reactivity, antibody, COVID-19

Introduction

SARS-CoV-2 is the causative agent of coronavirus disease 2019 (COVID-19), which caused a pandemic due to its rapid transmission and strong infectivity (1). The global epidemiological situation of COVID-19 remains serious. A rapid and accurate diagnosis is key in controlling the spread of the disease (2, 3). Serologic testing is complementary to nucleic acid screening for the identification of SARS-CoV-2 (4–6). As the pandemic developed, serologic testing was used to evaluate the effectiveness of vaccination and the prevalence of SARS-CoV-2 infection (7, 8). In addition, serologic tests have been employed to trace SARS-CoV-2 (9, 10). SARS-CoV-2 encodes four structural proteins, namely, the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, among which the spike and nucleocapsid proteins are most commonly detected in SARS-CoV-2 serologic assays (11). One hundred percent cross-reactivity with the full-length SARS nucleocapsid protein has been reported, suggesting that there are polyreactive antibodies in the natural immunoglobulin repertoire with affinity toward some epitopes shared by coronaviruses (12). However, no cross-reactivity in any healthy serum samples with the full-length SARS spike protein has been reported (12). The SARS-CoV-2 spike protein shares 76% homology with that of SARS-CoV-1 and only approximately 30% homology with those of seasonal Beta-CoVs (13). When testing for the presence of SARS-CoV-2 antibodies, researchers have utilized the full spike ectodomain as well as the receptor-binding domain (RBD) for antigens detection in serologic assays (14). However, this produces an unspecific reactivity, which causes difficulties in clinical diagnosis and treatment (1, 15, 16). Currently, the incidence, correlation factor and characteristics of unspecific reactivity in SARS-CoV-2 serologic tests based on RBD antigens was unclear. Investigating unspecific reactivity will greatly benefit serological diagnosis, epidemiological investigation, control of SARS-CoV-2 and even virus traceability (9, 17). Here, we retrospectively analysed samples from 46,777 subjects who were screened for

identify SARS-CoV-2 infection to investigate the incidence and characteristics of the unspecific reactivity. For comparison, we also investigated the unspecific reactivity in 1,114 pre-pandemic samples.

Materials and methods

Study design and participants

Consecutive patients who were screened for SARS-CoV-2 infection by serologic tests and RT-PCR between March 2020 and November 2021 in Zhongshan Hospital were retrospectively evaluated. Zhongshan Hospital is a large integrated Grade III-A hospital that provides approximately 2.50 million people with health care and outpatient medical and hospital services each year. A total of 46,777 subjects without vaccination were screened to identify SARS-CoV-2 infection based on epidemiological history, clinical symptoms, imaging findings and laboratory test results (Figure 1). All subjects underwent anti-SARS-CoV-2 total antibody (Ab) and PCR testing to identify SARS-CoV-2 infection. Those who were Ab-/PCR + and Ab + /PCR + were escorted directly to the hospital for a comprehensive evaluation and epidemiological investigation. Follow-up was performed 28 days later for PCR-/Ab- individuals. PCR-/Ab + individuals were assigned to a key screening population who were followed for 28 days and underwent multiple rounds of PCR testing during follow-up. The subjects with COVID-19 included asymptomatic COVID-19, symptomatic COVID-19 and convalescent patients. Subjects with unspecific reactivity subjects were the no infection subjects with positive serological test results. In unspecific reactivity subjects, PCR was performed at least three times despite negative results during a follow-up period of 28 days.

To investigate the unspecific reactivity before the date of the first COVID-19 case, total antibodies were measured in 1,114 frozen serum samples collected from January 2016 to July 2019 at the Center of Clinical

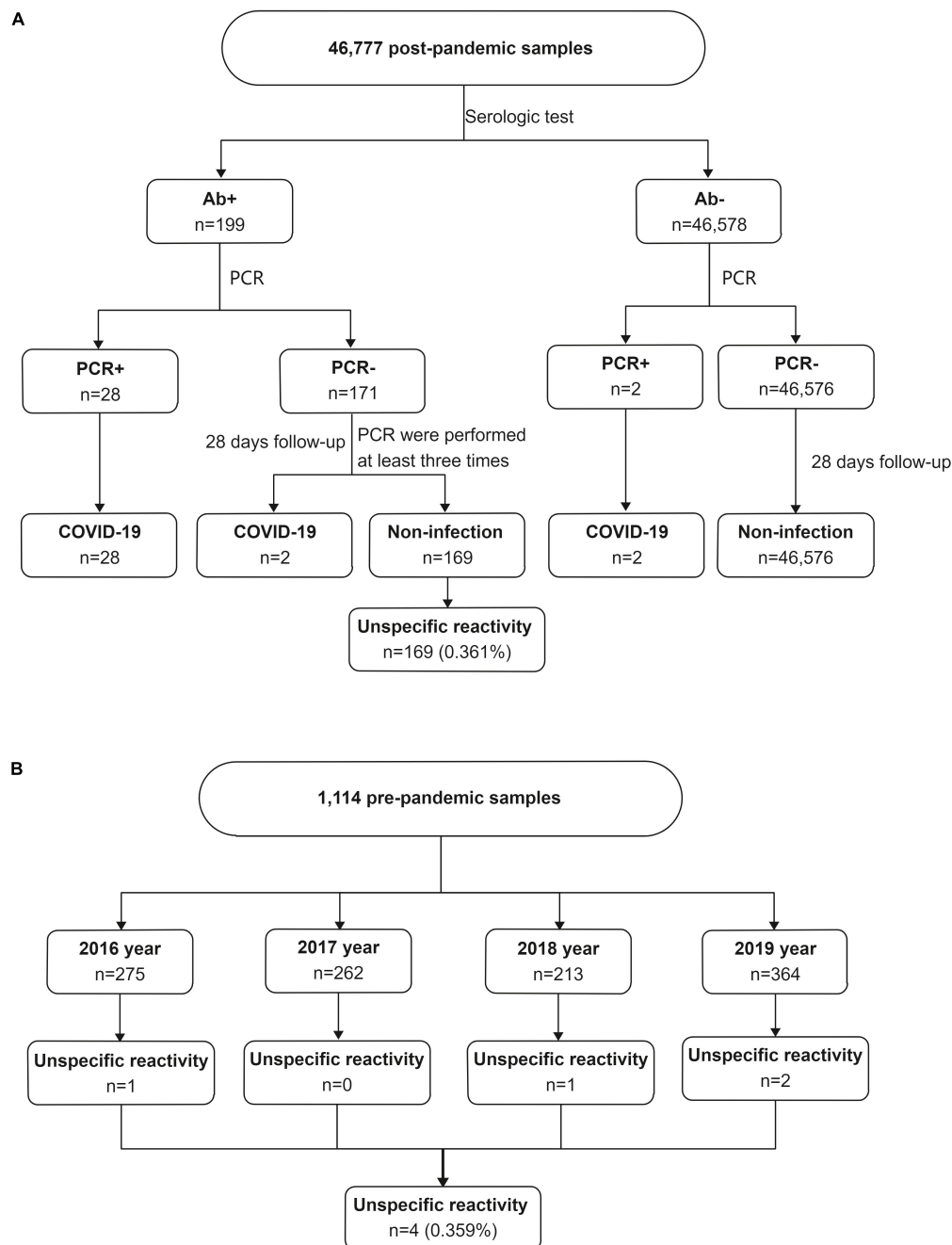


FIGURE 1

The unspecific reactivity in anti-SARS-CoV-2 total antibody testing. (A) The unspecific reactivity in 46,777 post-pandemic subjects who were screened for SARS-CoV-2 infection, (B) the unspecific reactivity in 1,114 pre-pandemic frozen serum samples. Ab, total anti-SARS-CoV-2 antibody; PCR, polymerase chain reaction.

Laboratory, Zhongshan Hospital. Based on the timeline of the first COVID-19 case and the clinical and follow-up data, a positive reaction in a frozen serum sample was considered an unspecific reactivity. Disease classification was based on the International Classification of Diseases, Revision 10 (ICD-10).

Serologic testing

Blood samples were centrifuged at $3,000 \times g$, and the upper serum layer was used for testing. The Wantai® Caris 200 system (a closed and fully automatic system) was used to measure the total SARS-CoV-2 antibody titer. The

detection experiments were performed using a kit from Wantai (Biological Pharmacy Enterprise Co., Ltd., Beijing, China) using a chemiluminescence microparticle immunoassay (CMIA) instrument (Wantai Biological Pharmacy Enterprise Co., Ltd., Beijing, China). Total antibody detection was based on a double-antigen sandwich immunoassay using two kinds of mammalian cell-expressed recombinant antigens containing the receptor-binding domain (RBD) of the spike protein of SARS-CoV-2 as the immobilized antigen and 2',6'-dimethyl-4'-(*N*-succinimidylloxycarbonyl)phenyl-10-methyl-acridinium-9-carboxylate-1-propanesulfonate inner salt (NSP-DMAE-NHS)-conjugated antigens. The antibody titer was calculated according to the signal to cut-off ratio and was recorded as the cut-off index (COI): a COI < 1.00 was considered negative, and a COI $\geq$ 1.00 was considered positive.

Polymerase chain reaction assays for SARS-CoV-2

Upper respiratory tract samples were collected from both oropharyngeal swabs and nasopharyngeal by medical personnel with regularity trained. For lower respiratory tract specimens, participants were given instructions the night before to collect first morning sputum samples (after gargling) in a specimen cup. The Allplex 2020-nCoV assay (Seegene, Seoul, South Korea) was used to perform PCR assays to detect SARS-CoV-2 infection *via* the identification of three genetic markers. These three genetic markers were the envelope (*env*) gene, RNA-dependent RNA polymerase (*RdRp*) gene, and nucleocapsid protein (*N*) gene. The cycle threshold (*Ct*) determined during RT-PCR testing refers to the cycle in which the detection of viral amplicons occurs, and it is inversely correlated with the amount of RNA present. When the cycle threshold values of all genes were less than 40 cycles, the results were considered positive. Double-site positives or two consecutive single-site positives were judged to indicate RT-PCR positivity according to the COVID-2019 Prevention and Control Plan (Eighth Edition).

Statistical analysis

Statistical analysis was conducted using SPSS 26.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). Continuous variables that did not follow a normal distribution are reported as medians with interquartile ranges (IQRs). The Pearson χ^2 test was used for analysis of the unspecific reactivity rate. The Mann-Whitney U test was applied for group comparisons. Factors were entered into a logistic regression model. A receiver operator characteristic curve (ROC) was constructed to analyse the total antibody titers in the unspecific reactivity and the COVID-19. Sensitivity and specificity were calculated from the ROC curve. The threshold for significance was a *p*-value < 0.05.

Results

Characteristics of unspecific reactivity

A total of 46,777 subjects without vaccination were investigated. Of them, 169 subjects had unspecific reactivity (Figure 1A). The incidence of the unspecific reactivity in the anti-SARS-CoV-2 total antibody was 0.361% (169/46,777). Logistic regression was used to analyse the effects of sex and age in the unspecific reactivity group, with no significance different between the sexes. Subjects $\geq$ 19 years old had a 2.753-fold (95% CI, 1.130–6.706) higher probability of a unspecific reactivity than subjects < 19 years old (*p* = 0.026) (Table 1).

A total of 1,114 frozen blood specimens were used to investigate the unspecific reactivity before the date of the first COVID-19 case. Based on the date of the first COVID-19 case, clinical data and the results of telephone follow-up, a positive reaction was considered a unspecific reactivity. The unspecific reactivity rate before the date of the first COVID-19 case was 0.359% (4/1,114) (Figure 1B), which was similar to that during the COVID-19 epidemic (*p* = 0.990). Patients with unspecific reactivity before the first COVID-19 case were diagnosed with ulcerative colitis, fever, systemic lupus erythematosus and testicular tumor.

Disease spectrum of unspecific reactivity

A total of 169 cases were classified into 14 categories within the disease spectrum according to the ICD-10. Among those with unspecific reactivity, the top three categories were diseases of the skin and subcutaneous tissue, respiratory system diseases and neoplasms, with incidence rates of 0.93%, 0.78%, and 0.76%, respectively. Among those with unspecific reactivity, diseases with proportions between 0.60%–0.70% were diseases of the eye and adnexa, the digestive system and the nervous system. Diseases with proportions between 0.40%–0.59% were diseases of the blood and blood-forming organs and certain disorders involving the immune mechanism, diseases of the genitourinary system and diseases of the musculoskeletal system and connective tissue (Table 2).

Unspecific reactivity titer

The titer of the unspecific reactivity group was 3.04 (1.74–5.05) COI, which was significantly lower than 58.34 (23.88–198.7) COI in the COVID-19 group (*p* < 0.001) (Figure 2). In the receiver operating curve (ROC) analyses of total antibody titers in unspecific reactivity and COVID-19 groups, the cut-off value was 13.87 COI, with 90.63% (95% CI: 73.83–97.55%) sensitivity and 92.31% (95% CI: 86.93–95.67%) specificity

TABLE 1 Factors associated with the occurrence of unspecific reactivity.

Risk factor	No infection	Unspecific reactivity (%)	OR	OR (95% CI)		P
				Lower	Upper	
Sex						
Female	23,597	83 (0.35)	1			
Male	23,148	86 (0.37)	0.947	0.700	1.280	0.722
Age						
<19	3,611	5 (0.14)	1			
≥19	43,134	164 (0.38)	2.753	1.130	6.706	0.026

TABLE 2 Disease spectrum associated with unspecific reactivity according to the International Classification of Diseases, Revision 10.

Sorting	Disease	No. of unspecific reactivity	The unspecific reactivity proportion (%)
1	Diseases of the skin and subcutaneous tissue	3	0.93 (3/322)
2	Diseases of the respiratory system	28	0.78 (28/3,568)
3	Neoplasms	40	0.76 (40/5,240)
4	Diseases of the eye and adnexa	5	0.63 (5/800)
5	Diseases of the digestive system	19	0.60 (19/3,145)
6	Diseases of the nervous system	4	0.60 (4/670)
7	Diseases of the blood and blood-forming organs and certain disorders involving the immune mechanism	3	0.49 (3/607)
8	Diseases of the genitourinary system	13	0.46 (13/2,838)
9	Diseases of the musculoskeletal system and connective tissue	9	0.41 (9/2,173)
10	Endocrine, nutritional and metabolic diseases	3	0.37 (3/802)
11	Diseases of the circulatory system	11	0.37 (11/2,947)
12	Pregnancy, childbirth, and puerperium	6	0.34 (6/1,759)
13	Injury, poisoning and certain other consequences of external causes	5	0.33 (5/1,505)
14	Symptoms, signs and abnormal clinical and laboratory findings not elsewhere classified	20	0.31 (20/6,514)
15	Other	0	0.00 (0/13,855)

(Table 3). The number of cases with titer ≥ 13.87 COI in unspecific reactivity was only 13 (7.69%) (Table 4). Among the 1,114 frozen blood specimens, all of the titers in unspecific reactivity were less than 13.87 COI.

Discussion

Serologic testing is a complementary to nucleic acid screening for the identification of SARS-CoV-2 infection (4–6). Currently, the incidence, correlative factors and characteristics of unspecific reactivity in SARS-CoV-2 serologic tests based on RBD antigens are unclear. In our study, 46,777 subjects were retrospectively investigated between March 2020 and November 2021. The incidence of unspecific reactivity in the anti-SARS-CoV-2 total antibody test was 0.361%, which was similar to the incidence of 0.359% among

1,114 blood specimens collected before the first COVID-19 case. Subjects ≥ 19 years old had a 2.753-fold (95% CI, 1.130–6.706) higher probability of a unspecific reactivity than subjects < 19 years old. There was no significance different between the sexes. The unspecific reactivity was associated with 14 categories within the disease spectrum. The three top categories were diseases of the skin and subcutaneous tissue, respiratory system and neoplasms. The percentage of subjects with titer ≥ 13.87 COI in the unspecific group was 7.69%.

Serologic testing has been used to elucidate the timeline of the COVID-19 pandemic. Some researchers have used the frozen blood specimens collected before the COVID-19 epidemic to screen for SARS-CoV-2 antibodies to trace the source of SARS-CoV-2 (9, 10). Basavaraju et al. reported that SARS-CoV-2 was present in the United States earlier

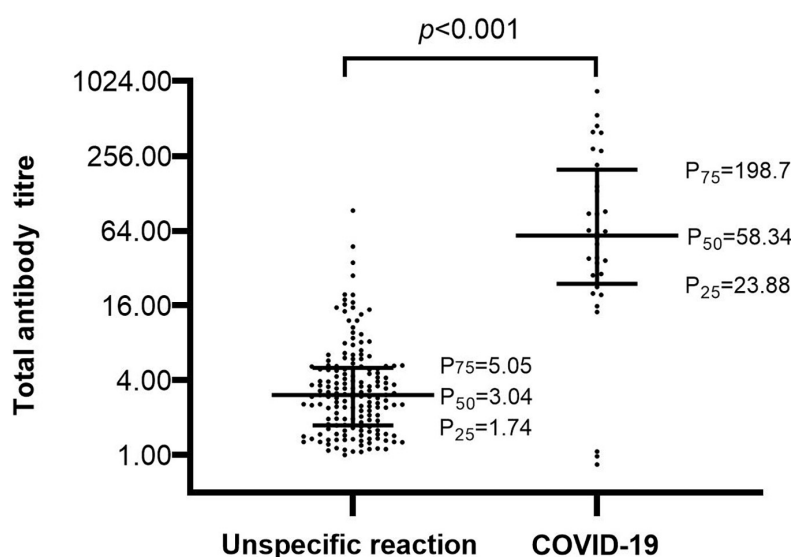


FIGURE 2

Comparison of total antibody titers between the unspecific reactivity and COVID-19. Bars represent the arithmetic median with the interquartile range.

than previously recognized (9). We used the CMIA double-antigen sandwich method to detect the total antibodies against SARS-CoV-2 in serum and found 169 unspecific

TABLE 3 Diagnostic efficacy of the total antibody titer in unspecific reactivity and COVID-19 according to the cut-off of 13.87 cut-off index (COI).

	COVID-19		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)
	+	-		
Total antibody	+	29	13	90.63
	-	3	156	(73.83–97.55)

TABLE 4 Disease spectrum in unspecific reactivity with a total antibody titer ≥ 13.87 cut-off index (COI).

Disease	No (%)	Antibody titer (COI)
Neoplasms	3 (23.10%)	14.81, 47.70, 93.20
Diseases of the skin and subcutaneous tissue	2 (15.38%)	19.47, 35.40
Diseases of the respiratory system	2 (15.38%)	15.38, 15.40
Diseases of the digestive system	2 (15.38%)	14.35, 19.59
Diseases of the musculoskeletal system and connective tissue	1 (7.69%)	27.84
Diseases of the nervous system	1 (7.69%)	17.84
Diseases of the circulatory system	1 (7.69%)	16.33
Pregnancy, childbirth and puerperium	1 (7.69%)	16.90
Total	13	

reactivity results; with an incidence of 0.361%. Pfluger et al. summarized three automatic serological total antibody detection methods for SARS-CoV-2, and all assays had false positive rates of 0.6% (2/320, ELISA), 0.3% (1/320, ECLIA) and 0.0% (0/320, CLIA) (18). Overall, the incidence rates of unspecific reactivity were low, never exceeding 1%. Furthermore, the unspecific reactivity incidence before the date of the first COVID-19 case was 0.359%, which was similar to 0.361% during the COVID-19 epidemic in our study. This result indicates that the unspecific reactivity are inevitable. The unspecific reactivity could confound the results of the source of SARS-CoV-2 according to tracing based on serologic testing.

The unspecific reactivity tendency has been shown to be higher in older populations with antibodies against other pathogens (19, 20). In this study, the unspecific reactivity was associated with age, with subjects ≥ 19 years having a higher probability. With increasing age, endogenous interfering substances such as rheumatoid inducers (RFs) and cross-antigens in the blood increase, which could affect antibody detection and lead to false-positive results (21, 22). In addition, we investigated the disease spectra of patients with unspecific reactivity and found that the three top categories were diseases of the skin and subcutaneous tissue, respiratory system, and neoplasms. The causes may be as follows. First, a high incidence of unspecific reactivity has been reported in those with diseases of the skin and subcutaneous tissue (19), and these diseases might be associated with potential autoimmune aetiologies that produce abnormal expression of IgE, IgG, or IL-1 (23–26), resulting in a unspecific reactivity (27). Second,

a high incidence of non-specific reactivity has been reported in those with diseases of the respiratory system, and an unspecific reactivity in SARS-CoV-2 antibody testing may be due to previous infection with other human coronaviruses (HCoVs); in fact, cross reaction between nucleocapsid or spike proteins of different HCoVs has been reported (28, 29). Finally, neoplasms, the third most common disease category, regularly induce adaptive immune responses in humans and may lead to abnormal protein expression (30, 31). The unspecific reactivity in a serologic test may result from endogenous interfering substances or cross reactivity antibodies. This result supports the notion that unspecific reactivity is unavoidable. Unspecific reactivity needs to be excluded when performing serologic antibody testing in SARS-CoV-2 tracing.

It is worth noting that the titer of unspecific reactivity was much lower than that of the COVID-19. If a risk assessment dictates an overriding concern, the cut-off can be set accordingly (11). In the ROC analysis of the total antibody titer in unspecific reactivity and COVID-19 groups, a cut-off value of 13.87 COI was established, with 90.63% sensitivity and 92.31% specificity. A low titer was one of the characteristics of unspecific reactivity. To exclude the unspecific reactivity, the cut-off value should be reevaluated according to the specific objective and population.

This study has some limitations. First, as a retrospective study, endogenous interfering substances in serum, such as RF, heterophile antibodies and cross-antigens, were not detected. Second, the unspecific reactivity in frozen serum were defined based on clinical data and not pathogen detection of SARS-CoV-2. Third, this retrospective study was conducted in a single hospital. Finally, the unspecific reactivity group was not checked according to PCR based on N gene mutations of SARS-CoV-2.

Conclusion

The incidence rate of unspecific reactivity was 0.361% and was similar to the incidence of 0.359% before the first COVID-19 case. Subjects ≥ 19 years old had a 2.753-fold higher probability of unspecific reactivity than subjects < 19 years old. There were 14 categories within the disease spectrum associated with unspecific reactivity. Titers in unspecific reactivity were generally low (COI < 13.87). Unspecific reactivity needs to be excluded when using serologic antibody testing for COVID-19 epidemiological analysis or virus tracing.

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 Institutional Ethics Committee of Zhongshan Hospital of Xiamen University, School of Medicine, Xiamen University (#xmzsyyky2021196). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

X-ML: conceptualization, formal analysis, project administration, study design, funding, writing, and manuscript submission. M-JC and JL: data analyses, interpretation, manuscript drafting, and critical revision of the manuscript. J-HZ: data acquisition. ZD and Y-QL: production of tables and figures. 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.

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References

1. Van Elslande J, Houben E, Depypere M, Brackenier A, Desmet S, Andre E, et al. Diagnostic performance of seven rapid IgG/IgM antibody tests and the Euroimmun IgA/IgG ELISA in COVID-19 patients. *Clin Microbiol Infect.* (2020) 26:1082–7. doi: 10.1016/j.cmi.2020.05.023
2. Fernandez-Barat L, Lopez-Aladid R, Torres A. The value of serology testing to manage SARS-CoV-2 infections. *Eur Respir J.* (2020) 56:2002411. doi: 10.1183/13993003.02411-2020
3. Perico L, Tomasoni S, Peracchi T, Perna A, Pezzotta A, Remuzzi G, et al. COVID-19 and lombardy: TESTing the impact of the first wave of the pandemic. *EBioMedicine.* (2020) 61:103069. doi: 10.1016/j.ebiom.2020.103069
4. Long QX, Liu BZ, Deng HJ, Wu GC, Deng K, Chen YK, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat Med.* (2020) 26:845–8.
5. Zou LR, Ruan F, Huang MX, Liang LJ, Huang HT, Hong ZS, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. *N Engl J Med.* (2020) 382:1177–9. doi: 10.1056/NEJMc2001737
6. Pan Y, Li X, Yang G, Fan J, Tang Y, Zhao J, et al. Serological immunochromatographic approach in diagnosis with SARS-CoV-2 infected COVID-19 patients. *J Infect.* (2020) 81:e28–32. doi: 10.1016/j.jinf.2020.03.051
7. Ng OT, Marimuthu K, Koh V, Pang J, Linn KZ, Sun J, et al. SARS-CoV-2 seroprevalence and transmission risk factors among high-risk close contacts: a retrospective cohort study. *Lancet Infect Dis.* (2021) 21:333–43. doi: 10.1016/S1473-3099(20)30833-1
8. Xu QY, Xue JH, Xiao Y, Jia ZJ, Wu MJ, Liu YY, et al. Response and duration of serum Anti-SARS-CoV-2 antibodies after inactivated vaccination within 160 days. *Front Immunol.* (2021) 12:786554. doi: 10.3389/fimmu.2021.786554
9. Basavaraju SV, Patton ME, Grimm K, Rasheed MAU, Lester S, Mills L, et al. Serologic testing of US blood donations to identify severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-reactive antibodies: december 2019-january 2020. *Clin Infect Dis.* (2021) 72:E1004–9. doi: 10.1093/cid/ciaa1785
10. Gragnani L, Monti M, Santini SA, Marri S, Madia F, Lorini S, et al. SARS-CoV-2 was already circulating in Italy, in early December 2019. *Eur Rev Med Pharmacol Sci.* (2021) 25:3342–9.
11. Zhang YV, Wiencek J, Meng QH, Theel ES, Babic N, Sepiashvili L, et al. AACC practical recommendations for implementing and interpreting SARS-CoV-2 emergency use authorization and laboratory-developed test serologic testing in clinical laboratories. *Clin Chem.* (2021) 67:1188–200. doi: 10.1093/clinchem/hvab051
12. Maache M, Komurian-Pradel F, Rajoharison A, Perret M, Berland JL, Pouzol S, et al. False-positive results in a recombinant severe acute respiratory syndrome-associated coronavirus (SARS-CoV) nucleocapsid-based western blot assay were rectified by the use of two subunits (S1 and S2) of spike for detection of antibody to SARS-CoV. *Clin Vaccine Immunol.* (2006) 13:409–14. doi: 10.1128/CVI.13.3.409-414.2006
13. Hofmann H, Hattermann K, Marzi A, Gramberg T, Geier M, Krumbiegel M, et al. S protein of severe acute respiratory syndrome-associated coronavirus mediates entry into hepatoma cell lines and is targeted by neutralizing antibodies in infected patients. *J Virol.* (2004) 78:6134–42. doi: 10.1128/JVI.78.12.6134-6142.2004
14. Stadlbauer D, Amanat F, Chromikova V, Jiang K, Strohmeier S, Arunkumar GA, et al. SARS-CoV-2 seroconversion in humans: a detailed protocol for a serological assay, antigen production, and test setup. *Curr Protoc Microbiol.* (2020) 57:e100. doi: 10.1002/cpmc.100
15. Kowitdamrong E, Puthanakit T, Jantarabenjakul W, Prompetchara E, Suchartlikitwong P, Putcharoen O, et al. Antibody responses to SARS-CoV-2 in patients with differing severities of coronavirus disease 2019. *PLoS One.* (2020) 15:e0240502. doi: 10.1371/journal.pone.0240502
16. Montesinos I, Gruson D, Kabamba B, Dahma H, Van den Wijngaert S, Reza S, et al. Evaluation of two automated and three rapid lateral flow immunoassays for the detection of anti-SARS-CoV-2 antibodies. *J Clin Virol.* (2020) 128:104413. doi: 10.1016/j.jcv.2020.104413
17. Xiao X, Zhou Q, Zhu J, Sun L, Zhang H, Sun Y, et al. False-positive colloidal gold-based immunochromatographic strip assay reactions for antibodies to SARS-CoV-2 in patients with autoimmune diseases. *Ann Transl Med.* (2021) 9:534. doi: 10.21037/atm-20-6509
18. Pfluger LS, Bannasch JH, Brehm TT, Pfeifferle S, Hoffmann A, Norz D, et al. Clinical evaluation of five different automated SARS-CoV-2 serology assays in a cohort of hospitalized COVID-19 patients. *J Clin Virol.* (2020) 130:104549. doi: 10.1016/j.jcv.2020.104549
19. Liu F, Liu L-L, Guo X-J, Xi Y, Lin L-R, Zhang H-L, et al. Characterization of the classical biological false-positive reaction in the serological test for syphilis in the modern era. *Int Immunopharmacol.* (2014) 20:331–6. doi: 10.1016/j.intimp.2014.03.011
20. Lin Y-Q, Gao Y-L, Wang M, Yan S-D, Lin L-R. Analysis of the characteristics of patients with false-positive HIV screening assay results. *Int Immunopharmacol.* (2022) 105:108556. doi: 10.1016/j.intimp.2022.108556
21. Mariller MM, Santos-Eggimann B. The prevalence of rheumatic diseases in the elderly in developed countries and its evolution over time. *Soz Präventivmed.* (2005) 50:45–51. doi: 10.1007/s00038-004-3139-2
22. Farnsworth CW, Anderson NW. SARS-CoV-2 serology: much hype, little data. *Clin Chem.* (2020) 66:875–7. doi: 10.1093/clinchem/hvaa107
23. Bracken SJ, Abraham S, MacLeod AS. Autoimmune theories of chronic spontaneous urticaria. *Front Immunol.* (2019) 10:627. doi: 10.3389/fimmu.2019.00627
24. Kanani A, Betschel SD, Warrington R. Urticaria and angioedema. *Allergy Asthma Clin Immunol.* (2018) 14(Suppl 2):59. doi: 10.1186/s13223-018-0288-z
25. Christoph T, Muller-Rover S, Audring H, Tobin DJ, Hermes B, Cotsarelis G, et al. The human hair follicle immune system: cellular composition and immune privilege. *Br J Dermatol.* (2000) 142:862–73. doi: 10.1046/j.1365-2133.2000.03464.x
26. Belkaid Y, Segre JA. Dialogue between skin microbiota and immunity. *Science.* (2014) 346:954–9. doi: 10.1126/science.1260144
27. Ye Q, Zhang T, Lu D. Potential false-positive reasons for SARS-CoV-2 antibody testing and its solution. *J Med Virol.* (2021) 93:4242–6. doi: 10.1002/jmv.26937
28. Ye ZW, Yuan S, Yuen KS, Fung SY, Chan CP, Jin DY. Zoonotic origins of human coronaviruses. *Int J Biol Sci.* (2020) 16:1686–97. doi: 10.7150/ijbs.45472
29. Adachi S, Koma T, Doi N, Nomaguchi M, Adachi A. Commentary: Origin and evolution of pathogenic coronaviruses. *Front Immunol.* (2020) 11:811. doi: 10.3389/fimmu.2020.00811
30. Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res.* (2009) 15:5323–37. doi: 10.1158/1078-0432.CCR-09-0737
31. Blankenstein T, Coulie PG, Gilboa E, Jaffee EM. The determinants of tumour immunogenicity. *Nat Rev Cancer.* (2012) 12:307–13. doi: 10.1038/nrc3246



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An attenuated vaccinia vaccine encoding the severe acute respiratory syndrome coronavirus-2 spike protein elicits broad and durable immune responses, and protects cynomolgus macaques and human angiotensin-converting enzyme 2 transgenic mice from severe acute respiratory syndrome coronavirus-2 and its variants

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As long as the coronavirus disease-2019 (COVID-19) pandemic continues, new variants of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) with altered antigenicity will emerge. The development of vaccines that elicit robust, broad, and durable protection against SARS-CoV-2 variants is urgently required. We have developed a vaccine consisting of the attenuated vaccinia virus Dairen-I (DIs) strain platform carrying the SARS-CoV-2 *S* gene (rDIs-S). rDIs-S induced neutralizing antibody and T-lymphocyte responses in cynomolgus macaques and human angiotensin-converting enzyme 2 (hACE2) transgenic mice, and the mouse model showed broad protection against

SARS-CoV-2 isolates ranging from the early-pandemic strain (WK-521) to the recent Omicron BA.1 variant (TY38-873). Using a tandem mass tag (TMT)-based quantitative proteomic analysis of lung homogenates from hACE2 transgenic mice, we found that, among mice subjected to challenge infection with WK-521, vaccination with rDIs-S prevented protein expression related to the severe pathogenic effects of SARS-CoV-2 infection (tissue destruction, inflammation, coagulation, fibrosis, and angiogenesis) and restored protein expression related to immune responses (antigen presentation and cellular response to stress). Furthermore, long-term studies in mice showed that vaccination with rDIs-S maintains S protein-specific antibody titers for at least 6 months after a first vaccination. Thus, rDIs-S appears to provide broad and durable protective immunity against SARS-CoV-2, including current variants such as Omicron BA.1 and possibly future variants.

KEYWORDS

SARS-CoV-2, DIs-based SARS-CoV-2 vaccine, animal model, SARS-CoV-2 variants, broad immune response, durable immune response, quantitative proteomics

Introduction

Coronavirus disease-2019 (COVID-19), which is caused by infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has spread worldwide due to the lack of specific immunity against SARS-CoV-2 in most humans (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020; Zhou et al., 2020). Since the outbreak began in December 2019, SARS-CoV-2 infection has been associated with more than 590 million cases, resulting in more than 6.4 million deaths worldwide.¹ The acquisition of memory immune responses against SARS-CoV-2 is required for preventing COVID-19 and severe symptoms that require hospitalization. Vaccination is considered an essential means of obtaining such lymphocytic responses prior to infection.

Severe acute respiratory syndrome coronavirus-2 is an enveloped, single-stranded, positive-sense RNA virus. The spike (S) protein on the virion surface mediates SARS-CoV-2 entry into target cells through binding to the host cell receptor, angiotensin-converting enzyme 2 (ACE2) (Hoffmann et al., 2020; Wrapp et al., 2020). Consistent with this fact, the S protein is a major target of both neutralizing antibodies (nAbs; Ju et al., 2020; Dai and Gao, 2021) and T-cell responses to SARS-CoV-2-infected cells (Grifoni et al., 2020; Peng et al., 2020), indicating that the S protein is important as a vaccine component to elicit protective immunity against SARS-CoV-2.

The global effort to develop an effective vaccine enabled the distribution of the first COVID-19 vaccines within a year of the start of the pandemic and the initial identification of SARS-CoV-2. Subsequently, several COVID-19 vaccines have

been approved for general or emergency use in multiple countries, including the United States, the United Kingdom, China, and Russia,² and more than 11 billion doses have been administered worldwide.³ Currently, mRNA vaccines (Jackson et al., 2020; Polack et al., 2020), adenovirus vector vaccines (Folegatti et al., 2020; Logunov et al., 2020; Sadoff et al., 2021), and inactivated whole-virus-particle vaccines (Jara et al., 2021; Tanriover et al., 2021) are in wide use, but the development of additional vaccines that are safe and effective is still of interest, especially given concerns about duration of protective efficacy, cross-reactivity against variants, vaccine cost, and the need for cold chains for distribution of the current vaccines.

Highly attenuated vaccinia viruses (VACVs) have gained attention as promising viral vectors owing to their safety and immunogenicity in humans, properties that have contributed to the eradication of smallpox (Altenburg et al., 2014). Among VACVs, the DIs strain was derived from the embryonated chicken egg adapted Dairen (DIE) strain of VACV through extensive serial passaging using one-day-old eggs (Tagaya et al., 1961). Notably, the DIs strain has a restricted host range because of a large-scale deletion (approximately 15.4 kb) representing 8% of the parental VACV genome; this deletion results in a loss of replication in most mammalian cells. The recombinant DIs strain also has been tested extensively as a platform for a candidate vaccine against severe acute respiratory syndrome (SARS), a previous coronavirus outbreak (Ishii et al., 2002, 2006). Thus, the DIs strain is considered a promising viral vector for the development of novel vaccines. In the present study, we investigated the protective efficacy of

¹ <https://covid19.who.int/>

² <https://extranet.who.int/pqweb/vaccines/covid-19-vaccines>

³ <https://coronavirus.jhu.edu/map.html>

rDIs-S, a recombinant DIs strain carrying the SARS-CoV-2 spike-encoding gene, against SARS-CoV-2 infection in both a nonhuman primate model and human ACE2 (hACE2) heterologous expressing mouse models.

Materials and methods

Ethics statement

This study was carried out in strict accordance with the “Guidelines for the Husbandry and Management of Laboratory Animals” of the Tokyo Metropolitan Institute of Medical Science and the Research Center for Animal Life Science at Shiga University of Medical Science, and with the “Standards Relating to the Care and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions” under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan. The animal experimental protocols were approved by the Ethics Committee of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science (Permission Nos. 20–085, 20–086, 21–079, and 21–080), and by the Shiga University of Medical Science Animal Experiment Committee (Permit No. 2020-6-20). In the macaque study, regular veterinary care and monitoring, balanced nutrition, and environmental enrichment were provided by personnel of the Research Center for Animal Life Science at Shiga University of Medical Science. The macaques were euthanized at the endpoint (7 days after SARS-CoV-2 virus inoculation) using ketamine/xylazine followed by intravenous injection of pentobarbital (200 mg/kg). Animals were monitored every day during the study to permit the calculation of clinical scores (Supplementary Table 3) and underwent daily veterinary examinations intended to help alleviate suffering. The animals were euthanized if their clinical score reached 15 (a humane endpoint).

Cells

Primary chicken embryonic fibroblasts were prepared for constructing and propagating the recombinant VACV DIs strain that carries the gene encoding the spike protein of SARS-CoV-2. Seven-day-old chicken embryos were collected in Hanks’ Balanced Salt Solution [HBSS (–)] supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, and 0.1% glucose. After removing the eyes, brain, beak, wings, and feet from each embryo, the rest of the body was minced with scissors and digested in TrypLE Select (Thermo Fisher Scientific, Waltham, MA, United States). The resulting chicken embryonic fibroblasts were grown in Dulbecco’s Modified Eagle Medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum and tryptose phosphate broth.

Vaccine construction

Codon optimization was performed for the spike protein-encoding gene sequence of SARS-CoV-2 (AI/I-004/2020 strain GISAID EPI_ISL_407084, or Delta variant hCoV/Japan/TY11-927-P1/2021 strain GISAID EPI_ISL_2158617) to facilitate stable expression in the context of DIs. Silent mutations were introduced in the sequences encoding nCoV-S to remove stop signal sequences (TTTTTTNT) for the vaccinia virus early promoter. The resulting synthetic DNA encodes a modified nCoV-S (mnCoV-S) or Delta variant-S and was designed to include flanking SbfI and AsiSI restriction sites upstream and downstream (respectively) of the S open reading frame (ORF). This synthetic DNA was cloned into the DIs vector plasmid pSMART-DIs-L3-GPTF (purchased from GenScript, Nanjing, China), which harbors the *Escherichia coli gpt* gene (encoding xanthine-guanine phosphoribosyltransferase, XGPRT) under control of the VACV *p7.5* early promoter. The resulting constructs, designated pSMART-DIs-L3-mnCoV-S-GPTF and pSMART-DIs-L3-mnCoV-Delta S-GPTF, were linearized by digestion with the Apa I restriction enzyme. The linearized plasmid was purified and transfected into primary chicken embryonic fibroblasts that had been infected with DIs at a multiplicity of infection of 10 for 1 h. After 20 h, the virus-cell mixture was harvested by scraping of the cell layer, and the resulting suspension was frozen at –80°C until use. rDIs-mnCoV-S and rDIs-mnCoV-Delta S [i.e., the rDIs-S and rDIs-S (Delta) used in this study] were purified in the presence of the selective reagent mycophenolic acid, an inhibitor of purine metabolism; the use of a vector containing the *E. coli gpt* gene in the presence of xanthine and hypoxanthine permitted the cultures to overcome the blocking of the pathway for GMP synthesis caused by mycophenolic acid, as described previously (Mortensen and Kingston, 2009). DIs was used as a control virus. The expression of spike protein of SARS-CoV-2 in VeroE6/TMPRSS2 cell infected with rDIs-S or rDIs-S (Delta) were detected by western blotting using rabbit polyclonal anti-SARS-CoV-2 S1 IgG (GeneTex, GTX135356, 1:10,000).

Severe acute respiratory syndrome coronavirus-2

Severe acute respiratory syndrome coronavirus-2 JP/TY-WK-521/2020 (WK-521; GenBank Sequence Accession: LC522975), SARS-CoV-2 hCoV-19/Japan/TY8-612/2021 (TY8-612; GISAID strain name: EPI_ISL_1123289), SARS-CoV-2 hCoV/Japan/TY11-927-P1/2021 (TY11-927; GISAID strain name: EPI_ISL_2158617), and SARS-CoV-2 hCoV-19/Japan/TY38-873/2021 (TY38-873; GISAID strain name: EPI_ISL_7418017) were used as challenge strains; these isolates were kindly provided by Drs. Masayuki Saijo, Mutsuyo Takayama-Ito, Masaaki Sato, and Ken Maeda, National Institute of Infectious Disease (NIID; Matsuyama et al., 2020). SARS-CoV-2 Japan/SHG-SUMS2/2020 (SUMS2;

GISAIID strain name: EPI_ISL_10434280), which was isolated from a patient hospitalized in the Shiga University of Medical Science Hospital, encodes an S protein with a D614G substitution. All virus strains used in this study are listed in [Supplementary Table 4](#). The nucleotide sequence of WK-521 has 99.9% similarity to that of the Wuhan-Hu-1 strain (NC_045512.2). The WK-521 virus was propagated twice at the NIID, and then once at the Shiga University of Medical Science or twice at The Tokyo Metropolitan Institute of Medical Science, using the VeroE6/TMPRSS2 cell line (JCRB Cell Bank, Osaka, Japan). The other variants also were propagated at the NIID, and then once at the Tokyo Metropolitan Institute of Medical Science or at the Shiga University of Medical Science, using VeroE6/TMPRSS2. The macaques were challenged with the WK-521 virus [2×10^7 mean tissue culture infectious dose (TCID₅₀)], which was inoculated into the animals' conjunctiva (0.05 ml $\times$ 2), nostrils (0.5 ml $\times$ 2), oral cavity (0.9 ml), and trachea (5 ml) with pipettes and catheters; the animals were placed under ketamine/xylazine anesthesia prior to inoculation. Experiments using the virus were performed in the Biosafety Level 3 facility of the Research Center for Animal Life Science, the Shiga University of Medical Science, and the Tokyo Metropolitan Institute of Medical Science.

VeroE6/TMPRSS2 cells were grown in DMEM supplemented with 10% inactivated fetal bovine serum (Capricorn Scientific GmbH, Ebsdorfergrund, Germany), penicillin (100 units/ml), streptomycin (100 µg/ml), and G418 (1 mg/ml; Nacalai Tesque). To assess viral replication, serial dilutions of swab samples and tissue homogenate samples (10% w/v in HBSS) were inoculated onto confluent VeroE6/TMPRSS2 cells. The VeroE6/TMPRSS2 cells were cultured in DMEM supplemented with 0.1% bovine serum albumin (BSA), penicillin, streptomycin, and gentamycin (50 µg/ml; Fujifilm, Tokyo, Japan). Cytopathic effects were determined by examination under a microscope 3 days later.

Mice

C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). hACE2 transgenic mice were obtained from the National Institute of Biomedical Innovation, Health and Nutrition as ACE2 Tg #17 (Strain nbio0298). To maintain the heterozygous (hACE2 Tg/+) hACE2 mice, C57BL/6 mice, and heterozygous (hACE2 Tg/+) hACE2 mice were mated. BALB/c mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Throughout the mouse studies, animals were provided with free access to food and water, and were maintained on a 12-h light/12-h dark cycle. Prior to inoculation, animals were anesthetized by intraperitoneal administration of a ketamine/xylazine mixture. Animals then were inoculated intratracheally with 20 TCID₅₀ per 50 µl of the WK-521 strain, 100 plaque-forming units (PFU) per 50 µl of the TY8-612 strain, or 50 PFU per 50 µl of the TY11-927 strain.

To evaluate the efficacy of rDIs-S (encoding spike derived either from an early-pandemic strain of SARS-CoV-2 or from a Delta variant) against an Omicron variant, we used an alternative

hACE2 transgenic mouse model. Specifically, we generated an adenoviral vector expressing the hACE2-encoding gene under the *EF1α* promoter with a leftward orientation (rAd5 pEF1α-hACE2-L) as a novel transgene vector to confer SARS-CoV-2 susceptibility in wild-type mice ([Matsumoto et al., 2022](#)). BALB/c mice were inoculated intranasally with 5×10^7 focus-forming units (FFU) per 50 µl of rAd5 pEF1-hACE2-L. Five days after the inoculation, the BALB/c mice were inoculated intranasally with 1×10^5 PFU per 50 µl of the TY38-837 strain of SARS-CoV-2. Body weight was monitored daily; mice that lost 30% or more of their initial body weight were humanely euthanized and scored as dead.

Macaques

Nine- to 18-year-old female and male cynomolgus macaques that were born in the Philippines or at the Shiga University of Medical Science were used; for animals bred in-house, the maternal macaques originated from Vietnam, and the paternal macaques originated from Indonesia or China. All procedures were performed under ketamine/xylazine anesthesia, and all efforts were made to minimize suffering. Food pellets of CMK-2 (CLEA Japan, Inc.) were provided once per day after recovery from the anesthesia, and drinking water was available *ad libitum*. The macaques were single-housed in cages equipped with climbable bars for environmental enrichment under controlled conditions of humidity (46–70%), temperature (22.3–23.9°C), and light (12-h light/12-h dark cycle, lights on at 8:00 a.m.). Two weeks before virus inoculation, two temperature data loggers (iButton, Maxim Integrated, San Jose, CA, United States) were implanted in the peritoneal cavity of each macaque under ketamine/xylazine anesthesia followed by isoflurane inhalation; the data loggers permitted monitoring of body temperature. The macaques used in the present study were confirmed, by testing, to be free of herpes B virus, hepatitis E virus, *Mycobacterium tuberculosis*, *Shigella* spp., *Salmonella* spp., and *Entamoeba histolytica*. Attenuated VACV (1×10^8 PFU) was injected intracutaneously twice using a syringe with a 29-G needle. Macaques were distinguished by identification numbers as follows: C1–C4, macaques inoculated with DIs; V1–V4, macaques inoculated with rDIs-S.

Using animals anesthetized with ketamine/xylazine, two cotton sticks (Eiken Chemical, Ltd., Tokyo, Japan) were used to collect fluid samples from the conjunctiva, nasal cavity, oral cavity, trachea, and rectum, and the sticks subsequently were immersed in 1 ml of DMEM supplemented with 0.1% BSA and antibiotics. A bronchoscope (MEV-2560; Machida Endoscope Co., Ltd., Tokyo, Japan) and cytology brushes (BC-203D-2006; Olympus Co., Tokyo, Japan) were used to obtain bronchial samples. Samples were collected on the indicated days.

Chest X-ray radiographs were obtained using the I-PACS system (Konica Minolta Inc., Tokyo, Japan) and a PX-20BT mini (Kenko Tokina Corporation, Tokyo, Japan). Saturation of

peripheral oxygen (SpO₂) was measured with a pulse oximeter (Nellcor™; Medtronic plc, Dublin, Ireland).

Extraction of RNA and quantitative reverse transcription-polymerase chain reaction for the detection of SARS-CoV-2 RNA

Total RNA samples were extracted from swab samples and tissue samples from the macaques using the QIAamp Viral RNA Mini Kit and RNeasy Plus Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The levels of RNA corresponding to the N protein-encoding gene of SARS-CoV-2 were measured using the TaqMan Fast Virus 1-step Master Mix (Thermo Scientific). Each 20-μl reaction mixture contained 5.0 μl of 4× TaqMan Fast Virus 1-Step Master Mix, 0.25 μl of 10 μM probe, 1.0 μl each of 10 μM forward and reverse primers, 7.75 μl of nuclease-free water, and 5.0 μl of nucleic acid extract. Amplification was carried out in 96-well plates using a CFX-96 cyclor equipped with CFX Maestro software (Bio-Rad, Hercules, CA, United States). The thermocycling conditions were as follows: 5 min at 50°C for reverse transcription, 20 s at 95°C for the inactivation of reverse transcriptase and initial denaturation, and 45 cycles of 5 s at 95°C and 30 s at 60°C for amplification. Each run included a no-template control reaction as well as reactions intended to provide a standard curve. The latter used *in vitro* transcribed RNA of the N protein-encoding gene (at 10⁰, 10¹, 10², 10³, 10⁴, 10⁶, and 10⁸ copies/reaction); this template was generated from the cDNA of SARS-CoV-2 AI/I-004/2020 using the T7 RiboMAX Express Large Scale RNA Production System (Promega, Madison, WI, United States). The primers and probe used for detecting the WK-521, TY8-612, or TY11-927 strain were as follows: forward primer, 5'-GACCCCAAAATCAGCGAAAT-3'; reverse primer, 5'-TCTGGTTACTGCCAGTTGAATCTG-3'; and probe, 5'-(FAM)-ACCCCGCATTACGTTTGGTGGACC-(BHQ-1)-3', where FAM and BHQ-1 are 6-fluorescein amidite and Black Hole Quencher-1, respectively. For detecting the TY38-873 strain, the primers and probe were as follows: forward primer, 5'-TTACAAACATTGGCCGCAAA-3'; reverse primer, 5'-GCGCGACATTCCGAAGAA-3'; and probe, 5'-(FAM)-ACAA TTTGCCCCAGCGCTTCAG-(BHQ-1)-3'.

Histopathological examination

Lungs were obtained at necropsy, and eight lung tissue slices were collected from each macaque: one slice from each upper lobe and middle lobe, and two slices from each lower lobe in the bilateral lungs. These slices were fixed in 10% neutral buffered formalin for approximately 72 h, embedded in paraffin, and cut into 3-μm-thick sections, which were mounted on glass slides. Sections were stained with hematoxylin and eosin and observed by light microscopy. Histological evaluation was performed by

two pathologists, both blinded to sample identification, based on criteria established for influenza virus infection (Ogiwara et al., 2014) as follows: 0, normal lung; 1, mild destruction of bronchial epithelium; 2, mild peribronchiolar inflammation; 3, inflammation in the alveolar walls resulting in alveolar thickening; 4, mild alveolar injury accompanied by vascular injury; 5, moderate alveolar injury and vascular injury; and 6 and 7, severe alveolar injury with hyaline membrane-associated alveolar hemorrhage (under or over 50% of the section area, respectively). The mean score for the eight sections was calculated for each macaque, and the mean score of the two pathologists was defined as the histological score. After autoclaving the slides in citrate buffer (pH 9) for antigen retrieval, SARS-CoV-2 N antigen was detected using monoclonal anti-N antibody 8G8A (Bioss, Inc., Boston, MA, United States) and a secondary antibody.

Blood cytokine and biochemical analyses

Levels of cytokines/chemokines in macaque plasma were measured using the Milliplex MAP Non-human Primate Cytokine Panel in combination with a Luminex 200 (Millipore Corp., Billerica, MA, United States) according to the manufacturer's instructions.

Virus neutralization assay

In the macaque study, complement in plasma samples was inactivated by heating at 56°C for 1 h. The diluted samples were mixed for 30 min with 100 TCID₅₀/well of the SARS-CoV-2 strains shown in [Supplementary Table 4](#). Then, each mixture was added onto a VeroE6/TMPRSS2 monolayer in 96-well plate. After 1 h of incubation, the cells were cultured in DMEM supplemented with 0.1% BSA. After incubation at 37°C for 3 days, the number of wells showing a cytopathic effect (CPE) was counted. Neutralization titers are expressed as the dilution at which CPEs were observed in 50% of the wells. This assay was performed in quadruplicate culture. In the mouse study, serial 4-fold dilutions of heat-inactivated sera were mixed with equal volumes of 100 PFU of SARS-CoV-2 WK-521 strain and incubated at 37°C for 1 h. VeroE6/TMPRSS2 cells were infected with 100 μl of the virus-serum mixtures in 6-well plate for 1 h and then cultured in DMEM supplemented with 10% inactivated fetal bovine serum and 0.6% agarose. After 2 days of infection, the neutralization titer was determined as the end point dilution of the serum at which there was a 50% reduction of the number of plaques formed by SARS-CoV-2.

Detection of cytokine-producing cells by enzyme-linked immunosorbent spot

After separation from red blood cells, peripheral blood mononuclear cells (PBMCs) were stored at -80°C until use.

Thawed cells [5×10^5 /well] were cultured overnight with a peptide pool of SARS-CoV-2 S protein (0.6 nmol/ml; PepTivator; Miltenyi Biotec, Bergisch Gladbach, Germany) in the presence of anti-CD28 antibody (0.1 μ g/ml); culturing was performed in ELISpot plates coated with anti-IFN- γ and anti-IL-2 antibodies (Cellular Technology, Ltd., Shaker Heights, OH, United States). The number of cytokine-producing cells was counted according to the manufacturer's instructions. Stimulation indices (S.I.) were calculated as follows: S.I. = number of spots in the culture of cells with peptides/number of spots in the culture of cells without peptides. In the mouse study, isolated single splenocytes were used for the ELISpot assay. The splenocytes were cultured with 1 μ g/ml of the peptide pool of SARS-CoV-2 S protein (PepTivator SARS-CoV-2 Prot_S, SARS-CoV-2 Prot_S1, and Prot_S+, which cover the full-length of the S protein; Miltenyi Biotec) for 24 h in ELISpot plates coated with anti-mouse-IFN- γ antibody (Mabtech AB, Nacka Strand, Sweden). The procedures were carried out according to the manufacturer's instructions (Mabtech, #3321-2H). After drying the ELISpot plates, the number of spots in each well was counted with an automated ELISpot plate reader (Advanced Imaging Devices GmbH, Strassberg, Germany).

In vivo cytotoxic T-lymphocyte assay

Single cells were isolated from the spleens of C57BL/6J mice. After the lysis of red blood cells, splenocytes were incubated with 1 μ g/ml of the peptide pool of SARS-CoV-2 S protein (PepTivator, as above) in a 37°C water bath for 1 h. After washing with HBSS, unpulsed splenocytes and the S-peptide-pulsed splenocytes were stained with 0.5 and 5 μ M (respectively) of 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; BioLegend, San Diego, CA, United States). The unpulsed and peptide-pulsed splenocytes were mixed at a 1:1 ratio (5×10^6 cells each), and the mixture was injected intravenously into DI- and rDI-S-inoculated mice. Twenty-four hours later, the spleens were harvested, and the percentages of cells positive for staining with CFSE (i.e., CFSE⁺ cells) that were CFSE^{low} and CFSE^{high} were assessed by flow cytometry. The percent specific killing was calculated as $[1 - (\text{Non-transferred control ratio}/\text{Experimental ratio})] \times 100$.

Detection of IgG specific for the SARS-CoV-2 spike protein

For ELISA, recombinant SARS-CoV-2 spike S1+S2 extracellular domain (ECD; Sino Biological, Inc., Beijing, China) was coated onto 96-well round-bottom plates, and the plates were incubated overnight at 4°C. The plates were blocked with 1% BSA in phosphate-buffered saline [PBS (–)] containing 0.5% Tween 20 and 2.5 mM ethylenediaminetetraacetic acid, then incubated with a

500-fold dilution of sera from C57BL/6 and hACE2 transgenic mice immunized with either rDI-S or DI, a 1,000-fold dilution of sera from BALB/c mice immunized with either rDI-S or DI, or a 1,000-fold dilution of plasmas from cynomolgus macaques immunized with either rDI-S or DI. After extensive washing, the plates were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG polyclonal antibody (GE Healthcare, Chicago, IL, United States) or goat anti-monkey IgG polyclonal antibody (NORDIC IMMUNOLOGICAL LABORATORIES, Eindhoven, The Netherlands). Antigen–antibody interactions were detected using o-phenylenediamine solution as the substrate (Nacalai Tesque), and the binding activity was measured by monitoring absorbance at 490 nm. For the bead array assay to detect IgG specific for S1, RBD, and S2 in plasma, the Milliplex SARS-CoV-2 Antigen Panel 1 IgG was used according to the manufacturer's instructions (Merck Millipore, Darmstadt, Germany).

TMTpro 11plex MS analysis

Lysates extracted from mouse lung tissues with a bead shaker were processed and digested using an EasyPep Mini MS Sample Prep Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Then, 25 μ g of peptides from each sample were labeled with 0.25 mg of the TMTpro TMT-labeling reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. After TMT labeling, aliquots from the 11 sample channels were combined in an equal ratio, dried using a vacuum concentrator, and resuspended in 0.1% trifluoroacetic acid (TFA). Samples were separated into eight fractions using a High-pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Then, 1 μ g of peptides from each fraction were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) on an EASY-nLC 1,200-connected Orbitrap Fusion Lumos Tribrid MS (Thermo Fisher Scientific) equipped with a High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS)-Pro ion mobility interface (Thermo Fisher Scientific). Peptides were separated on an analytical column (C18, 1.6- μ m particle size $\times$ 75 μ m diameter $\times$ 250 mm; Ion-Opticks, VIC, Australia) using a gradient of 0–28% acetonitrile over 240 min at a constant flow rate of 300 nl/min. Peptide ionization was performed using a Nanospray Flex Ion Source (Thermo Fisher Scientific). The FAIMS-Pro was set to three phases (–40, –60, and –80 CV); a “1-s cycle for a phase” data-dependent acquisition method was used, in which the most intense ions for every 1-s interval were selected for MS/MS fragmentation by higher-energy C-trap dissociation (HCD). MS raw files were analyzed using the Sequest HT search program in Proteome Discoverer 2.4 (Thermo Fisher Scientific). MS/MS spectra were searched against the Swiss-Prot-reviewed mouse reference proteome database (UniProt). TMTpro-based protein quantification was performed using the Reporter Ions Quantifier node in Proteome Discoverer 2.4.

Volcano plot

The volcano plot was prepared using VolcaNoseR software (Goedhart and Luijsterburg, 2020).

Enrichment analysis

GO term enrichment related to BP was analyzed by Metascape (<http://metascape.org>; Zhou et al., 2019). Terms with a value of $p < 0.05$, a minimum count of 3, and an enrichment factor > 2.0 were collected and grouped into clusters based on their membership similarities.

Statistical analyses

Data plotted on a linear scale were expressed as the mean $\pm$ SD, except for the mean $\pm$ SEM of body weight change in Figure 1J. Data plotted on logarithmic scales were expressed as the geometric mean $\pm$ geometric SD. Inferential statistical analysis was performed using a two-tailed non-paired Student's *t*-test, Mann-Whitney U test, One-way ANOVA followed by Tukey's test, or Chi-squared test, as appropriate. Statistical significance was set at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, # $p < 0.05$, ## $p < 0.01$). The Prism software package (version 9.1; GraphPad Software, San Diego, CA, United States) was used for all statistical analyses.

Results

Immunization with rDIs-S induces both humoral and cellular immune responses, and protects hACE2 transgenic mice from lethal challenge with SARS-CoV-2 and its variants

rDIs-S was constructed by homologous gene recombination in primary chicken embryonic fibroblasts infected with DIs and transfected with the pSMART-DIs-L3 plasmid vector (Figure 1A). This plasmid carries the full-length spike protein-encoding S gene of an early-pandemic SARS-CoV-2 strain (AI/I-004/2020 strain GISAID EPI_ISL_407084). The recombinant rDIs-S, like rDIs-S (Delta) described in Figure 2, had a slightly low propagation rate compared to the parental DIs (Supplementary Figure 1). Western blotting confirmed the expression of S protein in VeroE6/TMPRSS2 cells infected with rDIs-S (Figure 1B). To determine the immunogenicity of rDIs-S, S protein-specific humoral and cellular immune responses were analyzed in C57BL/6 mice that had been immunized intradermally with either rDIs-S or DIs; immunization was performed two times with a 3-week interval between injections (Figure 1C). In rDIs-S-inoculated mice, immunoglobulin (Ig) G specific for SARS-CoV-2 S protein and

nAb were detected 1 week after the first vaccination, and IgG and nAb levels increased after the second vaccination (Figures 1D,E). In contrast, no S protein-specific antibodies were detected in the DIs-inoculated mice (control group). S protein-specific cellular immune responses were analyzed by *in vivo* cytotoxic T-lymphocyte (CTL) assays. The *in vivo* number of target cells carrying SARS-CoV-2 S protein peptides was significantly decreased in the rDIs-S-inoculated mice compared to the DIs-inoculated mice (Figure 1F).

Next, we examined the protective efficacy of rDIs-S against lethal challenge infection of hACE2 transgenic mice with an early-pandemic SARS-CoV-2 strain (Figures 1G–J). The immunized hACE2 transgenic mice were infected intratracheally with SARS-CoV-2 (TY/WK-521/2020) 1 week after the second vaccination (Figure 1G). All rDIs-S-inoculated mice survived the challenge with SARS-CoV-2 without any decrease in body weight, whereas DIs-inoculated mice succumbed to the SARS-CoV-2 infection, showing a drastic decrease in the body weight 4 days or more after the infection (Figures 1H,I). When assessed 7 days after infection, the titer of infectious SARS-CoV-2 in the lungs of rDIs-S-inoculated mice was below the detection limit; in contrast, the virus was detected in the lungs of 3 of 4 DIs-inoculated mice (Figure 1J). In rDIs-S-inoculated hACE2 transgenic mice, nAb was detected 1 week after the first vaccination, and the nAb level increased after the second vaccination (Figure 1K). The number of T cells specifically producing interferon (IFN) γ was elevated significantly in the rDIs-S-inoculated group compared to the DIs-inoculated group when assessed 7 days after infection with SARS-CoV-2 (Figure 1L).

As a next step, the cross-protective efficacy of rDIs-S against variants, including Beta, Delta, and Omicron BA.1, was investigated. The rDIs-S-inoculated hACE2 transgenic mice were infected intranasally with the Beta or Delta variant 1 week after the second vaccination (Figure 2A). All rDIs-S-inoculated hACE2 mice, but not control [phosphate-buffered saline (PBS)-immunized] mice, survived the lethal challenge with the Beta variant of SARS-CoV-2 (TY8-612 strain) without any decrease in body weight (Figures 2B,C). At Day 7 after infection, the infectious SARS-CoV-2 titer in the lungs of rDIs-S-inoculated mice was below the detection limit in three of four mice, whereas the virus was detected in the lungs of all unvaccinated mice (Figure 2D). Vaccination with rDIs-S also protected mice from lethal challenge with a Delta variant (TY11-927; Figures 2E,F). At Day 10 after infection, the infectious SARS-CoV-2 titer was below the detection limit in the lungs of all of the rDIs-S-inoculated mice (Figure 2G).

As we have reported elsewhere (Matsumoto et al., 2022), we recently generated an adenoviral vector expressing the hACE2-encoding gene under control of the *EF1 α* promoter with a leftward orientation (rAd5 pEF1 α -hACE2-L); this novel transgene vector confers SARS-CoV-2 susceptibility in wild-type mice. Using this model, we investigated the ability of rDIs-S carrying an S gene, from either an early-pandemic strain of SARS-CoV-2 or a Delta variant, to provide protection against the Omicron BA.1 variant (TY38-873; Figure 2H). BALB/c mice were inoculated twice with

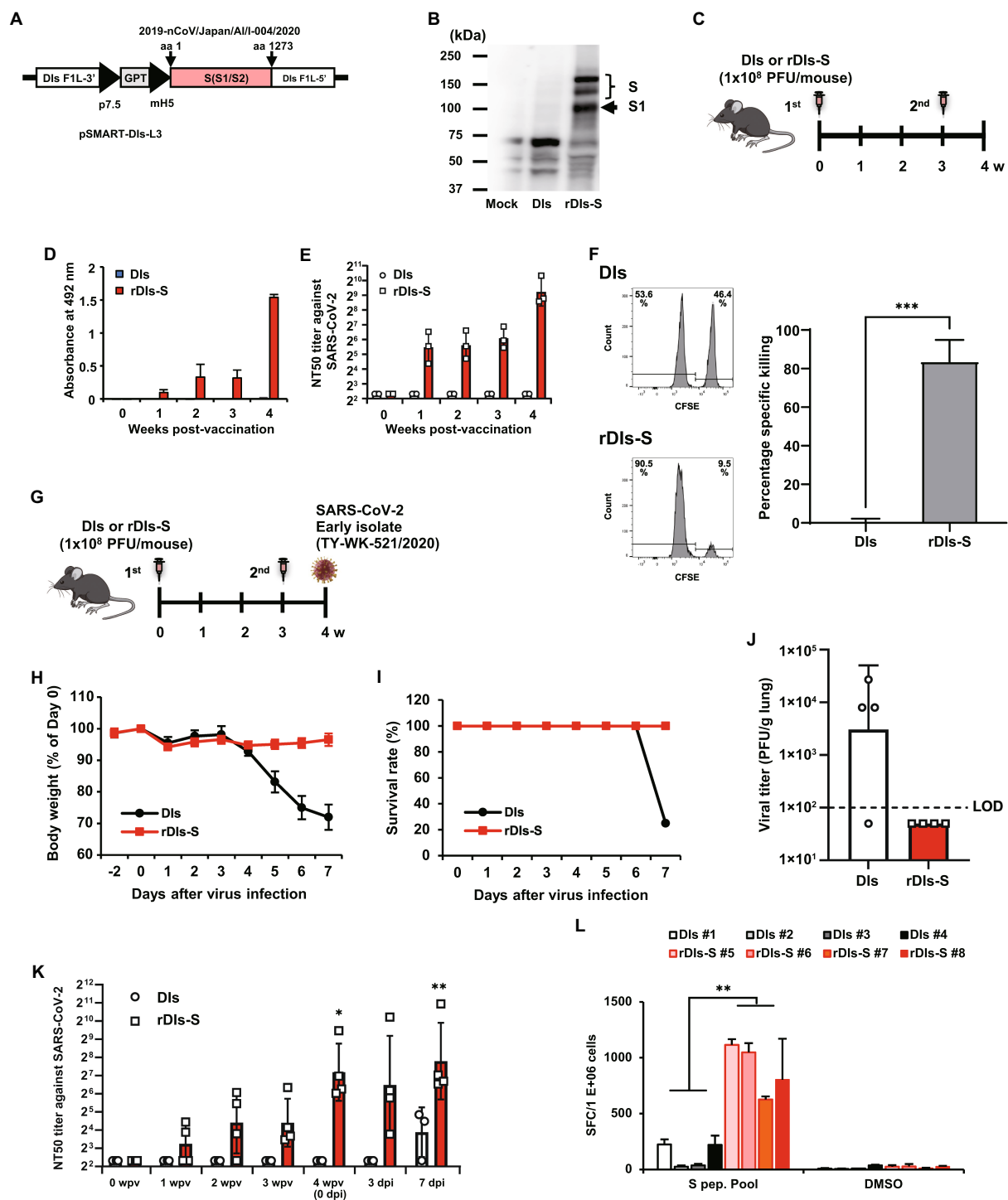


FIGURE 1

Immunization with rDIs-S induces both cellular and humoral immune responses. (A) Construction of the plasmid vector used for generating rDIs-S. (B) Expression of SARS-CoV-2 S protein as detected by western blot analysis. (C) Vaccination schedule in C57BL/6J mice. Nine- to 10-week-old C57BL/6J mice were inoculated twice intradermally with 1×10^8 PFU of rDIs-S or DIs with a 3-week interval. (D) Time course of the production of IgG specific for SARS-CoV-2 S protein as measured by ELISA ($n=3$ per group). (E) Temporal changes in the neutralization titer against the SARS-CoV-2 WK-521 strain ($n=3$ per group). (F) An *in vivo* CTL assay specific for SARS-CoV-2 S protein peptides. The left panels are representative flow cytometry histograms. The right graph shows the mean $\pm$ SD of the specific killing of target cells ($n=3$ per group). Value of p s was calculated using a two-tailed non-paired Student's t -test *** $p < 0.001$. DIs, vaccinia virus DIs strain; rDIs-S, recombinant DIs carrying S gene of SARS-CoV-2; PFU, plaque-forming unit; CTL, cytotoxic T lymphocyte; GPT, gene-encoding xanthine-guanine phosphoribosyltransferase; p7.5, vaccinia virus early promoter; ELISA, enzyme-linked immunosorbent assay; hACE2, human angiotensin-converting enzyme 2; SD, standard deviation. (G) Experimental schedule in hACE2 transgenic mice. Six- to 10-week-old hACE2 transgenic mice were inoculated twice intradermally with 1×10^8 PFU of rDIs-S or DIs with a

(Continued)

FIGURE 1 (Continued)

3-week interval, and then infected intratracheally with 20 TCID₅₀ of SARS-CoV-2 (TY/WK-521/2020) 1 week after the second vaccination. **(H–J)** Protective effect of rDIs-S against challenge with SARS-CoV-2 WK-521 strain ($n=4$ per group). **(H)** Temporal changes in the body weight of hACE2 transgenic mice with or without vaccination after infection with SARS-CoV-2 WK-521 strain. **(I)** Survival rate of hACE2 transgenic mice after SARS-CoV-2 infection. **(J)** Infectious viral titers in left lung homogenates were measured by a plaque assay. The dashed line indicates the limit of detection (LOD; 100PFU/g lung). Viral titers below the LOD are shown as 50. Value of p s was calculated using a two-tailed non-paired Student's t -test ($p=0.1106$). **(K)** Time course of the neutralization titers against TY/WK-521/2020 before and after SARS-CoV-2 infection in hACE2 transgenic mice inoculated with either rDIs-S or DIs. Value of p s was calculated using a two-tailed Kruskal–Wallis test, followed by a Dunn's multiple comparison test (vs. 0 wpv). wpv, weeks post-vaccination; dpi, days post-infection; NT₅₀, 50% neutralization titer; and TCID₅₀, 50% tissue culture infectious dose * $p<0.05$ and ** $p<0.01$. **(L)** The number of IFN- γ -producing cells specific for SARS-CoV-2 S protein peptides was examined by the ELISpot assay. Splenocytes were isolated from SARS-CoV-2-infected animals at 7dpi. Splenocytes (2×10^5 cells/well) were treated for 24h with either DMSO (no-stimulus control) or 1 μ g/ml of spike protein peptides. Triplicate wells for each sample were counted. Results are shown as mean $\pm$ SD of the number of IFN- γ secreting cells per 1,000,000 splenocytes. p values were calculated using a two-tailed non-paired Student's t -test. ** $p<0.01$. IFN, interferon. SFC: spot-forming cell.

a 4-week interval by epicutaneous immunization (skin scarification) with rDIs-S encoding spike protein from either an early-pandemic strain of SARS-CoV-2 or a Delta variant. The expression of Delta variant spike protein was confirmed by western blotting using VeroE6/TMPRSS2 cells infected with rDIs-S (Delta; Figure 2I). Five weeks after the second vaccination, the mice were inoculated intranasally with 5×10^7 focus-forming units (FFU) of rAd5-pEF1 α -hACE2 and challenged (5 days later) with the TY38-873 strain of SARS-CoV-2. Importantly, immunization with rDIs-S, which encodes spike derived from an early-pandemic strain of SARS-CoV-2, prevented the propagation of the virus, demonstrating an efficacy greater than that of rDIs-S encoding spike derived from a Delta variant (Figure 2J). Taken together, these results indicated that rDIs-S efficiently protects mice from challenges not only with “classic” SARS-CoV-2 but also with viral variants.

Protection from SARS-CoV-2 pneumonia in a nonhuman primate model vaccinated with rDIs-S

Human angiotensin-converting enzyme 2 transgenic mice infected with SARS-CoV-2 show much more severe acute morbidity than do human patients. Therefore, we next evaluated the efficacy of rDIs-S in a nonhuman primate model (Ishigaki et al., 2021). Cynomolgus macaques were immunized intracutaneously with rDIs-S or DIs, administered twice with a 3-week interval between injections. To evaluate the protective efficacy of rDIs-S, the immunized macaques were infected with SARS-CoV-2 TY-WK-521/2020 via the conjunctiva, nasal cavity, oral cavity, and trachea 1 week after the second vaccination; this infection protocol is known to induce clinical signs of disease in unvaccinated macaques (Ishigaki et al., 2021). Of the four macaques immunized with DIs, two and three macaques displayed infectious virus in nasal swab samples and lung tissue samples, respectively, at Day 7 after SARS-CoV-2 inoculation; in contrast, no infectious virus was detected in macaques immunized with rDIs-S, either in nasal swab samples at Day 3 or in lung tissue samples at Day 7 (Figure 3A;

Supplementary Tables 1, 2). The virus titer area under the curve (AUC) of the macaques immunized with rDIs-S was significantly lower than that of macaques inoculated with DIs (Figure 3B). Viral RNA was detected in the nasal swab samples, oral swab samples, and lung tissues of all four of the DIs-immunized macaques 7 days after infection with SARS-CoV-2 virus (Figure 3C; Supplementary Figure 2). In contrast, in macaques immunized with rDIs-S, the levels of SARS-CoV-2 viral RNA in the trachea, bronchus, and a part of the lung tissues were below the detection limit. Thus, vaccination with rDIs-S prevented the propagation of SARS-CoV-2 in cynomolgus macaques.

We also examined the vaccinated macaques for clinical signs of disease after infection. All four of the DIs-immunized macaques showed increases in body temperature after infection with SARS-CoV-2 (Supplementary Figure 3). Among these four macaques, three showed a body temperature higher than 39°C during the daytime. In contrast, of four rDIs-S-immunized macaques (V1–V4), three did not show an increase in body temperature during the daytime, while the remaining animal (V4) showed a body temperature increase for the first 3 days after infection. Among the infected macaques, the clinical scores (which were determined based on body temperature, appetite, posture, and behavior; Supplementary Table 3) were lower in the macaques immunized with rDIs-S than in those immunized with DIs (Supplementary Figures 4A–D). Thus, vaccination with rDIs-S attenuated the clinical signs of disease after SARS-CoV-2 infection in cynomolgus macaques.

Next, the effects of vaccination with rDIs-S on viral pneumonia were examined by X-ray radiography and histological examination of post-mortem samples. On chest X-ray radiography, all four macaques immunized with DIs showed a ground glass appearance in areas of the lungs by Day 3 after infection with SARS-CoV-2 (Figure 3D). However, no apparent radiographic changes were detected in the lungs of macaques immunized with rDIs-S and subsequently infected with SARS-CoV-2. Macroscopic observations at necropsy revealed dark red lesions on the surfaces of the lungs in the macaques immunized with DIs and subsequently infected (Supplementary Figure 5A), whereas a very mild reddish change was seen in the lung of only

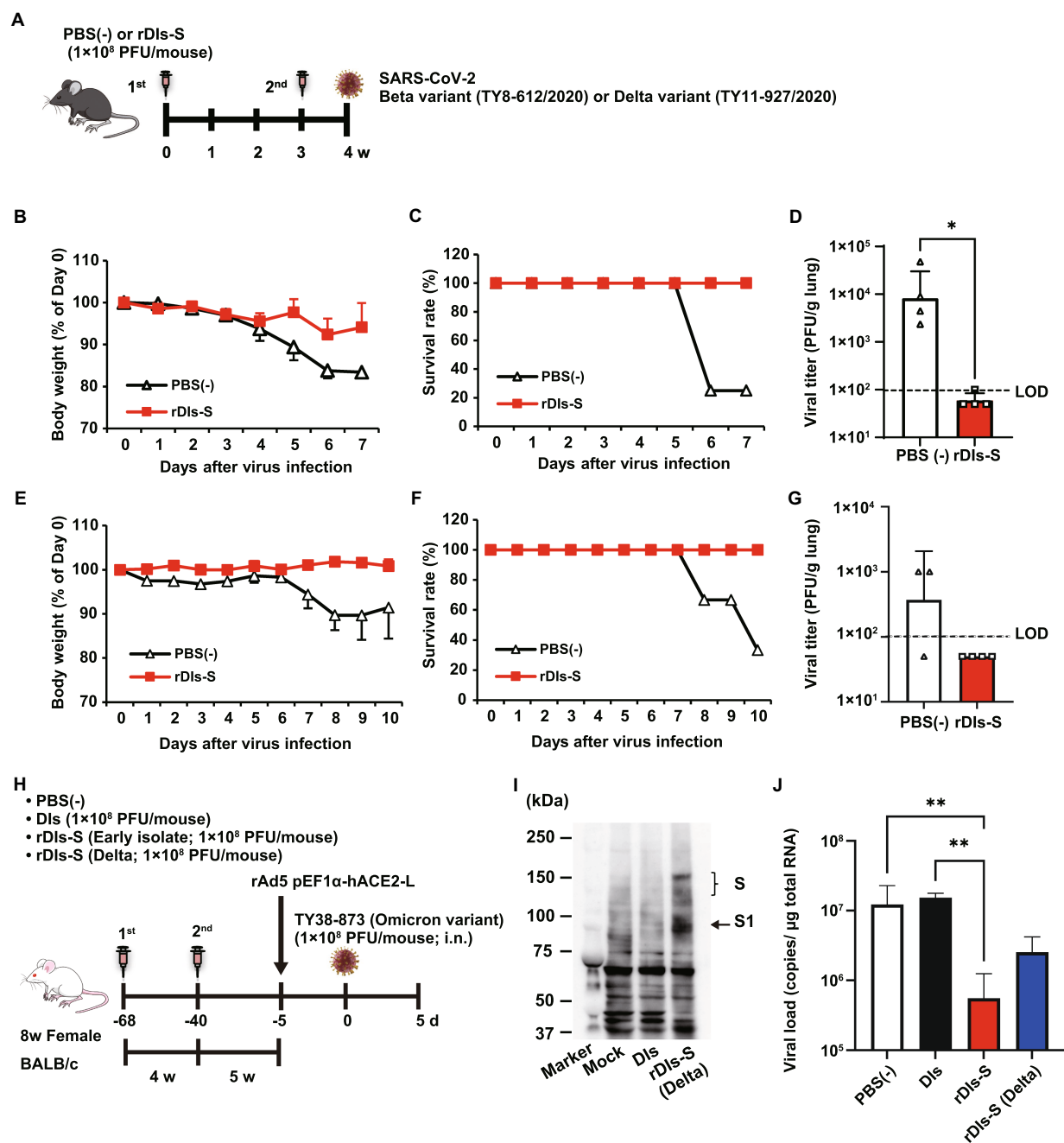


FIGURE 2

Immunization with rDIs-S protects mice from lethal challenge with SARS-CoV-2. (A) Experimental schedule in human angiotensin-converting enzyme 2 (hACE2) transgenic mice. hACE2 transgenic mice were inoculated twice intradermally with 1×10^8 PFU of rDIs-S or PBS(-) with a 3-week interval, and then infected intratracheally with 100PFU of a Beta variant (TY8-612) or 50PFU of a Delta variant (TY11-927) SARS-CoV-2 strain 1week after the second vaccination. (B–D) Protective effect of rDIs-S against challenge with a Beta variant of SARS-CoV-2 (TY8-612 strain; $n=4$ per group). (B) Temporal changes in the body weight of hACE2 transgenic mice, with or without vaccination, after infection with the Beta variant. (C) Survival rate of hACE2 transgenic mice after Beta variant infection. (D) Infectious viral titers in left lung homogenates were measured by a plaque assay. The dashed line indicates the limit of detection (LOD; 100PFU/g lung). Viral titers below the LOD are shown as 50. Value of p was calculated using a two-tailed non-paired Student's t -test (* $p=0.0286$). (E–G) Protective effect of rDIs-S against challenge with a Delta variant of SARS-CoV-2 (TY11-927 strain; $n=3$ –4 per group). (E) Temporal changes in the body weight of hACE2 transgenic mice after infection with the TY11-927 strain. (F) Survival rates after infection with the TY11-927 strain. (G) Infectious viral titers in left lung homogenates were measured by a plaque assay. The dashed line indicates the LOD (100PFU/g lung). The pulmonary viral titers of unvaccinated mice are shown at the respective necropsy (8, 9, or 10dpi). Viral titers below the LOD are shown as 50. (H) Experimental schedule in BALB/c mice. BALB/c mice were inoculated twice by epicutaneous immunization (skin scarification) with 1×10^8 PFU of rDIs-S (derived from an early-pandemic strain of SARS-CoV-2 or a Delta variant), DIs, or PBS(-) with a 4-week interval. Five weeks after the second vaccination, the mice were intranasally inoculated with 5×10^7 FFU of rAd5-pEF1 α -hACE2 and challenged with 1×10^8 PFU of an Omicron variant of SARS-CoV-2 5days later. (I) Expression of the S protein derived from the SARS-CoV-2 Delta variant following infection with rDIs-S (Delta) was confirmed by western blot analysis. (J) Viral RNA in left lung homogenates was quantified by qRT-PCR. Value of p s was calculated using a two-tailed Kruskal–Wallis test, followed by a Dunn's multiple comparison test. ** $p < 0.01$; ns, not significant; FFU, focus-forming unit; PFU, plaque-forming unit; and PBS, phosphate-buffered saline.

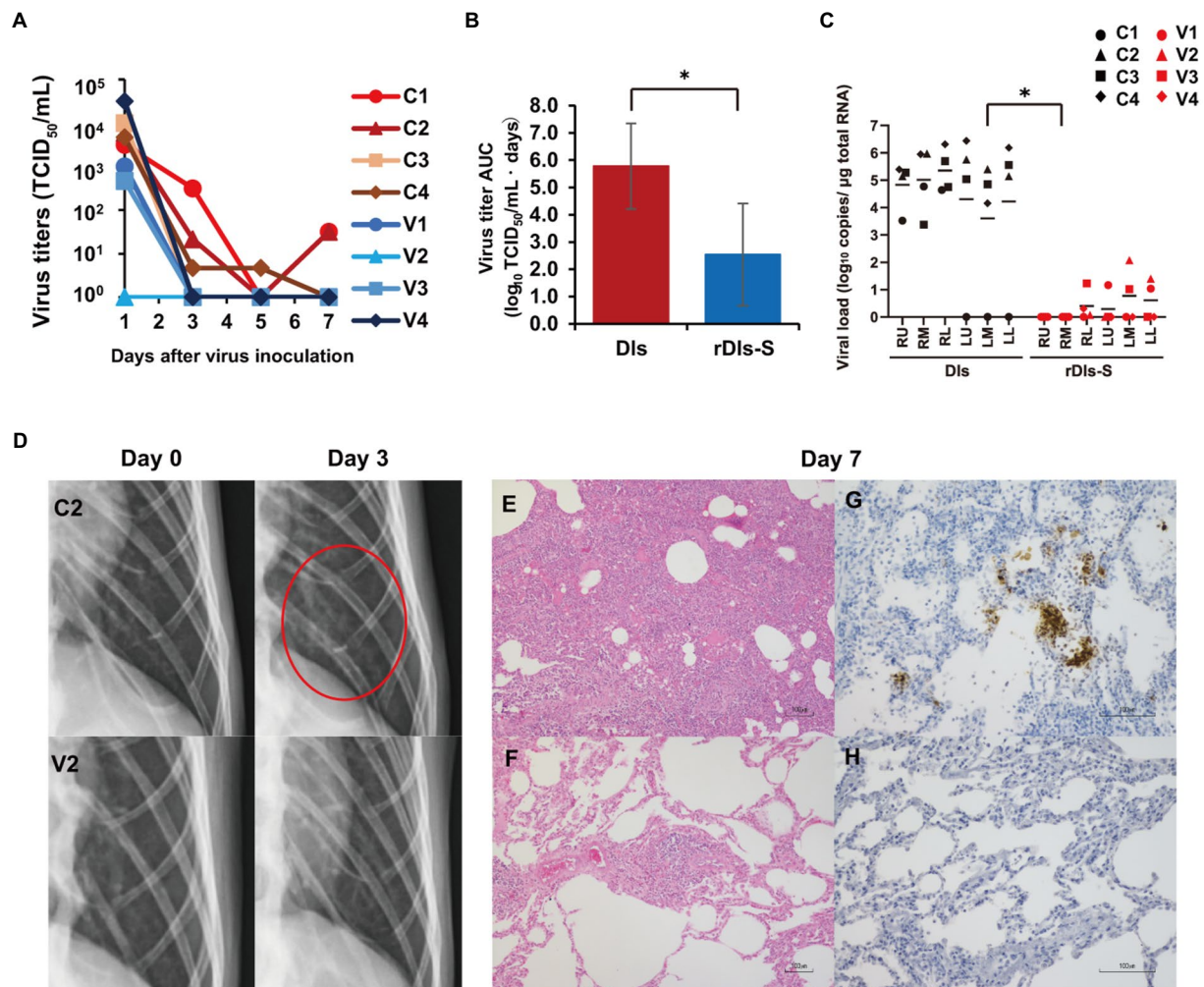


FIGURE 3

Protection from SARS-CoV-2 pneumonia in macaques vaccinated with rDIs-S. Cynomolgus macaques were immunized intradermally with DIs (C1–C4) or rDIs-S (V1–V4). One week after the second vaccination, SARS-CoV-2 strain WK-521 was inoculated into the conjunctiva, nostril, oral cavity, and trachea of each macaque at Day 0. (A) Nasal swab samples were collected on the indicated days after virus inoculation. Viral titers below the limit of detection (LOD; $0.67\log_{10}\text{TCID}_{50}/\text{mL}$) are shown as 10^0 . (B) Means and standard deviations of virus titer areas under the virus titer time curve (virus titer AUCs) in nasal swab samples were calculated based on the titers shown in (A) and [Supplementary Table 1](#). Virus titers under the detection limit were treated as 0 in the calculations. Means and standard deviations across each quartet of macaques are shown. Red: DIs; blue: rDIs-S. Value of p was calculated using a two-tailed Student's t -test ($*p=0.039$). (C) Lung tissues were collected 7 days after virus inoculation. Viral RNA was quantified by qRT-PCR. RU, right upper lobe; RM, right middle lobe; RL, right lower lobe; LU, left upper lobe; LM, left middle lobe; and LL, left lower lobe. Viral loads of all lung lobes were compared between DIs and rDIs-S using a two-tailed Student's t -test ($*p=0.012$). (D) X-ray radiography was performed at Day 0 before infection and at Day 3 after infection. Representative photos are shown. The red circle indicates the ground glass appearance. (E,F) Hematoxylin- and eosin-stained sections of lung. (G,H) Immunohistochemical staining of lung sections for SARS-CoV-2 N antigen (brown color). Nuclei were stained with hematoxylin. Bars indicate $100\mu\text{m}$ in E–H. (E,G) C2: a macaque immunized with DIs. (F,H) V2: a macaque immunized with rDIs-S. Bars indicate $100\mu\text{m}$.

one of the four macaques (V4) vaccinated with rDIs-S and subsequently infected ([Supplementary Figure 5B](#)). In macaques immunized with DIs, thickened alveolar walls, exudates, and hyaline membrane formation were observed in the lung tissues 7 days after infection with SARS-CoV-2; these changes were attenuated in the macaques immunized with rDIs-S and subsequently infected, as confirmed by the histological scoring ([Figures 3E,F](#); [Supplementary Figure 5C](#)). At necropsy of the infected macaques, the relative (body weight-normalized) lung weight of the macaques immunized with rDIs-S was nominally

(though not significantly) lower than that of the macaques immunized with DIs ($p=0.065$; [Supplementary Figure 5E](#)), consistent with histological observations indicating pneumonia ([Supplementary Figure 5D](#)). Additionally, SARS-CoV-2 nucleoprotein (N protein)-positive cells formed clusters that were distributed sparsely in the lungs of the macaques immunized with DIs, whereas no N protein-positive cells were detected in the lung tissues of the macaques immunized with rDIs-S ([Figures 3G,H](#)). Thus, vaccination with rDIs-S prevented viral pneumonia in cynomolgus macaques.

Immune responses in a nonhuman primate model following vaccination with rDIs-S

We next examined the acquired immune responses responsible for the protection in macaques immunized with rDIs-S. Among macaques immunized with rDIs-S, IgG antibodies specific for the SARS-CoV-2 S protein, including those with specificity for the receptor-binding domain (RBD) and domains 1 and 2 of the S protein (S1 and S2), were detected in the plasma 10 days after the first vaccination (Figures 4A,C), and the levels of those antibodies increased after the second vaccination. No IgG specific for S1, S2, or RBD was detected in the plasma of macaques inoculated with DIs 1 week after the second vaccination and 7 days after the challenge infection (Figure 4D). SARS-CoV-2-specific nAbs against the challenge strain WK-521 (Clade S) and variant strains SUMS2 (Clade GR, Pango lineage B.1.1), QHN001 (Clade GRY, Pango lineage B.1.1.7), TY7-501 (Clade GR/501Y.V3, Pango lineage P.1), and TY8-612 (Clade GH/501Y.V2, Pango lineage B.1.351; Supplementary Table 4) were detected in the plasma of the macaques immunized with rDIs-S, and the neutralization titers increased after challenge infection with WK-521, indicating the activation of memory responses after infection (Figures 4B,E). On the other hand, no nAb specific for SARS-CoV-2 was detected in the plasma of the DIs-immunized macaques at Day 7 after challenge infection. T-cell responses specific for SARS-CoV-2 S protein peptides were detected 7 days after the second vaccination with rDIs-S (Figures 4F,G). The ratio of IFN- γ - and interleukin (IL)-2-producing cells increased after the second rDIs-S vaccination and challenge infection. Thus, humoral and cellular immunity specific for SARS-CoV-2 was induced effectively in macaques immunized with rDIs-S.

Prevention of inflammatory responses in macaques and hACE2 transgenic mice vaccinated with rDIs-S

The levels of systemic and local inflammation after SARS-CoV-2 infection and the effects of vaccination on cytokine responses were examined in macaques and hACE2 transgenic mice. The levels of the inflammatory cytokine IL-6 and the chemokine monocyte chemoattractant protein-1 (MCP-1) in the plasma of the macaques inoculated with DIs were increased at Day 1 after challenge infection, whereas no such increase was seen in the plasma of the macaques vaccinated with rDIs-S and subjected to challenge infection (Supplementary Figure 6). The levels of IL-15, granulocyte colony-stimulating factor (G-CSF), and IFN- γ in the plasma of the macaques immunized with DIs showed a similar increase at Day 1 after challenge infection, and the slight elevation persisted at Days 3 and 5. On the other hand, no significant increase in IL-15, G-CSF, or IFN- γ levels was detected

following challenge infection in the rDIs-S-vaccinated macaques. Thus, vaccination with rDIs-S prevented inflammatory responses in cynomolgus macaques.

Changes in protein expression levels in the lungs of hACE2 transgenic mice 7 days after virus infection were analyzed comprehensively by mass spectrometry (MS)-based quantitative proteomics using a tandem mass tag (TMT) reagent (Supplementary Figure 7A). In the lungs of DIs-immunized hACE2 transgenic mice infected with WK-521, a total of 177 proteins showed significantly increased expression (mean fold-change ≥ 2.0 , value of $p < 0.05$), and 251 proteins showed significantly decreased expression (mean fold-change ≤ 0.5 , value of $p < 0.05$) compared to lung tissue from uninfected mice (Figure 5A, top). On the other hand, the expression levels of 278 and 32 proteins were increased and decreased, respectively, in the lungs of infected mice that had been vaccinated with rDIs-S, compared to the uninfected mice (Figure 5A, middle). The expression levels of 36 and 82 proteins were increased and decreased, respectively, in the lungs of infected mice that had been vaccinated with rDIs-S, compared to the infected mice that had been immunized with DIs (Figure 5A, bottom).

The proteins with increased and decreased levels in the DIs-immunized mice following infection were submitted for gene ontology (GO) term enrichment analyses using Metascape for terms related to biological processes (BP; Zhou et al., 2019). The proteins with increased levels in the infected mice that had been immunized with DIs were significantly enriched in terms related to phagocytosis, blood coagulation, and inflammatory response (Figure 5B, upper), consistent with results obtained for COVID-19 patients. On the other hand, the proteins with decreased levels in the infected mice that had been immunized with DIs were significantly enriched in terms related to cytoplasmic translation, ribosome biogenesis, and negative regulation of chromatin silencing (Figure 5B, lower), indicating that the *de novo* synthesis of proteins was significantly suppressed by SARS-CoV-2 infection. Of the 177 proteins with increased levels in DIs-immunized mice following infection, 57 showed decreases of greater than 2-fold in rDIs-S-vaccinated mice following infection (Figure 5A, bottom and Figure 5C). In comparison, of the 251 proteins that were depleted in the DIs-immunized mice following infection, 28 showed increases of greater than 2-fold in the rDIs-S-vaccinated mice following infection (Figure 5A, bottom and Figure 5C). GO enrichment terms related to BP were analyzed for the 57 and 28 proteins that showed divergent changes in expression between the infected DIs-immunized mice and infected rDIs-S-vaccinated mice (Figure 5D). All of the proteins listed under the top-13 GO enrichment terms of the upregulated proteins are shown in Supplementary Figure 7B. Furthermore, overlap analysis of the proteins listed under the top-13 GO enrichment terms showed that these proteins include multiple GO enrichment terms (Supplementary Figure 7C). The expression levels of proteins associated with fibrinolysis (coagulation), inflammatory

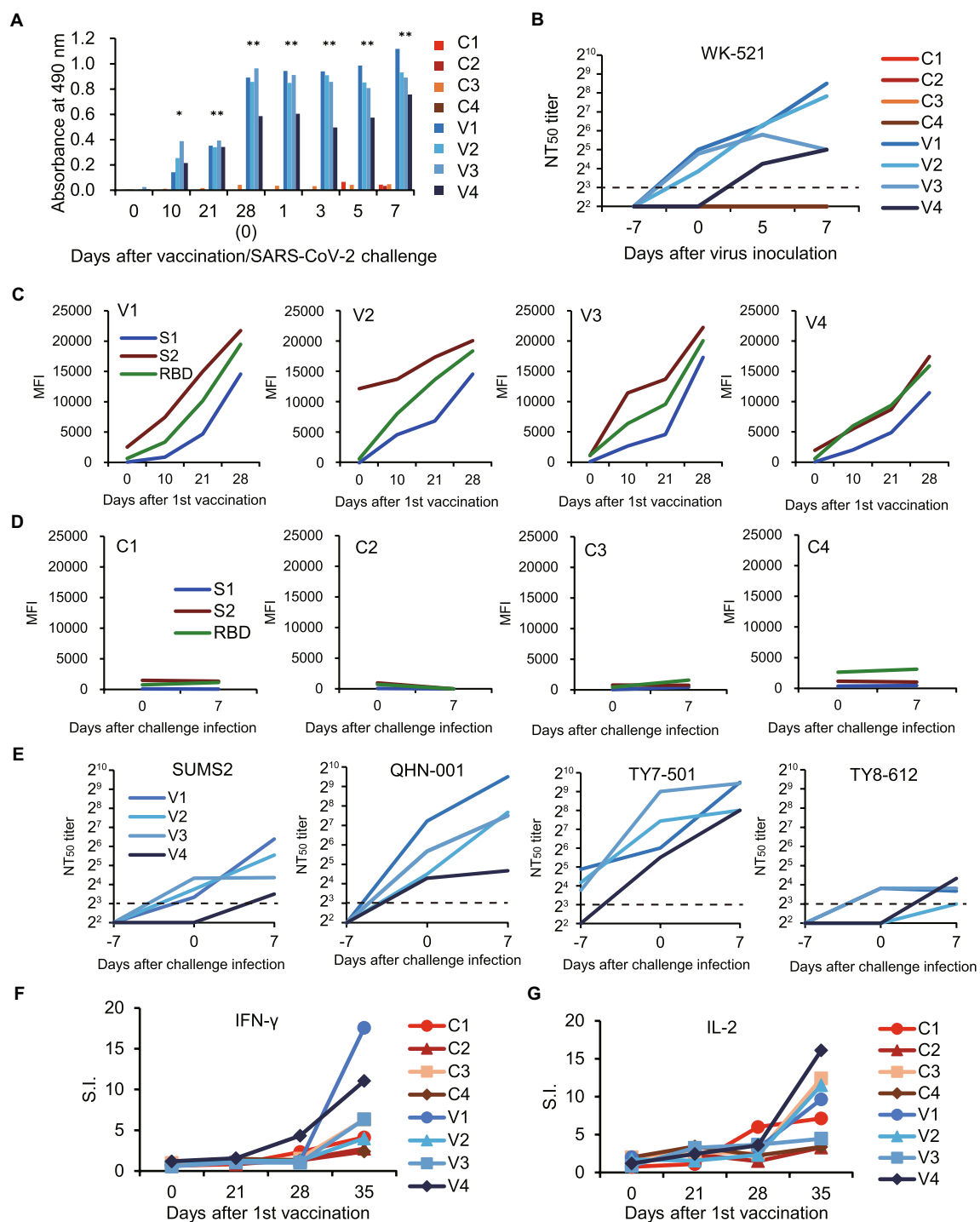


FIGURE 4

Immune responses in macaques vaccinated with rDIs-S. The macaques were immunized intradermally with DIs (C1–C4) or rDIs-S (V1–V4). One week after the second vaccination, SARS-CoV-2 strain WK-521 was inoculated at Day 0. **(A)** The levels of IgG specific for SARS-CoV-2 S protein in the plasma of macaques immunized with DIs (C1–C4) and macaques immunized with rDIs-S (V1–V4) were analyzed using ELISA. Plasma was collected after the first vaccination (Days 0–28) and after challenge infection (Days 1–7). Significant differences were calculated by a two-tailed unpaired Student's *t*-test (**p* < 0.05, ***p* < 0.01). **(B)** 50% neutralization titers (NT₅₀) of plasma against strain WK-521. Plasma was collected from macaques on the indicated days after the first vaccination. Day 7: the day of the second vaccination and 7 days before challenge infection. Day 0: the day of challenge infection. Day 0 samples were collected before infection. Days 5 and 7: 5 and 7 days (respectively) after challenge infection. **(C,D)** Plasma IgG specific for SARS-CoV-2 S1, S2, and receptor-binding domain (RBD). MFI, mean fluorescence intensity. **(E)** Levels of neutralizing antibodies against variants of SARS-CoV-2. NT₅₀ of plasma against variant strains with amino acid changes in the S protein were measured. **(F,G)** The numbers of IFN-γ-producing cells **(F)** and IL-2-producing cells **(G)** specific for SARS-CoV-2 S protein peptides were examined by the ELISpot assay. Stimulation indices (S.I.) were calculated as follows: S.I. = number of spots in the culture of cells with peptides/number of spots in the culture of cells without peptides.

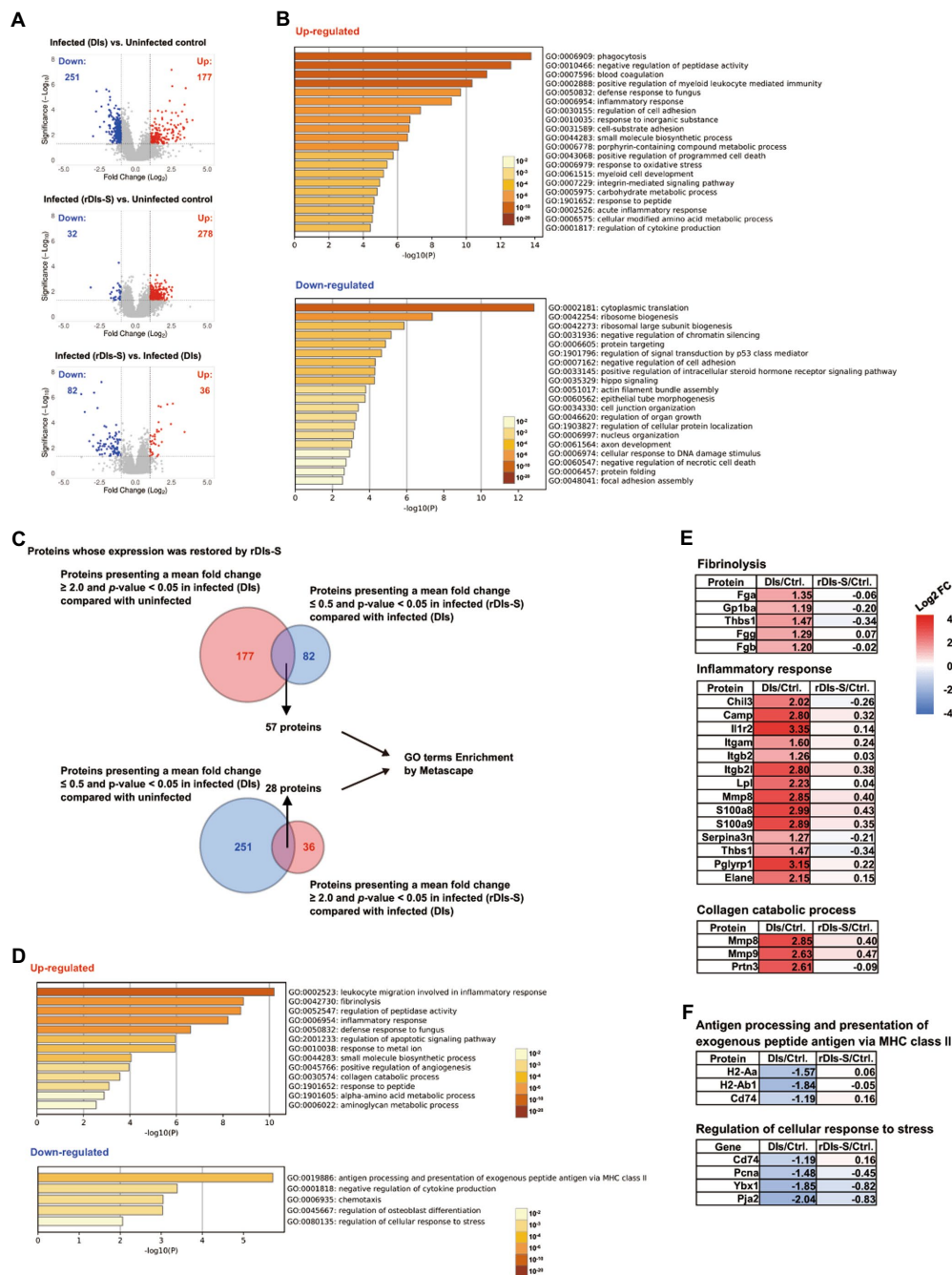


FIGURE 5

Proteomic analysis in vaccinated mice following SARS-CoV-2 infection. The protein expression levels in the lung tissues of hACE2 transgenic mice 7 days after challenge infection were analyzed using multiplex peptide labeling and mass spectrometry (MS). (A) Volcano plot for differentially expressed proteins. Comparison of the protein expression levels between uninfected mice and infected mice inoculated with DIs (top), between uninfected mice and infected mice inoculated with rDIs-S (middle), and between infected mice inoculated with DIs and infected mice inoculated with rDIs-S (bottom). x-axis: expression ratios. y-axis: p values of the comparisons. Vertical dotted lines indicate a 2-fold increase or decrease in the protein level in mice inoculated with DIs or rDIs-S. Horizontal lines indicate a p value of 0.05 from a Chi-squared test. Red circles: proteins with concentration increases of more than 2.0-fold in each comparison (DIs vs. Uninfected, rDIs-S vs. Uninfected, and rDIs-S vs. DIs); blue circles: proteins with concentrations decreased to less than half in each comparison (DIs vs. Uninfected, rDIs-S vs. Uninfected, and rDIs-S vs. DIs). (B) The top-20 gene ontology (GO) enrichment terms related to biological processes (BP) of the proteins that were upregulated (upper) and downregulated (lower), as analyzed by Metascape. (C) Number of proteins with altered expression in infected mice without vaccination for which expression was restored by rDIs-S vaccination. (D) GO enrichment terms related to BP of the genes encoding the proteins that were restored by rDIs-S vaccination among the upregulated (upper) and downregulated (lower) proteins in the infected (DIs-inoculated) mice. (E) Representative cluster of GO enrichment terms related to BP of the genes encoding the proteins that were restored by rDIs-S vaccination, among the upregulated proteins in the infected (DIs-inoculated) mice. (F) Representative cluster of GO enrichment terms related to BP of the genes encoding the proteins that were restored by rDIs-S vaccination, among the downregulated proteins in the infected (DIs-inoculated) mice.

proteins, and collagen catabolism (tissue destruction) were significantly lower in the rDIs-S-vaccinated mice than in the DIs-immunized mice, as were the expression levels of proteins related to leukocyte migration involved in inflammatory response, peptidase activity, defense responses to fungus, apoptotic signaling, responses to metal ions, small-molecule biosynthetic processes, angiogenesis, responses to peptides, α -amino acid metabolic processes, and aminoglycan metabolic processes (Figure 5E; Supplementary Figure 7D). On the other hand, the expression levels of proteins involved in antigen presentation, negative regulation of cytokine production, chemotaxis, osteoblast differentiation, and cellular response to stress were decreased in the DIs-immunized mice compared to the rDIs-S-vaccinated mice and uninfected mice (Figure 5F; Supplementary Figure 8). Although the expression levels of proteins involved in phagocytosis, oxidative stress, and protein transport were increased in the infected mice (whether DIs-immunized and rDIs-S-vaccinated; Supplementary Figure 9), the expression levels tended to be lower in the rDIs-S-vaccinated mice than in the DIs-immunized mice. The expression levels of proteins involved in tissue repair processes, such as gene expression, cell junction assembly, and cellular response to growth stimulus, were decreased even in the rDIs-S-vaccinated mice (Supplementary Figure 10), but the number of proteins in this category was only 14. The magnitude of the decrease in the levels of these proteins was smaller in rDIs-S-vaccinated mice than in DIs-immunized mice. Taken together, these results indicated that, among mice subjected to challenge infection with WK-521, vaccination with rDIs-S prevents gene expression indicative of tissue destruction and of lung inflammation, and restores gene expression indicative of immune responses and tissue repair processes, changes that are otherwise observed in DIs-immunized mice upon infection.

Long-term humoral immune responses following vaccination with rDIs-S

To investigate the ability of rDIs-S to establish a long-lived immunological memory, 8-week-old BALB/c mice were vaccinated twice with a 3-week interval, and the antibody responses specific to S protein were monitored by enzyme-linked immunosorbent assay (ELISA) using the S protein ectodomain trimer as an antigen. As shown in Figure 6, S protein-specific IgG was detected 3 weeks after the first vaccination, and the IgG titer was increased significantly after the second vaccination. Importantly, the titer of S protein-specific IgG was maintained at the same level from 4 to 24 weeks after the second vaccination (7–27 weeks after the first vaccination), indicating that the titers of S protein-specific IgG induced by rDIs-S were maintained for at least 6 months after vaccination. This result raises the possibility that rDIs-S confers long-term protection against SARS-CoV-2 infection (Levin et al., 2021).

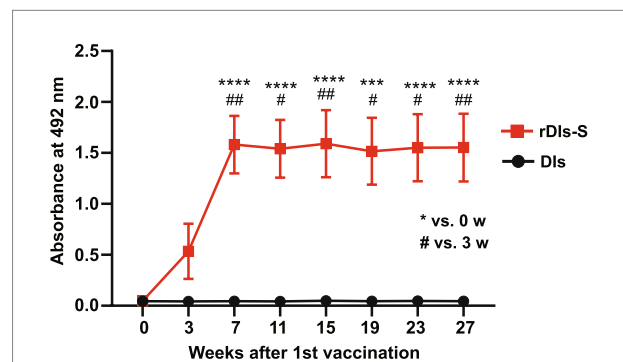


FIGURE 6

Time course of antibody responses after vaccination with rDIs-S in mice. BALB/c mice were inoculated twice intradermally with 1×10^8 PFU of rDIs-S or DIs with a 3-week interval. Time course of the production of IgG specific for SARS-CoV-2 S protein as measured by ELISA ($n = 12$ per group). p values were calculated using a two-tailed Kruskal–Wallis test, followed by a Dunn's multiple comparison test.

Discussion

This study demonstrated the efficacy of rDIs-S, an attenuated vaccinia virus vaccine engineered to encode the SARS-CoV-2 S protein, against SARS-CoV-2 infection; the efficacy was assessed in mouse and macaque models. Two vaccinations with rDIs-S induced nAbs against not only the “classic” (original) SARS-CoV-2 strain isolated in early 2020 but also variant strains, while also inducing IFN- γ -producing T cells specific for SARS-CoV-2 S antigen. These effects resulted in a decrease in SARS-CoV-2 virus titers, along with protection from lethal infection in hACE2 transgenic mice and protection from pneumonia in cynomolgus macaques. A comprehensive analysis of protein levels in SARS-CoV-2-infected mice showed that the expression of proteins involved in tissue damage and inflammation was attenuated in the DIs-S-vaccinated mice compared to the DIs-immunized animals.

In the present study, we immunized hACE2 transgenic mice and the cynomolgus macaques twice with rDIs-S. After the second vaccination, nAb titers against SARS-CoV-2 and the amounts of IFN- γ produced by T lymphocytes were increased from the pre-immunization baseline levels. In addition, we previously reported that a second vaccination with a vaccinia virus carrying the SARS-CoV S gene enhanced nAb responses against SARS-CoV and vaccinia virus in rabbits that had nAb against these viruses after the first vaccination (Kitabatake et al., 2007). These observations suggest that rDIs-S will induce immune responses specific for SARS-CoV-2 in people who previously have been immunized with the attenuated vaccinia virus and may have antibodies against vaccinia virus, and a repeated vaccination is expected to enhance and maintain immunological memory against SARS-CoV-2. This effect may make rDIs-S advantageous compared to SARS-CoV-2 vaccines employing other virus vectors, such as the adenovirus vaccine encoding the SARS-CoV-2 S protein that is recommended as a single-dose vaccination.

We note, however, that a booster effect was reported in aged mice vaccinated twice with the adenovirus vaccine carrying the SARS-CoV-2 S gene (Mercado et al., 2020; Silva-Cayetano et al., 2021).

The results of the present study, including the induction of nAbs against the early-pandemic SARS-CoV-2 strain and protective efficacy, are consistent with the results of previous studies in which mice (Chiuppesi et al., 2020; García-Arriaza et al., 2021; Liu et al., 2021; Pan et al., 2021; Tscherne et al., 2021) and rhesus macaques (Routhu et al., 2021) were immunized with modified vaccinia Ankara strains carrying the SARS-CoV-2 S gene. Those studies, like ours, confirmed the safety of vaccinia-based vaccines and their immunogenicity in animals vaccinated repeatedly, indicating that vaccinia-based vaccines may be usable even in younger populations and in the elderly with pre-existing immunity against smallpox (Kitabatake et al., 2007). Furthermore, our results demonstrated the efficacy of rDIs-S against variant strains, since neutralization activity was seen against the variant strains in macaques, and an improved survival rate was seen in hACE2 transgenic mice, a model that had not been examined in other studies. Therefore, we expect that rDIs-S will confer broad protection against multiple variants of SARS-CoV-2, at least as indicated by broadly reactive nAbs (Yasui et al., 2016).

Using TMT-based quantitative proteomic analysis of lung homogenates from uninfected, DIs-immunized, and rDIs-S-immunized mice, we found that inoculation with rDIs-S protected the mice from the severe pathogenic effects of SARS-CoV-2 infection, such as tissue destruction, inflammation, coagulation, fibrosis, and angiogenesis. These changes in protein expression, which were observed in the control (DIs-immunized) mice after infection with SARS-CoV-2, also are seen in critical COVID-19 patients (Nie et al., 2021), indicating the utility of the hACE2 transgenic mouse model for evaluating the potential protective efficacy of vaccines against severe COVID-19 symptoms. In addition, since the TMT-proteomic analysis detects changes in protein levels in a comprehensive and sensitive manner, this technology also may serve as a safety evaluation system to identify factors related to the adverse events that have been seen with the current vaccines (Cines and Bussel, 2021). Of note, coagulation factors are thought to be activated by COVID-19 and vaccination (Kollias et al., 2020; Cines and Bussel, 2021), but the expression levels of the coagulation factors in the rDIs-S-vaccinated mice were comparable to those in the uninfected mice.

We demonstrated that rDIs-S provides long-lived humoral immune responses for at least 6 months after vaccination in mice. Recent reports have shown that the antibody levels induced by the current mRNA vaccine decline dramatically 6 months after the second vaccination (Levin et al., 2021; Kato et al., 2022). Thus, additional periodic vaccination would be required for the control of COVID-19 using the current vaccines. However, since the current mRNA vaccines may cause undesirable adverse events, the long-term immune memory response conferred by rDIs-S may be a useful advantage for the development of new vaccines.

In the present study, we demonstrated the efficacy of rDIs-S, an attenuated vaccinia virus carrying the SARS-CoV-2 S gene.

Furthermore, given that vaccination with rDIs-S effectively induced antibody and T-lymphocyte responses that also reacted with variant strains, rDIs-S may be useful for conferring protection against new variants by use as a booster after vaccination with first-generation SARS-CoV-2 vaccines.

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

This study was carried out in strict accordance with the “Guidelines for the Husbandry and Management of Laboratory Animals” of the Tokyo Metropolitan Institute of Medical Science and the Research Center for Animal Life Science at Shiga University of Medical Science, and with the “Standards Relating to the Care and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions” under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan. The animal experimental protocols were approved by the Ethics Committee of Animal Experiments of the Toyo Metropolitan Institute of Medical Science (Permission Nos. 20–85, 20–86, 21–79, and 21–080), and by the Shiga University of Medical Science Animal Experiment Committee (Permit No. 2020-6-20). In the macaque study, regular veterinary care and monitoring, balanced nutrition, and environmental enrichment were provided by personnel of the Research Center for Animal Life Science at Shiga University of Medical Science.

Author contributions

YI and MK conceived the study. HI, FY, MN, AE, NY, KY, CN, YK, TS, TH, TM, MH, ST, RK, AT, YM, AK, KH, and MS performed the experiments. KI and YS supervised the study. HI, FY, MN, AE, NY, CN, TS, TH, TM, YM, YS, YI, and MK participated in data analysis, interpretation, and manuscript review. YI, FY, and MK wrote 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.

References

- Altenburg, A. F., Kreijtz, J., de Vries, R., Song, F., Fux, R., Rimmelzwaan, G., et al. (2014). Modified vaccinia virus Ankara (MVA) as production platform for vaccines against influenza and other viral respiratory diseases. *Viruses* 6, 2735–2761. doi: 10.3390/v6072735
- Chiappesi, F., Salazar, M. A., Contreras, H., Nguyen, V. H., Martinez, J., Park, Y., et al. (2020). Development of a multi-antigenic SARS-CoV-2 vaccine candidate using a synthetic poxvirus platform. *Nat. Commun.* 11:6121. doi: 10.1038/s41467-020-19819-1
- Cines, D. B., and Bussell, J. B. (2021). SARS-CoV-2 vaccine-induced immune thrombotic thrombocytopenia. *N. Engl. J. Med.* 384, 2254–2256. doi: 10.1056/NEJMe2106315
- Coronaviridae Study Group of the International Committee on Taxonomy of Viruses (2020). The species severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat. Microbiol.* 5, 536–544. doi: 10.1038/s41564-020-0695-z
- Dai, L., and Gao, G. F. (2021). Viral targets for vaccines against COVID-19. *Nat. Rev. Immunol.* 21, 73–82. doi: 10.1038/s41577-020-00480-0
- Folegatti, P. M., Ewer, K. J., Aley, P. K., Angus, B., Becker, S., Belij-Rammerstorfer, S., et al. (2020). Safety and immunogenicity of the ChAdOx1 nCoV-19 vaccine against SARS-CoV-2: a preliminary report of a phase 1/2, single-blind, randomised controlled trial. *Lancet* 396, 467–478. doi: 10.1016/S0140-6736(20)31604-4
- García-Arriaza, J., Garaigorta, U., Pérez, P., Lázaro-Frías, A., Zamora, C., Gastaminza, P., et al. (2021). COVID-19 vaccine candidates based on modified vaccinia virus Ankara expressing the SARS-CoV-2 spike induce robust T- and B-cell immune responses and full efficacy in mice. *J. Virol.* 95, e02260–e02320. doi: 10.1128/JVI.02260-20
- Goedhart, J., and Luijsterburg, M. S. (2020). Volca NoseR is a web app for creating, exploring, labeling and sharing volcano plots. *Sci. Rep.* 10:20560. doi: 10.1038/s41598-020-76603-3
- Grifoni, A., Weiskopf, D., Ramirez, S. I., Mateus, J., Dan, J. M., Moderbacher, C. R., et al. (2020). Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. *Cells* 181, 1489–1501.e15. doi: 10.1016/j.cell.2020.05.015
- Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cells* 181, 271–280.e8. doi: 10.1016/j.cell.2020.02.052
- Ishigaki, H., Nakayama, M., Kitagawa, Y., Nguyen, C. T., Hayashi, K., Shiohara, M., et al. (2021). Neutralizing antibody-dependent and -independent immune responses against SARS-CoV-2 in cynomolgus macaques. *Virology* 554, 97–105. doi: 10.1016/j.virol.2020.12.013
- Ishii, K., Hasegawa, H., Nagata, N., Mizutani, T., Morikawa, S., Tashiro, M., et al. (2006). Highly attenuated vaccinia virus DIs as a potential SARS vaccine. *Adv. Exp. Med. Biol.* 581, 593–596. doi: 10.1007/978-0-387-33012-9_107
- Ishii, K., Ueda, Y., Matsuo, K., Matsuura, Y., Kitamura, T., Kato, K., et al. (2002). Structural analysis of vaccinia virus DIs strain: application as a new replication-deficient viral vector. *Virology* 302, 433–444. doi: 10.1006/viro.2002.1622
- Jackson, L. A., Anderson, E. J., Roupael, N. G., Roberts, P. C., Makhene, M., Coler, R. N., et al. (2020). An mRNA vaccine against SARS-CoV-2—preliminary report. *N. Engl. J. Med.* 383, 1920–1931. doi: 10.1056/NEJMoa2022483
- Jara, A., Undurraga, E. A., González, C., Paredes, F., Fontecilla, T., Jara, G., et al. (2021). Effectiveness of an inactivated SARS-CoV-2 vaccine in Chile. *N. Engl. J. Med.* 385, 875–884. doi: 10.1056/NEJMoa2107715
- Ju, B., Zhang, Q., Ge, J., Wang, R., Sun, J., Ge, X., et al. (2020). Human neutralizing antibodies elicited by SARS-CoV-2 infection. *Nature* 584, 115–119. doi: 10.1038/s41586-020-2380-z
- Kato, H., Miyakawa, K., Ohtake, N., Yamaoka, Y., Yajima, S., Yamazaki, E., et al. (2022). Vaccine-induced humoral response against SARS-CoV-2 dramatically declined but cellular immunity possibly remained at 6 months post BNT162b2 vaccination. *Vaccine* 40, 2652–2655. doi: 10.1016/j.vaccine.2022.03.057
- Kitabatake, M., Inoue, S., Yasui, F., Yokochi, S., Arai, M., Morita, K., et al. (2007). SARS-CoV spike protein-expressing recombinant vaccinia virus efficiently induces neutralizing antibodies in rabbits pre-immunized with vaccinia virus. *Vaccine* 25, 630–637. doi: 10.1016/j.vaccine.2006.08.039
- Kollias, A., Kyriakoulis, K. G., Dimakakos, E., Poulakou, G., Stergiou, G. S., and Syrigos, K. (2020). Thromboembolic risk and anticoagulant therapy in COVID-19 patients: emerging evidence and call for action. *Br. J. Haematol.* 189, 846–847. doi: 10.1111/bjh.16727
- Levin, E. G., Lustig, Y., Cohen, C., Fluss, R., Indenbaum, V., Amit, S., et al. (2021). Waning immune Humoral response to BNT162b2 Covid-19 vaccine over 6 months. *N. Engl. J. Med.* 385:e84. doi: 10.1056/NEJMoa2114583
- Liu, R., Americo, J. L., Cotter, C. A., Earl, P. L., Erez, N., Peng, C., et al. (2021). One or two injections of MVA-vectored vaccine shields hACE2 transgenic mice from SARS-CoV-2 upper and lower respiratory tract infection. *Proc. Natl. Acad. Sci. U. S. A.* 118:e2026785118. doi: 10.1073/pnas.2026785118
- Logunov, D. Y., Dolzhikova, I. V., Zubkova, O. V., Tukhvatulin, A. I., Shcheblyakov, D. V., Dzharullaeva, A. S., et al. (2020). Safety and immunogenicity of an rAd26 and rAd5 vector-based heterologous prime-boost COVID-19 vaccine in two formulations: two open, non-randomised phase 1/2 studies from Russia. *Lancet* 396, 887–897. doi: 10.1016/S0140-6736(20)31866-3
- Matsumoto, Y., Yasui, F., Endo, A., Sanada, T., Munakata, T., Takagi, A., et al. (2022). Early circulating strain of SARS-CoV-2 causes severe pneumonia distinct from that caused by variants of concern. Research Square [Preprint]. doi: 10.21203/rs.3.rs-1267705/v1

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.967019/full#supplementary-material>

- Matsuyama, S., Nao, N., Shirato, K., Kawase, M., Saito, S., Takayama, I., et al. (2020). Enhanced isolation of SARS-CoV-2 by TMPRSS2-expressing cells. *Proc. Natl. Acad. Sci. U. S. A.* 117, 7001–7003. doi: 10.1073/pnas.2002589117
- Mercado, N. B., Zahn, R., Wegmann, F., Loos, C., Chandrashekar, A., Yu, J., et al. (2020). Single-shot Ad26 vaccine protects against SARS-CoV-2 in rhesus macaques. *Nature* 586, 583–588. doi: 10.1038/s41586-020-2607-z
- Mortensen, R. M., and Kingston, R. E. (2009). Selection of transfected mammalian cells. *Curr. Protoc. Mol. Biol.* 9:86. doi: 10.1002/0471142727.mb0905s86
- Nie, X., Qian, L., Sun, R., Huang, B., Dong, X., Xiao, Q., et al. (2021). Multi-organ proteomic landscape of COVID-19 autopsies. *Cells* 184, 775–791.e14. doi: 10.1016/j.cell.2021.01.004
- Ogiwara, H., Yasui, F., Munekata, K., Takagi-Kamiya, A., Munakata, T., Nomura, N., et al. (2014). Histopathological evaluation of the diversity of cells susceptible to H5N1 virulent avian influenza virus. *Am. J. Pathol.* 184, 171–183. doi: 10.1016/j.ajpath.2013.10.004
- Pan, Y., Liu, L., Tian, T., Zhao, J., Park, C. O., Lofftus, S. Y., et al. (2021). Epicutaneous immunization with modified vaccinia Ankara viral vectors generates superior T cell immunity against a respiratory viral challenge. *NPJ Vaccines* 6:1. doi: 10.1038/s41541-020-00265-5
- Peng, Y., Mentzer, A. J., Liu, G., Yao, X., Yin, Z., Dong, D., et al. (2020). Broad and strong memory CD4(+) and CD8(+) T cells induced by SARS-CoV-2 in UK convalescent individuals following COVID-19. *Nat. Immunol.* 21, 1336–1345. doi: 10.1038/s41590-020-0782-6
- Polack, F. P., Thomas, S. J., Kitchin, N., Absalon, J., Gurtman, A., Lockhart, S., et al. (2020). Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. *N. Engl. J. Med.* 383, 2603–2615. doi: 10.1056/NEJMoa2034577
- Routhu, N. K., Cheedarla, N., Gangadhara, S., Bollimpelli, V. S., Boddapati, A. K., Shiferaw, A., et al. (2021). A modified vaccinia Ankara vector-based vaccine protects macaques from SARS-CoV-2 infection, immune pathology, and dysfunction in the lungs. *Immunity* 54, 542–556.e9. doi: 10.1016/j.immuni.2021.02.001
- Sadoff, J., Gray, G., Vandebosch, A., Cárdenas, V., Shukarev, G., Grinsztajn, B., et al. (2021). Safety and efficacy of single-dose Ad26.COV2.S vaccine against Covid-19. *N. Engl. J. Med.* 384, 2187–2201. doi: 10.1056/NEJMoa2101544
- Silva-Cayetano, A., Foster, W. S., Innocentin, S., Belij-Rammerstorfer, S., Spencer, A. J., Burton, O. T., et al. (2021). A booster dose enhances immunogenicity of the COVID-19 vaccine candidate ChAdOx1 nCoV-19 in aged mice. *Medicine* 2, 243–262.e8. doi: 10.1016/j.medj.2020.12.006
- Tagaya, I., Kitamura, T., and Sano, Y. (1961). A new mutant of dermiovaccinia virus. *Nature* 192, 381–382. doi: 10.1038/192381a0
- Tanriover, M. D., Doğanay, H. L., Akova, M., Güner, H. R., Azap, A., Akhan, S., et al. (2021). Efficacy and safety of an inactivated whole-virion SARS-CoV-2 vaccine (CoronaVac): interim results of a double-blind, randomised, placebo-controlled, phase 3 trial in Turkey. *Lancet* 398, 213–222. doi: 10.1016/S0140-6736(21)01429-X
- Tscherne, A., Schwarz, J. H., Rohde, C., Kupke, A., Kalodimou, G., Limpinsel, L., et al. (2021). Immunogenicity and efficacy of the COVID-19 candidate vector vaccine MVA-SARS-2-S in preclinical vaccination. *Proc. Natl. Acad. Sci. U. S. A.* 118:e2026207118. doi: 10.1073/pnas.2026207118
- Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C. L., Abiona, O., et al. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 367, 1260–1263. doi: 10.1126/science.abb2507
- Yasui, F., Itoh, Y., Ikejiri, A., Kitabatake, M., Sakaguchi, N., Munekata, K., et al. (2016). Sensitization with vaccinia virus encoding H5N1 hemagglutinin restores immune potential against H5N1 influenza virus. *Sci. Rep.* 6:37915. doi: 10.1038/srep37915
- Zhou, P., Yang, X. L., Wang, X. G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579, 270–273. doi: 10.1038/s41586-020-2012-7
- Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A. H., Tanaseichuk, O., et al. (2019). Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat. Commun.* 10:1523. doi: 10.1038/s41467-019-09234-6



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Development of a colloidal gold immunochromatographic assay strip using monoclonal antibody for rapid detection of porcine deltacoronavirus

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Porcine deltacoronavirus (PDCoV) cause diarrhea and dehydration in newborn piglets and has the potential for cross-species transmission. Rapid and early diagnosis is important for preventing and controlling infectious disease. In this study, two monoclonal antibodies (mAbs) were generated, which could specifically recognize recombinant PDCoV nucleocapsid (rPDCoV-N) protein. A colloidal gold immunochromatographic assay (GICA) strip using these mAbs was developed to detect PDCoV antigens within 15min. Results showed that the detection limit of the GICA strip developed in this study was 10^3 TCID₅₀/ml for the suspension of virus-infected cell culture and 0.125μg/ml for rPDCoV-N protein, respectively. Besides, the GICA strip showed high specificity with no cross-reactivity with other porcine pathogenic viruses. Three hundred and twenty-five fecal samples were detected for PDCoV using the GICA strip and reverse transcription-quantitative real-time PCR (RT-qPCR). The coincidence rate of the GICA strip and RT-qPCR was 96.9%. The GICA strip had a diagnostic sensitivity of 88.9% and diagnostic specificity of 98.5%. The specific and efficient detection by the strip provides a convenient, rapid, easy to use and valuable diagnostic tool for PDCoV under laboratory and field conditions.

KEYWORDS

porcine deltacoronavirus, colloidal gold immunochromatographic assay (GICA) strip, monoclonal antibodies, real-time PCR, cross-species transmission

1. Introduction

Porcine deltacoronavirus (PDCoV), which belongs to the genus *Deltacoronavirus* in the family *Coronaviridae* of the order *Nidovirales* (Walker et al., 2019), is a emerging swine enteropathogenic coronavirus that causes acute diarrhea, vomiting, and dehydration in newborn piglets (Chen et al., 2015; Vitosh-Sillman et al., 2016). PDCoV was initially reported in Hong Kong during a territory-wide molecular epidemiology study in mammals and birds in 2012 (Woo et al., 2012). Subsequently, in early 2014, the first outbreak of PDCoV-associated diarrhea was emerged in swine in Ohio (United States; Wang et al., 2014a) and then spread to other US states (Wang et al., 2014b). Subsequently, the virus has been detected in fecal samples from piglets in Canada (Marthaler et al., 2014), South Korea (Lee et al., 2016), Japan (Suzuki et al., 2018), Thailand (Lorsirigool et al., 2017), Vietnam (Saeng-Chuto et al., 2017), and Lao PDR (Lorsirigool et al., 2016). In 2014, PDCoV was first detected in domestic pigs in mainland China (Zhao et al., 2017). Even independent infections of PDCoV among Haitian children have been reported (Lednicky et al., 2021). Experimental infection studies showed that calves, chickens, turkey poults, mice are susceptible to infection with PDCoV, standing for its potential for cross-species transmission (Woo et al., 2012; Duan, 2021). The PDCoV outbreak has exhibited a global spread and caused significant economic losses in pig industry worldwide.

The complete genome of PDCoV is approximately 25.4 kb in length (Zhang et al., 2019; Tang et al., 2021), making it the smallest genome known among Coronaviruses (CoVs). The genome arrangements of PDCoV are as follows: 5'UTR-ORF1a-ORF1b-S-E-M-NS6-N-NS7-3'UTR (Duan, 2021; Jin et al., 2021; Tang et al., 2021). ORF1a and ORF1b occupy the 5'-proximal two-thirds of the complete genome and code for two overlapping replicase precursor polypeptides, pp1a and pp1ab, which are cleaved into non-structural proteins which involved in viral replication and transcription. The 3'-proximal last third of the genome encodes four structural proteins (S, E, M and N), and at least three nonstructural proteins (NS6, NS7 and NS7a; Zhang et al., 2019; Duan, 2021; Jin et al., 2021). The N protein is a highly immunogenic protein and the most abundant viral protein expressed in virus-infected cells, which makes it a suitable candidate for the detection of virus-specific antibodies and disease diagnosis (Wang et al., 2019; Tang et al., 2021).

The epidemiological, clinical, and pathological features are similar among PDCoV, porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV; Ding et al., 2020; Tang et al., 2021), leading to difficulties in the clinical differential diagnosis. Although several detection methods, including virus neutralization tests, virus isolation, and indirect immunofluorescence assay (IFA), are available for the detection of viruses, these methods are not applicable for detection in large-scale samples and point-of-care testing (POCT; Zhang, 2016; Ding et al., 2020). Currently, reverse transcriptase real-time PCR (RT-qPCR; Pan et al., 2020) or RT-PCR (Wang et al., 2014a; Ding

et al., 2020) assays and sandwich enzyme-linked immunosorbent assay (ELISA; Wang et al., 2021) for PDCoV detection have been reported. However, these methods are labor-intensive and time-consuming, also requiring qualified personnel and appropriate biosafety facilities.

Colloidal gold immunochromatographic assay (GICA) is a highly useful tool in diagnostics based on the specific antigen-antibody immunoreactions, and has been successfully used for rapidly detection in kinds of samples especially specific antigens or antibodies of multiple diseases (Sheng et al., 2012; Liu et al., 2021). Compared with other laboratory-based diagnostic platform analyses, the assay results are directly visible to the naked eye, and without requiring specialized equipment, untrained personnel, and complicated handling procedures, which provide convenience for rapid testing. However, the GICA strip for detection of PDCoV has not been described. So, this study aimed to establish a GICA-based test strip as a supplementary technique for rapidly detecting PDCoV in fecal samples from pigs. This method was simple, rapid, and specific for detecting PDCoV, which is suitable for pathogen detection in laboratory and clinical samples.

2. Materials and methods

2.1. Viruses and cell culture

PDCoV CZ2020 strain (GenBank accession number: OK546242) was isolated and maintained in our laboratory. The LLC-PK1 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, MA, United States) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Tianhang, China) and antibiotics (0.25 µg/ml of amphotericin B, 100 µg/ml of streptomycin, and 100 U/ml of penicillin; Thermo Fisher Scientific). LLC-PK1 cells were purchased from the China Institute of Veterinary Drug Control, which maintained in DMEM (containing 7.5 µg/ml trypsin) and used to propagate PDCoV. When cytopathic effects (CPE) were observed (over 85% cells were split), the infected cell cultures were collected and freeze-thawed, and cell debris was removed by centrifugating at 4,000 ×g at 4°C for 10 min. The supernatant were collected and stored at -80°C until used.

PEDV/AH2010 (The virus was cultured in Vero cells and titer was 10^{6.5} TCID₅₀/ml), TGEV/JS2012 (The virus was cultured in ST cells and titer was 10^{8.0} TCID₅₀/ml), porcine rotavirus (PoRV/NING86 was cultured in Marc145 cells and titer was 10^{7.5} TCID₅₀/ml), porcine reproductive and respiratory syndrome virus (PRRSV/NF was cultured in Marc145 cells and titer was 10^{6.0} TCID₅₀/ml), classical swine fever virus (CSFV/C was cultured in ST cells and titer was 10^{6.0} TCID₅₀/ml), porcine circovirus type 2 (PCV2/2010AHCY was cultured in PK15 cells and titer was 10^{7.0} TCID₅₀/ml), and pseudorabies virus (PRV/AH02LA was cultured in ST cells and titer was 10^{8.0} TCID₅₀/ml) were conserved in the laboratory. PEDV/AH2010, TGEV/JS2012, PoRV/NING86, PRRSV/NF and

PCV2/2010AHCY were isolated in our lab. CSFV/C was obtained from commercial vaccine. PRV/AH02LA was obtained from Jichun Wang's lab of Institute of Veterinary Immunology and Engineering, JAAS. Besides, the titer of these viruses had been detected to make sure these viruses were present and enough viral load for using to analyse the specificity of the GICA strip.

2.2. Preparation of monoclonal antibody and rPDCoV-N protein

rPDCoV-N protein and two monoclonal antibodies (mAb-32[#] and mAb-33[#]) against the protein were prepared according to our previous study (Wang et al., 2021), and the two mAbs were identified by western blot and IFA in our laboratory.

Following the procedures described previously with slight modifications (Wang et al., 2021), the purified rPDCoV-N protein were separated by SDS-PAGE and transferred to PVDF membranes using a Bio-Rad Mini Trans-Blot Cell (Bio-Rad). The membranes were, respectively, incubated with mAb-32[#] (5.1 µg/ml for final concentration) or mAb-33[#] (3.9 µg/ml for final concentration) against PDCoV, followed by goat anti-mouse serum conjugated to horseradish peroxidase (HRP, 1:5000), and the target protein was visualized by enhanced chemiluminescence (ECL).

Indirect IFA was performed as described previously with slight modifications (Yu et al., 2019). Briefly, 10^{7.0} TCID₅₀/ml of PDCoV CZ2020 strain was diluted into 10^{4.0} TCID₅₀/ml with DMEM (7.5 µg/ml trypsin). Then, 500 µl of 10^{4.0} TCID₅₀/ml PDCoV was inoculated into LLC-PK1 cells (approximately 90, % confluent) cultured in 24-well plates, and the virus was adsorbed for 2 h. Subsequently, the liquid of the plates was discarded, and the plates were washed twice with DMEM (7.5 µg/ml trypsin). Finally, 1 ml DMEM (7.5 µg/ml trypsin) was added to each plate. Twelve hours post-inoculation, the cells were washed twice with PBS, fixed with methyl alcohol for 1 h at 4°C, then blocked with 5% skim milk (in PBS) for 2 h at 4°C, and subsequently incubated with mAb-32[#] (10.2 µg/ml for final concentration) or mAb-33[#] (7.8 µg/ml for final concentration) for 1 h at 37°C. Cells were washed thrice with PBST and incubated with goat anti-mouse IgG conjugated with FITC (Boster, China; 1:500) for additional 1 h at 37°C. Finally, the cells were washed thrice with PBST and observed under a fluorescence microscope (Olympus IX-51). Uninfected cells served as negative control.

2.3. Synthesis of colloidal gold

To prepare colloidal gold, 1 ml of 1% chloroauric acid (HAuCl₄) was added to the Erlenmeyer flask with 99 ml ddH₂O which was stirred and heating to boiling for 2 min. Then 2 ml of 1% sodium citrate aqueous solution was added accurately under constant agitation, followed to boiling for another

10 min. The colloidal gold suspension was cooled down to room temperature, and volume was fixed to 100 ml by adding ddH₂O.

2.4. Preparation of the GICA strip

As previously described (Zhang et al., 2008; Lin et al., 2011), a colloidal gold solution was prepared. The colloidal gold solution was adjusted to pH 7.0 with potassium carbonate (K₂CO₃, 0.2 M) to prepare the detector reagent. The mAb-32[#] was coupled to colloidal gold particles as previously described (Zeng et al., 2019; Liu et al., 2021). Briefly, purified mAb-32[#] (45 µg/ml) was added to 1 ml of a 40 nm colloidal gold solution with gentle stirring. After 40 min, 10% bovine serum albumin (BSA) in PBS (w/v) was added to a final concentration of 0.2% and the solution was stabilized for 30 min. The solution was then centrifuged at 8,500 ×g at 4°C for 10 min and the soft pellet was resuspended with PBS (0.02 M, pH 7.4) containing 1.0% BSA. The resuspended solution was stored at 4°C.

The immunochromatography strip was constructed as in previously studies (Xu et al., 2010; Lin et al., 2011; Yu et al., 2019). Colloidal gold-labeled antibody conjugate was jetted onto glass fiber and dried at 37°C. Goat anti-mouse IgG antibody (1.0 mg/ml) was dispensed onto a nitrocellulose (NC) membrane on the upper line (C line) for control with a volume of 1 µl per 1 cm line, and for another epi-position strain mAb-33[#] (1.0 mg/ml) in PBS was jetted into the lower part for test line (T line); the dispensed volume was also of 1 µl per 1 cm line. The remaining active sites on the membrane were blocked by incubation with 2% BSA in PBS (1 ml/cm membrane) for 30 min at room temperature. The membrane was washed once with PBS and again with ddH₂O and then, dried at 37°C. Finally, the sample pad, pre-treated conjugate pad, NC membrane, and absorbent pad adhered to a plate in the proper order, which was subsequently cut into 0.3 cm × 6 cm strips (Figure 1A).

2.5. Sensitivity of the GICA strip

To evaluate the sensitivity of the GICA strip, the PDCoV CZ2020 strain cell culture virus (10⁷ TCID₅₀/ml) was serially diluted to 10⁶, 10⁵, 10⁴, 10³, 10², 10 and 1 TCID₅₀/ml by using PBS. Then these samples were detected using the strips and RT-qPCR. Otherwise, different concentrations of purified rPDCoV-N protein (diluted to 5.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.0313 µg/ml by using PBS) were tested using the strips. PBS and DMEM (containing 7.5 µg/ml trypsin and 10% FBS) were used as blank controls. Approximately 100 µl of sample was added to the sample pad and waiting for 15 min. When red-purple bands appeared at both the test and control lines, the result was considered positive. When a red-purple band only appeared at the control line, the result was considered negative (Figure 1B). The same procedure was repeated 3 times with different operators.

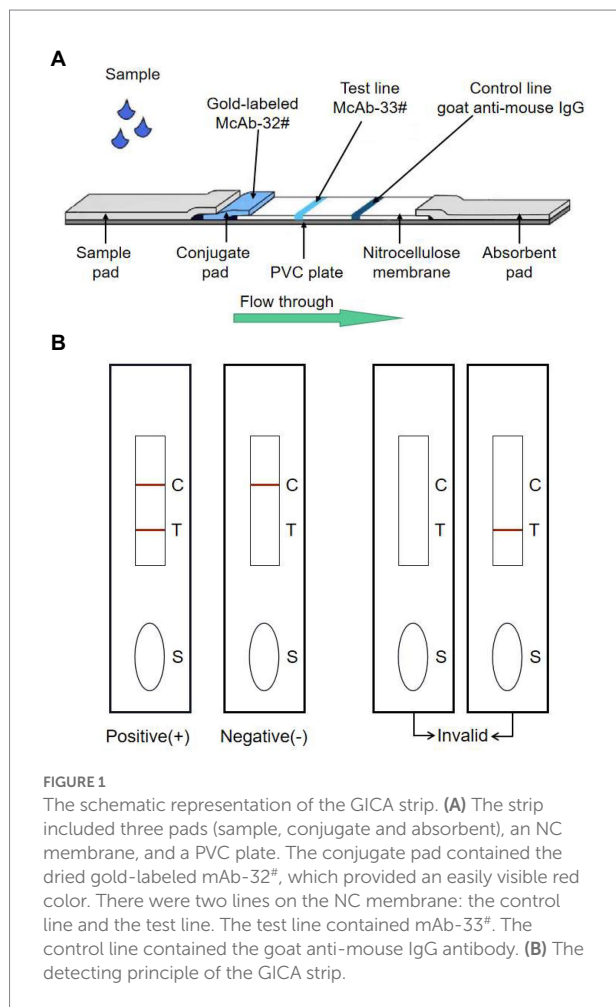


TABLE 1 The information on clinical field samples from swine farms.

The position of swine farms	Amount of fecal samples	Symptoms of neonatal piglets
Taian, Shangdong	50	Diarrhea
Yancheng, Jiangsu	82	Diarrhea
Huaian, Jiangsu	86	Diarrhea and vomiting
Taizhou, Jiangsu	50	Diarrhea
Yixing, Jiangsu	57	Diarrhea and vomiting

The RNA of PDCoV serially diluted samples and blank controls were extracted, and cDNA was synthesized by commercial kits (HiScript II Q RT SuperMix for qPCR, Vazyme, China). Then the cDNA of these samples was detected by qPCR. The qPCR primers of PDCoV M gene (forward, ATCGACCACATGGCTCCAA; reverse primer, CAGCTC TTGCCCATGTAGCTT) and a probe (FAM-CACACCAG TCGTTAAGCATGGCAAGCT-BHQ1) was run on QuantStudio 6 Real-Time PCR System (ThermoFisher, Carlsbad, CA, United States) with the following conditions: 5 min at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at

60°C. Assign a cycle threshold (C_t) value to each PCR reaction from a scan of all amplification plots (a plot of the fluorescence signal versus cycle number). If test samples have a C_t value ≥ 35.0 , it is considered the samples are negative; and if test samples have a C_t value < 35.0 , it means the samples are positive (strongly positive samples have a C_t value < 25.0).

2.6. Specificity of the GICA strip

PEDV, TGEV, PoRV, PRRSV, CSFV, PCV2 and PRV were tested with the strip to evaluate the specificity of the GICA strip. PDCoV CZ2020 strain cell culture supernatant and LLC-PK1 cells were detected as positive and negative control, respectively.

2.7. Comparison of the GICA strip and RT-qPCR in clinical field samples detection

A total of 325 fecal samples obtained from different swine farms (Table 1) were examined by using the GICA strip and RT-qPCR. The fecal swabs were stirred into PBS solution, and then stood for 1–2 min. The diagnostic sensitivity, specificity and accuracy were calculated using the following formulas: diagnostic sensitivity = true positive / (true positive + false negative) $\times 100\%$; diagnostic specificity = true negative / (true negative + false positive) $\times 100\%$; consistency = (true positive + true negative) / (true positive + false positive + true negative + false negative) $\times 100\%$. The agreement between the GICA strip and RT-qPCR was measured with the kappa statistic value (Tang et al., 2015).

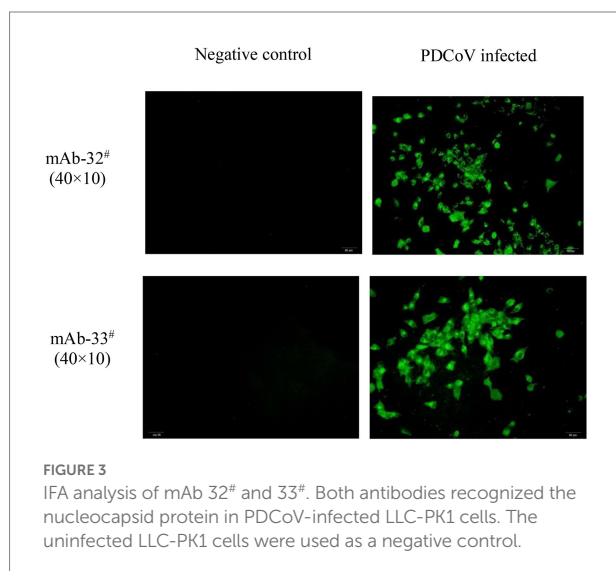
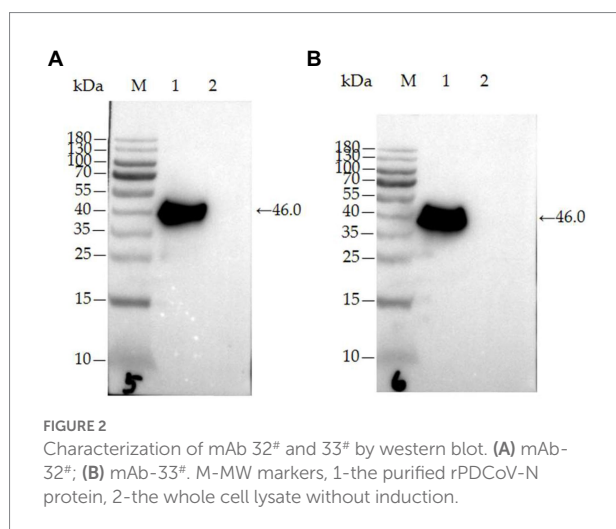
2.8. Ethics statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the Jiangsu Academy of Agricultural Sciences Experimental Animal Ethics Committee (NKYVET 2015-0127).

3. Results

3.1. Identification of mAbs

The two mAbs were identified by western blot and IFA. Purified rPDCoV-N proteins were subjected to western blot analysis, and the results demonstrated that the two mAbs could recognize the nucleocapsid protein (approximately 46.0 kDa) of PDCoV (Figures 2A,B). IFA showed that the mAbs could specifically react with PDCoV (Figure 3), thus indicating that the two mAbs are applicable for developing diagnostic methods to detect PDCoV antigens.



3.2. Sensitivity of the GICA strip

To evaluate the sensitivity of the GICA strip, the assay's detection limit was determined by testing against dilutions of PDCoV CZ2020 strain and rPDCoV-N protein. Results of chromogenic reaction revealed that the strip was able to detect PDCoV CZ2020 strain at a level of 10^3 TCID₅₀/ml (Figure 4) and rPDCoV-N protein at a level of 0.125 µg/ml (Figure 5). In parallel, the RT-qPCR assay detected the viral genome at a limit of 10^2 TCID₅₀/ml (Table 2), which was 10-fold more sensitive than the GICA strip.

3.3. Specificity of the GICA strip

The specificity of the GICA strip was evaluated using common swine pathogens, such as PEDV, TGEV, PoRV, PRRSV, PCV2,

CSFV and PRV. While PDCoV cell culture supernatant yielded positive result, all other samples showed negative results (Figure 6). These data convincingly demonstrated that the strip could be used to detect PDCoV specifically.

3.4. Clinical field samples detection

A total of 325 fecal samples were examined by using the GICA strip and RT-qPCR (Table 3). The GICA strip was found to have 88.9% diagnostic sensitivity [$48/(48+6)$] and 98.5% diagnostic specificity [$267/(4+267)$] relative to RT-qPCR. The consistency of these two detection methods was [$(48+267)/(52+273)$] = 96.9%. An example of detection of a fecal sample using GICA strip is shown in Supplementary Figure S1. No bands were identified at low virus titers, but bands were detected at high virus titers. In addition, the kappa value was 0.887, which is considered 'almost perfect' agreement between the two detection methods. The positive rate of PDCoV using the GICA strip was $(48+4)/325 = 16.0\%$ versus $(48+6)/325 = 16.6\%$ detected by RT-qPCR.

This result showed that PDCoV infection had been become one of swine farm's most important enteropathogenic pathogens. Also, these results show it is a good agreement for PDCoV detection between the GICA strip and RT-qPCR, and the developed strip would be effective in rapidly identifying of PDCoV antigens in fecal samples from swine farms.

4. Discussion

Coronaviruses (CoVs) are existed widely among mammals and birds (Tang et al., 2021). As globally important pathogens, zoonotic CoVs have a higher risk for cross-species transmission to humans and animals (Thakor et al., 2022). We found that PDCoV can infect swines of different ages, while piglets are more susceptible. In experimental infection researches, we also confirmed that calves, chickens, mice, turkey poults are susceptible to infecting PDCoV (Duan, 2021). Even in November 2021, Lednicky et al. (2021) first reported that cross-species transmission of PDCoV may have occurred from swines to children in Haiti. It has been posing a threat to the swine population and persons with direct exposure to pigs (e.g., pig farm workers and slaughterhouse workers). Besides, PDCoV infections have resulted in economic losses for the global swine industry (Ma et al., 2015; Zhang et al., 2020). So, rapid and early diagnosis is crucial to prevent and control PDCoV for swine health.

Currently, many methods for PDCoV detection have been developed, which were divided into serological and virological methods. Common virological methods include the detection of viral nucleic acid (various RT-PCRs (Marthaler et al., 2014; Wang et al., 2014b) and *in situ* hybridization (Jung et al., 2015)), viral antigen (immunofluorescence staining (Chen et al., 2015; Zhang et al., 2020), immunohistochemistry (Ma et al., 2015;

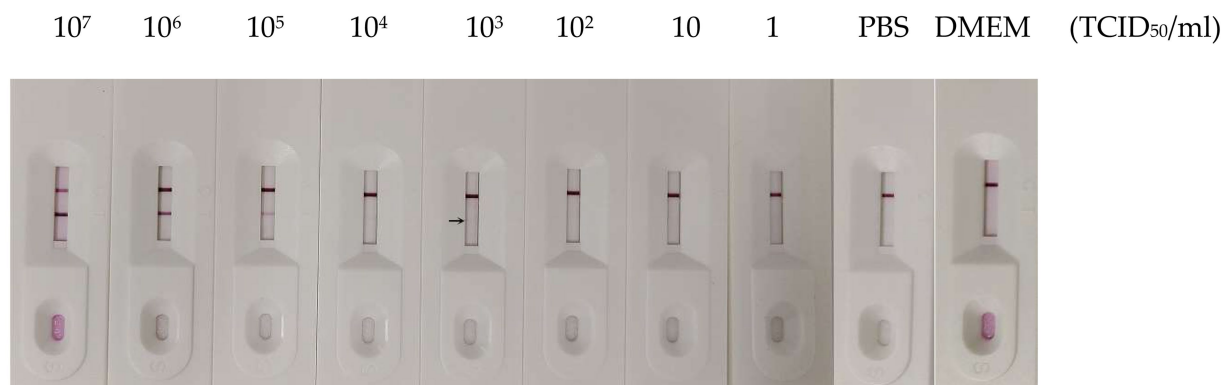


FIGURE 4

Sensitivity of the GICA strip for detecting PDCoV. Different virus titers of PDCoV CZ2020 strain were detected by the strip. PBS and DMEM were used as the negative control.

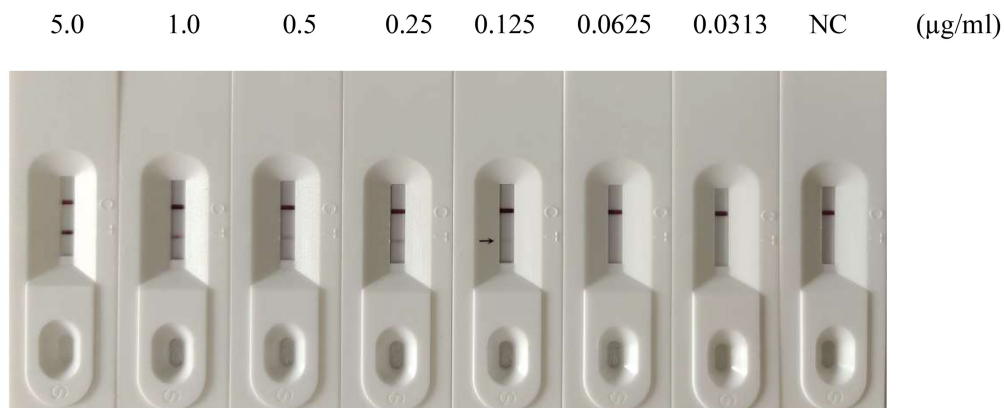


FIGURE 5

Sensitivity of the GICA strip for detecting rPDCoV-N protein. Different concentrations of the protein (5.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.0313 µg/ml) were detected by the strip. PBS was used as the negative control (NC).

Zhang et al., 2020) and sandwich ELISA (Wang et al., 2021)), virus particles (electron microscopy (Ma et al., 2015)) and virus isolation (Ma et al., 2015). The most commonly used serological assays include virus neutralization test (VNT; Zhang et al., 2020) and ELISA (Su et al., 2016; Zhang et al., 2020). However, these assays require to spend several hours even several days and need qualified personnel or expensive specialized equipment, which is often unaffordable for the mass detection in swine farms, specially POCT. To detect PDCoV from fecal samples in lesser time and achieve the control of this disease in swine farms, we have developed an antigen-capture colloidal GICA strip method, based on the use of a mAb conjugated with colloidal gold particles, which do not require special training or tools and yields rapid results within 15 min. The virus detection capacity of the GICA strip was systematically evaluated in this study, and all the obtained results suggested that the strip was a convenient method to detect and control the PDCoV infection.

To get a more specific and sensitive GICA strip, we first systematically studied the characterization of two mAbs (32[#], 33[#]) by western blot and IFA. Then, the reaction conditions of the GICA strip were optimized, including the pH of the colloidal gold fluid, the amount of labeled mAb-33[#] used, and the concentrations of colloidal gold-mAb-32[#] conjugate and goat anti-mouse IgG (Data not shown). After optimization, the GICA strip gave an accurate and clear result, visualized within 15 min by the naked eye. We further examined the accuracy of the result, including specificity, sensitivity, and coincidence rate with RT-qPCR.

During the sensitivity evaluation, the GICA strip detected PDCoV at 10³ TCID₅₀/ml (*C_t* value is 30.81 by RT-qPCR), whereas RT-qPCR could detect 100 TCID₅₀/ml (*C_t* is 33.30 by RT-qPCR). Although the sensitivity of the GICA strip was lower than that of RT-qPCR for the detection of clinical samples, the coincidence rates with RT-qPCR were confirmed to be over 96%. The data suggested that the GICA strip could detect PDCoV in fecal samples effectively.

TABLE 2 Sensitivity of the qPCR for detecting PDCoV.

Samples	PDCoV CZ2020 strain (TCID ₅₀ /ml)								Negative control	
	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10	1	PBS	DMEM
C _t value	15.95	19.66	23.05	26.5	30.81	33.3	36.91	Undetermined	Undetermined	Undetermined
Determination of results	++	++	++	+	+	+	—	—	—	—

“++” indicates strongly positive results, and “+” indicates the positive results by RT-qPCR, while “—” indicates the negative results.

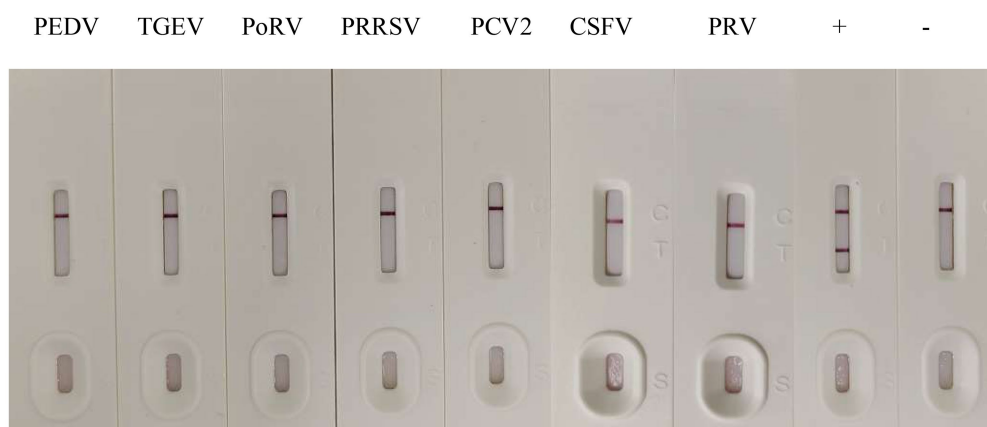


FIGURE 6

Specificity of the GICA strip. PDCoV cell culture supernatant as a positive control (+), LLC-PK1 cells as negative control (–), PEDV, TGEV, PoRV, PRRSV, PCV2, CSFV and PRV were tested with the strip.

TABLE 3 Comparison of RT-qPCR and the GICA strip for detecting PDCoV in fecal samples.

Fecal samples		GICA strip			Kappa value
		Positive	Negative	Total	
RT-qPCR	Positive	48	6	54	0.887
	Negative	4	267	271	
	Total	52	273	325	

The GICA strip was used to detect PDCoV in 325 clinical fecal samples to examine its practicability. Among them, the results obtained from the strip agreed with RT-qPCR up to 96.9%. Eight samples which were identified as positive by RT-qPCR but missed by the GICA strip. These results are attributed to the excessively low virus content in the samples. Five other samples were PDCoV-negative by RT-qPCR but PDCoV-positive by the GICA strip. The reason of this disagreement might be PCR-suppression effect and degradation of nucleic acids in assays, which affected the accuracy of qPCR. This finding suggests that the developed strips effectively identify PDCoV in swine farms.

PDCoV was often involved in co-infection with other porcine viruses in previous studies (Zhang, 2016), such as PEDV (Song et al., 2015) and TGEV (Fang et al., 2021). Seven different DNA or RNA porcine viruses were used in this study to evaluate the

specificity of the GICA strip. It showed that the strips were positive only for PDCoV cell culture supernatant, which indicated that the strips could be used to differentiate PDCoV from other porcine viruses, including PEDV, TGEV, PoRV, PRRSV, PCV2, CSFV and PRV.

In summary, the GICA strip developed in this study represents a means for the rapid and inexpensive detection of viral antigens to confirm PDCoV infection. The GICA strip exhibited high coincidence rates compared to RT-qPCR while taking only 15 min to yield results, which would allow a rapid diagnosis and early control of the disease.

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 at: <https://www.ncbi.nlm.nih.gov/genbank/>, OK546242.

Ethics statement

The animal study was reviewed and approved by the Jiangsu Academy of Agricultural Sciences Experimental Animal Ethics Committee (NKYVET 2015-0127).

Author contributions

WW took part in all the experiments and wrote the manuscript. BF helped to design the whole project and draft the manuscript. RG, YZ, and JiZ conducted cell culture, virus proliferation, RNA isolation and RT-qPCR detection. JuZ and QP conducted data analysis. XZ and MZ revised the English language of this article. BL and JL contributed essential ideas and discussion. 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.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.1074513/full#supplementary-material>

References

- Chen, Q., Gauger, P., Stafne, M., Thomas, J., Arruda, P., Burrough, E., et al. (2015). Pathogenicity and pathogenesis of a United States porcine deltacoronavirus cell culture isolate in 5-day-old neonatal piglets. *Virology* 482, 51–59. doi: 10.1016/j.virol.2015.03.024
- Ding, G. M., Fu, Y. G., Li, B. Y., Chen, J. N., Wang, J. L., Yin, B. S., et al. (2020). Development of a multiplex RT-PCR for the detection of major diarrhoeal viruses in pig herds in China. *Transbound. Emerg. Dis.* 67, 678–685. doi: 10.1111/tbed.13385
- Duan, C. (2021). An updated review of porcine deltacoronavirus in terms of prevalence, pathogenicity, pathogenesis and antiviral strategy. *Front. Vet. Sci.* 8:811187. doi: 10.3389/fvets.2021.811187
- Fang, P., Fang, L., Zhang, H., Xia, S., and Xiao, S. (2021). Functions of coronavirus accessory proteins: overview of the state of the art. *Viruses* 13:1139. doi: 10.3390/v13061139
- Jin, X. H., Zhang, Y. F., Yuan, Y. X., Han, L., Zhang, G. P., and Hu, H. (2021). Isolation, characterization and transcriptome analysis of porcine deltacoronavirus strain HN2K-02 from Henan Province, China. *Mol. Immunol.* 134, 86–99. doi: 10.1016/j.molimm.2021.03.006
- Jung, K., Hu, H., Eyerly, B., Lu, Z., Chepngeno, J., and Saif, L. (2015). Pathogenicity of 2 porcine Deltacoronavirus strains in Gnotobiotic pigs. *Emerg. Infect. Dis.* 21, 650–654. doi: 10.3201/eid2104.141859
- Lednický, J. A., Tagliamonte, M. S., White, S. K., Elbadry, M. A., Alam, M. M., Stephenson, C. J., et al. (2021). Independent infections of porcine deltacoronavirus among Haitian children. *Nature* 600, 133–137. doi: 10.1038/s41586-021-04111-z
- Lee, J. H., Chung, H. C., Nguyen, V. G., Moon, H. J., Kim, H. K., Park, S. J., et al. (2016). Detection and phylogenetic analysis of porcine deltacoronavirus in Korean swine farms, 2015. *Transbound. Emerg. Dis.* 63, 248–252. doi: 10.1111/tbed.12490
- Lin, T., Shao, J. J., Du, J. Z., Cong, G. Z., Gao, S. D., and Chang, H. (2011). Development of a serotype colloidal gold strip using monoclonal antibody for rapid detection type Asia1 foot-and-mouth disease. *Virol. J.* 8:418. doi: 10.1186/1743-422X-8-418
- Liu, F., Cao, Y., Yan, M., Sun, M., Zhang, Q., Wang, J., et al. (2021). Development of a colloidal gold Immunochromatographic assay for duck enteritis virus detection using monoclonal antibodies. *Pathogens* 10:365. doi: 10.3390/pathogens10030365
- Lorsirigool, A., Saeng-chuto, K., Madapong, A., Temeeyasen, G., Tripipat, T., Kaewprommal, P., et al. (2017). The genetic diversity and complete genome analysis of two novel porcine deltacoronavirus isolates in Thailand in 2015. *Virus Genes* 53, 240–248. doi: 10.1007/s11262-016-1413-z
- Lorsirigool, A., Saeng-Chuto, K., Temeeyasen, G., Madapong, A., Tripipat, T., Wegner, M., et al. (2016). The first detection and full-length genome sequence of porcine deltacoronavirus isolated in Lao PDR. *Arch. Virol.* 161, 2909–2911. doi: 10.1007/s00705-016-2983-8
- Ma, Y., Zhang, Y., Liang, X., Lou, F., Oglesbee, M., Krakowka, S., et al. (2015). Origin, evolution, and virulence of porcine deltacoronaviruses in the United States. *mBio* 6:e00064. doi: 10.1128/mBio.00064-15
- Marthaler, D., Raymond, L., Jiang, Y., Collins, J., Rossow, K., and Rovira, A. (2014). Rapid detection, complete genome sequencing, and phylogenetic analysis of porcine deltacoronavirus. *Emerg. Infect. Dis.* 20, 1347–1350. doi: 10.3201/eid2008.140526
- Pan, Z., Lu, J., Wang, N., He, W. T., Zhang, L., Zhao, W., et al. (2020). Development of a TaqMan-probe-based multiplex real-time PCR for the simultaneous detection of emerging and reemerging swine coronaviruses. *Virulence* 11, 707–718. doi: 10.1080/21505594.2020.1771980
- Saeng-Chuto, K., Lorsirigool, A., Temeeyasen, G., Vui, D. T., Stott, C. J., Madapong, A., et al. (2017). Different lineage of porcine deltacoronavirus in Thailand, Vietnam and Lao PDR in 2015. *Transbound. Emerg. Dis.* 64, 3–10. doi: 10.1111/tbed.12585
- Sheng, X. Z., Song, J. L., and Zhan, W. B. (2012). Development of a colloidal gold immunochromatographic test strip for detection of lymphocystis disease virus in fish. *J. Appl. Microbiol.* 113, 737–744. doi: 10.1111/j.1365-2672.2012.05389.x
- Song, D., Zhou, X., Peng, Q., Chen, Y., Zhang, F., Huang, T., et al. (2015). Newly emerged porcine deltacoronavirus associated with diarrhoea in swine in China: identification, prevalence and full-length genome sequence analysis. *Transbound. Emerg. Dis.* 62, 575–580. doi: 10.1111/tbed.12399

- Su, M., Li, C., Guo, D., Wei, S., Wang, X., Geng, Y., et al. (2016). A recombinant nucleocapsid protein-based indirect enzyme-linked immunosorbent assay to detect antibodies against porcine deltacoronavirus. *J. Vet. Med. Sci.* 78, 601–606. doi: 10.1292/jvms.15-0533
- Suzuki, T., Shibahara, T., Imai, N., Yamamoto, T., and Ohashi, S. (2018). Genetic characterization and pathogenicity of Japanese porcine deltacoronavirus. *Infect. Genet. Evol.* 61, 176–182. doi: 10.1016/j.meegid.2018.03.030
- Tang, P., Cui, E., Song, Y., Yan, R., and Wang, J. (2021). Porcine deltacoronavirus and its prevalence in China: a review of epidemiology, evolution, and vaccine development. *Arch. Virol.* 166, 2975–2988. doi: 10.1007/s00705-021-05226-4
- Tang, W., Hu, J., Zhang, H., Wu, P., and He, H. (2015). Kappa coefficient: a popular measure of rater agreement. *Shanghai Arch. Psychiatry* 27, 62–67. doi: 10.11919/j.issn.1002-0829.215010
- Thakor, J. C., Dinesh, M., Manikandan, R., Bindu, S., Sahoo, M., Sahoo, D., et al. (2022). Swine coronaviruses (SCoVs) and their emerging threats to swine population, inter-species transmission, exploring the susceptibility of pigs for SARS-CoV-2 and zoonotic concerns. *Vet. Q.* 42, 125–147. doi: 10.1080/01652176.2022.2079756
- Vitosh-Sillman, S., Loy, J. D., Brodersen, B., Kelling, C., Doster, A., Topliff, C., et al. (2016). Experimental infection of conventional nursing pigs and their dams with porcine deltacoronavirus. *J. Vet. Diagn. Investig.* 28, 486–497. doi: 10.1177/1040638716654200
- Walker, P. J., Siddell, S. G., Lefkowitz, E. J., Mushegian, A. R., Dempsey, D. M., Dutilh, B. E., et al. (2019). Changes to virus taxonomy and the international code of virus classification and nomenclature ratified by the international committee on taxonomy of viruses (2019). *Arch. Virol.* 164, 2417–2429. doi: 10.1007/s00705-019-04306-w
- Wang, L., Byrum, B., and Zhang, Y. (2014a). Detection and genetic characterization of deltacoronavirus in pigs, Ohio, USA, 2014. *Emerg. Infect. Dis.* 20, 1227–1230. doi: 10.3201/eid2007.140296
- Wang, L., Byrum, B., and Zhang, Y. (2014b). Porcine coronavirus HKU15 detected in 9 US states, 2014. *Emerg. Infect. Dis.* 20, 1594–1595. doi: 10.3201/eid2009.140756
- Wang, W., Li, J., Fan, B., Zhang, X., Guo, R., Zhao, Y., et al. (2021). Development of a novel double antibody sandwich ELISA for quantitative detection of porcine deltacoronavirus antigen. *Viruses* 13:2403. doi: 10.3390/v13122403
- Wang, Q., Vlasova, A. N., Kenney, S. P., and Saif, L. J. (2019). Emerging and re-emerging coronaviruses in pigs. *Curr. Opin. Virol.* 34, 39–49. doi: 10.1016/j.coviro.2018.12.001
- Woo, P. C., Lau, S. K., Lam, C. S., Lau, C. C., Tsang, A. K., Lau, J. H., et al. (2012). Discovery of seven novel mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *J. Virol.* 86, 3995–4008. doi: 10.1128/JVI.06540-11
- Xu, Y., Huang, Z. B., He, Q. H., Deng, S. Z., Li, L. S., and Li, Y. P. (2010). Development of an immunochromatographic strip test for the rapid detection of deoxynivalenol in wheat and maize. *Food Chem.* 119, 834–839. doi: 10.1016/j.foodchem.2009.08.049
- Yu, M., Bao, Y., Wang, M., Zhu, H., Wang, X., Xing, L., et al. (2019). Development and application of a colloidal gold test strip for detection of avian leukosis virus. *Appl. Microbiol. Biotechnol.* 103, 427–435. doi: 10.1007/s00253-018-9461-z
- Zeng, Y., Liang, D., Zheng, P., Peng, T., Sun, S., Mari, G. M., et al. (2019). Immunochromatographic fluorometric determination of clenbuterol with enhanced sensitivity. *Mikrochim. Acta* 186:225. doi: 10.1007/s00604-019-3326-8
- Zhang, J. (2016). Porcine deltacoronavirus: overview of infection dynamics, diagnostic methods, prevalence and genetic evolution. *Virus Res.* 226, 71–84. doi: 10.1016/j.virusres.2016.05.028
- Zhang, J., Chen, J., Liu, Y., Da, S., Shi, H., Zhang, X., et al. (2020). Pathogenicity of porcine deltacoronavirus (PDCoV) strain NH and immunization of pregnant sows with an inactivated PDCoV vaccine protects 5-day-old neonatal piglets from virulent challenge. *Transbound. Emerg. Dis.* 67, 572–583. doi: 10.1111/tbed.13369
- Zhang, Y., Cheng, Y., Xing, G., Yu, J., Liao, A., Du, L., et al. (2019). Detection and spike gene characterization in porcine deltacoronavirus in China during 2016–2018. *Infect. Genet. Evol.* 73, 151–158. doi: 10.1016/j.meegid.2019.04.023
- Zhang, G., Wang, X., Zhi, A., Bao, Y., Yang, Y., Qu, M., et al. (2008). Development of a lateral flow immunoassay strip for screening of sulfamonomethoxine residues. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 25, 413–423. doi: 10.1080/02652030701561452
- Zhao, F., Sun, Y., Qian, B., Zhang, X., and Wu, Y. (2017). Complete genome characterization of Chinese porcine deltacoronavirus strain CHN/Tianjin/2016. *Genome Announc.* 5:e00237-17. doi: 10.1128/genomeA.00237-17



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Molecular determinants associated with temporal succession of SARS-CoV-2 variants in Uttar Pradesh, India

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The emergence and rapid evolution of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) caused a global crisis that required a detailed characterization of the dynamics of mutational pattern of the viral genome for comprehending its epidemiology, pathogenesis and containment. We investigated the molecular evolution of the SARS-CoV-2 genome during the first, second and third waves of COVID-19 in Uttar Pradesh, India. Nanopore sequencing of the SARS-CoV-2 genome was undertaken in 544 confirmed cases of COVID-19, which included vaccinated and unvaccinated individuals. In the first wave (unvaccinated population), the 20A clade (56.32%) was superior that was replaced by 21A Delta in the second wave, which was more often seen in vaccinated individuals in comparison to unvaccinated (75.84% versus 16.17%, respectively). Subsequently, 21A delta got outcompeted by Omicron (71.8%), especially the 21L variant, in the third wave. We noticed that Q677H appeared in 20A Alpha and stayed up to Delta, D614G appeared in 20A Alpha and stayed in Delta and Omicron variants (got fixed), and several other mutations appeared in Delta and stayed in Omicron. A cross-sectional analysis of the vaccinated and unvaccinated individuals during the second wave revealed signature combinations of E156G, F157Del, L452R, T478K, D614G mutations in the Spike protein that might have facilitated vaccination breach in India. Interestingly, some of these mutation combinations were carried forward from Delta to Omicron. *In silico* protein docking showed that Omicron had a higher binding affinity with the host ACE2 receptor, resulting in enhanced infectivity of Omicron over the Delta variant. This work has identified the combinations of key mutations causing vaccination breach in India and provided insights into the change of [virus's] binding affinity with evolution, resulting in more virulence in Delta and more infectivity in Omicron variants of SARS-CoV-2. Our findings will help in understanding the COVID-19 disease biology and guide further surveillance of the SARS-CoV-2 genome to facilitate the development of vaccines with better efficacies.

KEYWORDS

SARS-CoV-2 genome, COVID-19, vaccination breach, spike mutations, Delta variant, omicron variant, COVID-19 waves

1. Introduction

Comprehending the unremitting molecular evolution of SARS-CoV-2 genome is essential to control the devastating surge of the COVID-19 pandemic. The pace of research has to match the pace of SARS-CoV-2 evolution to tackle the spread of virus. Accelerated research and concrete efforts worldwide resulted in the development of vaccines to combat the pandemic; nevertheless, new variants have rendered vaccines ineffective at some points (Dubey et al., 2022). Emerging variants, being facilitated by new signature mutations, have rapidly outcompeted the prior circulating variants (MacLean et al., 2021; Purushotham et al., 2021). In India, the first surge of COVID-19 gained its momentum in March 2020, which declined in the late July 2020. Later, in March 2021, the noxious variant Delta broke out in India for the first time and conquered the prior circulating variants to dominate the second wave (Gupta, 2021; Jha et al., 2021). In late November 2021, the first Omicron variant was detected in South Africa, which after its first detection in India in December 2021, gradually replaced Delta. Interestingly, although Delta is more virulent in terms of COVID-19 disease severity, Omicron replaced Delta by acquiring increased transmissibility, generating neutralizing antibodies and mutational fitness over natural selection pressure (Singh and Yi, 2021; Petersen et al., 2022).

COVID-19 vaccination in India started in mid-January, 2021. The vaccine was offered free of cost by the Government of India in staggered phases at various centers across the country, starting with the frontline and healthcare workers. This was followed by the next phase of COVID-19 vaccination to the elderly population starting March, 2021. Vaccination to the general population was opened in May 2021 (Purohit et al., 2022). In the beginning, people did not actively take vaccine despite efforts from the government. Apart from significant mortality in the first and the second waves in the elderly population, the second wave of COVID-19 resulted in a much higher death rate in below 45 years age group in comparison to the first wave (Purohit et al., 2022). The breakout of Delta variant with a very high morbidity and mortality rate forced people to actively seek COVID-19 vaccination (Vishvkarma and Rajender, 2020). Eventually, vaccination was in full swing in the months of June–July, 2021. However, only one fourth of Indian population had received the first dose of vaccine and only 6% of population had received both the doses by July 2021 (Choudhary et al., 2021). However, In Uttar Pradesh, 13% of the population had received at least one dose and 3% had completed two doses by July 2021.

After its first appearance in China in the late 2019, the virus has continuously evolved by either substitutions or deletions, resulting in significant and unexpected changes in its virulence and infectivity (Abraham, 2021). Particularly, mutations in the Spike protein have driven this evolution and have caught attention (Banerjee et al., 2021; Chan and Zhan, 2022). Some of these mutational events have driven regional spread the virus, causing havoc in closed territories, certain countries, or throughout the world (Rochman et al., 2021; Williams and Burgers, 2021). Among factors that could affect COVID-19 presentation, severity and eventual outcome, viral genome variations remain one of the most prominent and interesting factors. In order to understand the molecular determinants associated with mutation-driven evolution, we sequenced SARS-CoV-2 genomes from the first, second and third wave of COVID-19 in Uttar Pradesh, India.

2. Material and methodology

2.1. Sample collection

COVID-19 research was approved by the Institutional Biosafety Committee of the Central Drug Research Institute, Lucknow and the Institutional Ethics Committee of Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGI), Lucknow. The samples for this study were collected from Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow and the COVID-19 testing facility of the Central Drug Research Institute (CDRI), Lucknow. SGPGI is the largest state hospital in Uttar Pradesh and served as the biggest COVID-19 facility during the pandemic. The hospital received COVID-19 samples from the patients visiting the COVID-19 clinic for diagnosis, treatment or emergency care. The COVID-19 testing facility of CDRI is a government approved facility for testing of samples collected by various government approved centers for COVID-19 surveillance. The facility during its peak operation received 1,000 samples per day. Both of these facilities received patients or samples from Uttar Pradesh only. A total of 544 RNA samples from confirmed COVID-19 cases arising from different urban and rural areas of Uttar Pradesh (2020–2022), India, were subjected to Oxford Nanopore Technology (ONT) sequencing and clade analysis.

During the first wave (May 2020 to August 2020), 87 RNA samples having RT-PCR Ct value <30 were collected from different places of Lucknow and Jhansi. This sample cohort consisted of 78.65% male and 21.83% female patients.

During the second wave (April 2021–July 2021), 218 RNA samples with RT-PCR Ct value <30 patients were collected. The samples were from different cities of Uttar Pradesh, including Lucknow, Jhansi, Lalitpur, Ayodhya, and Orai. The age of the patients ranged from 12 years to 80 years, consisting of 87% males and 13% females. This cohort consisted of 64% fully vaccinated and 46% partially vaccinated (completed only the first dose) and 18% unvaccinated individuals. In total population, 24% were asymptomatic and rest 76% had symptoms like cold fever, body pain, breathing problem, abdominal disturbances.

During the third surge of COVID-19 in Uttar Pradesh (December 2021–January 2022), 239 samples were collected from symptomatic individuals from different districts, which consisted of 59.8% males and 40.2% females, and the entire group was vaccinated. The patient age ranged from 11 to 75 years.

2.2. SARS-CoV-2 genome sequencing

2.2.1. Library preparation

RNA samples were amplified by using primers of ARTIC nCov-2019 (version 3). Briefly, the RNA template was converted into complementary DNA (cDNA) using the high capacity cDNA reverse transcription kit (Applied Biosystems, United States) by keeping the sample in a thermocycler initially for 10 min at 25°C, followed by 60 min at 37°C, again 60 min at 37°C and finally 5 min at 85°C. The second run of PCR was performed by using AmpliTaq Gold™ 360 Master Mix while exposing the cDNA samples initially at 95°C for 10 min, followed by 35 cycles at 95°C for 45 s, 59°C for 5 min and 72°C for 45 s, followed by a final incubation at 72°C for 7 min. The expected PCR product size was 450 bp. The samples showing good quality bands on 1.8% agarose gel were considered for downstream library preparation.

Sequencing of the Spike region was targeted for sequencing in maximum number of the cases. The libraries for the first 120 samples were prepared by Oxford Nanopore native barcode kit (NBD104 and EXP-NBD114), where samples were first cleaned (by AMPureXP beads) after performing end prep, and the barcodes were ligated. After barcoding, all the samples were pooled in a single Eppendorf tube and adapter ligation was carried out at room temperature and the final washing was done. The libraries for the rest of the samples were prepared using Oxford Nanopore rapid barcode 96 kit (SQK-RBK110.96). Briefly, the samples were prepared by first ligating them in the barcode plate and then pooling all the samples together for magnetic bead wash. Adapters were ligated at room temperature before priming the flow cell.

2.2.2. Flow cell priming and sequencing on MinION

For Nanopore MinION sequencing, spot-on flow cells of R9 version were used (FLO-MIN106D). The flow cells were primed according to the manufacturer's instructions, using flush buffer and flush tether. 800–1,000 ng of the library was premixed with sequencing buffer and loading beads just prior to loading on spot on port of the flow cell. The base quality filter cut off value of 8 was used for accurate base calling. The sequencing was continued upto >900 Mb for a batch of 96 samples to generate approximately 300X coverage, giving nearly 25,000–30,000 reads per sample.

2.2.3. Post sequencing read filtering and functional annotation

After completion of the sequencing process, barcoded reads were analyzed by ARTIC nCoV pipeline.¹ Briefly, the ARTIC environment was created first and then the reads having 400–600 bp length were filtered from unwanted reads using guppy commands. Consensus sequences were made from the amplicons by nanopolish and subjected to EPI2ME to check the coverage quality. The final sequences were submitted to annotate the ORFs in VIGOR₄ (Viral Genome ORF Reader; Wang et al., 2010).

2.3. Clade analysis and mutation tracking

The trimmed sequences were checked in Pango, Nextclade and GISAID-CoVsurver mutation app for clade and lineage characterization. GISAID (Khare et al., 2021) employs EpiCoV database to assign phylogenetic clades and lineages to the sequences. Nextclade works by identifying the differences between the query sequence and the original SARS-CoV-2 Wuhan sequence to identify matches and mutations and characterizes the clades.

2.4. Neighborhood homology mapping and phylogeny

The circular representations of homologous comparison of SARS-CoV-2 Spike sequences from different time frames (consensus of the highest abundant variants from the 1st, 2nd and 3rd waves) were conducted by MUSCLE2 (Edgar, 2004) and Proksee (Grant and Stothard, 2008),²

with visualization of GC skewness, ORF distribution, annotation and blast comparison. Phylogeny tree was constructed and visualized by MEGA 11 (Tamura et al., 2021), based on the maximum likelihood, followed by heat map plotting of Pearson coefficient of mean distance matrix between amino acid placements.

2.5. *In silico* docking with ACE2 receptor and binding free energy calculation

The consensus genome sequences, covering all major mutations with the highest quality score, were selected as representative genomes of the second and third waves. The pdb files of Spike trimeric glycoproteins from the ViGor annotated file were created by Phyre2. Pdb file was also generated for angiotensin converting enzyme 2 (ACE2, NCBI Gene ID: 59272), which is the universal receptor for human coronavirus HCoV-NL63 and severe acute respiratory syndrome coronaviruses (SARS-CoV) and SARS-CoV-2. *In silico* docking was performed by HADDOCK 2.4 (Roel-Touris et al., 2019),³ Spoton (Moreira et al., 2017) and Hawkdock server (Weng et al., 2019).⁴ The highest score model was selected from the top 10 solutions provided by the servers. The binding free energy in terms of Gibb's free energy ($-\Delta G$) and dissociation constant K_d were calculated by PRODIGY (Xue et al., 2016).⁵ For visualization, PyMOL platform was used and all.pdb files were checked for model authenticity, Z score and Ramachandran plot stability by ProSA, ProQ, and PdbSum (Laskowski et al., 2018).

3. Results

All the sequences are publicly available in the GISAID database under Asia/India/Uttar Pradesh/subhead CDRI submission. The spike gene region was covered in all the cases, spike region with other regions was covered in 20% of the cases and complete genome coverage was achieved in 36% of the cases. However, we have largely focused on the Spike region only. For a comparative account, the phylogenetic analysis of SARS-CoV-2 variants in India during the period of January 2020–December 2022 is presented in Figure 1 with variant distribution and transmission data corresponding to the first, second and third waves presented in Figure 2.

3.1. Evolution of SARS-CoV-2 clades

3.1.1. First wave

In the first wave, 56.32% of the samples were found to have 20A Alpha, followed by 37.93% with 20B and 5.74% with 19A, presenting 20A Alpha to be the most dominant clade in Uttar Pradesh (Figure 3). Since no vaccine was available at that time, these samples were not classified according to the vaccination status.

3.1.2. Second wave

During the second wave, the vaccination drive was in full swing in India and this provided us with the opportunity to classify the samples into vaccinated and unvaccinated groups. The overall prevalence of

1 <https://github.com/artic-network/artic-ncov2019>

2 <https://proksee.ca/>

3 <https://wenmr.science.uu.nl/haddock2.4/>

4 <http://cadd.zju.edu.cn/hawkdock/>

5 <https://wenmr.science.uu.nl/prodigy/>

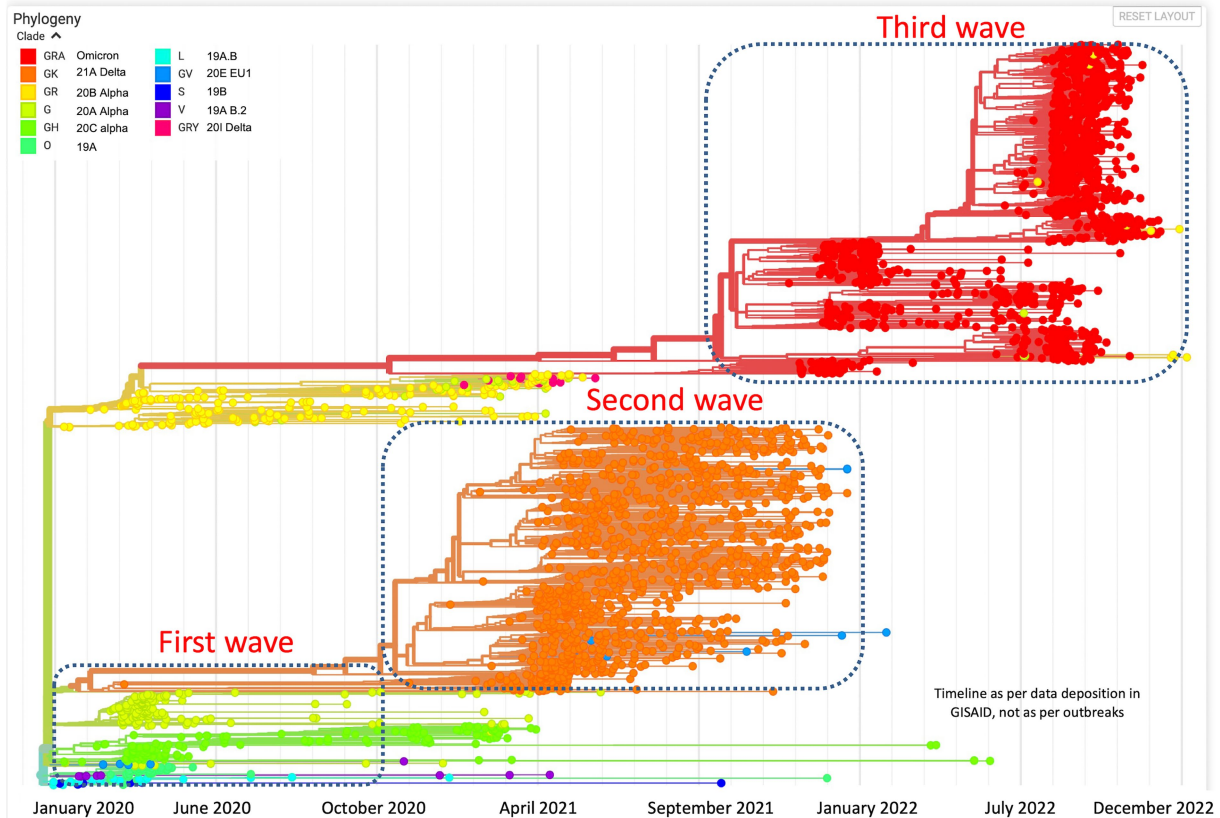


FIGURE 1
Phylogenetic analysis of SARS-CoV-2 variants in India during the period of January 2020–December 2022.

21A Delta in Uttar Pradesh at this time was 56.88%, with a relatively much higher frequency of 21A Delta (75.84%) in vaccinated people. The unvaccinated pool majorly carried 20A (29.41%), 20B clade (16.17%) and 21A Delta (16.17%) variants. Other delta variants (21 J + 21I) were more common in unvaccinated individuals (8.83%) in comparison to vaccinated individuals (3.96%; [Figure 3](#); [Table 1](#)).

3.1.3. Third wave

The situation took a turn when Omicron started replacing other variants in the mid of December 2021 in Uttar Pradesh. The cumulative percentage of Omicron was found to be 71.8%, which outcompeted Delta (16.19%) by the end of January 2022. Although the ratio of the sister lineages of 21 K and 21 L of Omicron differed from state to state, 21 L Omicron was found to be dominant (78.43%) over 21 K sub lineage (18.62%) in Uttar Pradesh. Interestingly, the frequency of 21 K Omicron was comparable to the frequency of Delta variant during this period ([Figure 3](#)).

3.2. Evolution of SARS-CoV-2 genome mutations

3.2.1. First wave

During the first wave, 20A Alpha carried the Spike protein mutations I285S and D614G. Other mutations were Q168H, M169V, S171stop, D172H, L174stop and N176S in NSP14; Q57H and G254stop in NS3; I199L, I210V, M211L, L212S, V213I, Y214del, C215A and F216L in NSP6; and L27F mutation in Envelop E.

20B carried D614G and Q677H mutations in the Spike protein. Other mutations included I124V, V149F in NSP6; R203K, G204R, D371V in Nucleocapsid N, K412N in NSP3, P323L in the NSP12 protein, L21F in the Envelop E protein.

Similarly, the 19A clade carried I285S, D614G and Q677H in the Spike protein. Other mutations included A185V, V381A in NSP12 and V1762F in NSP3 proteins. Interestingly, out of the three Spike mutations, only D614G and Q677H were carried forward to the second wave and only D614G was passed to the third wave ([Figure 4](#)).

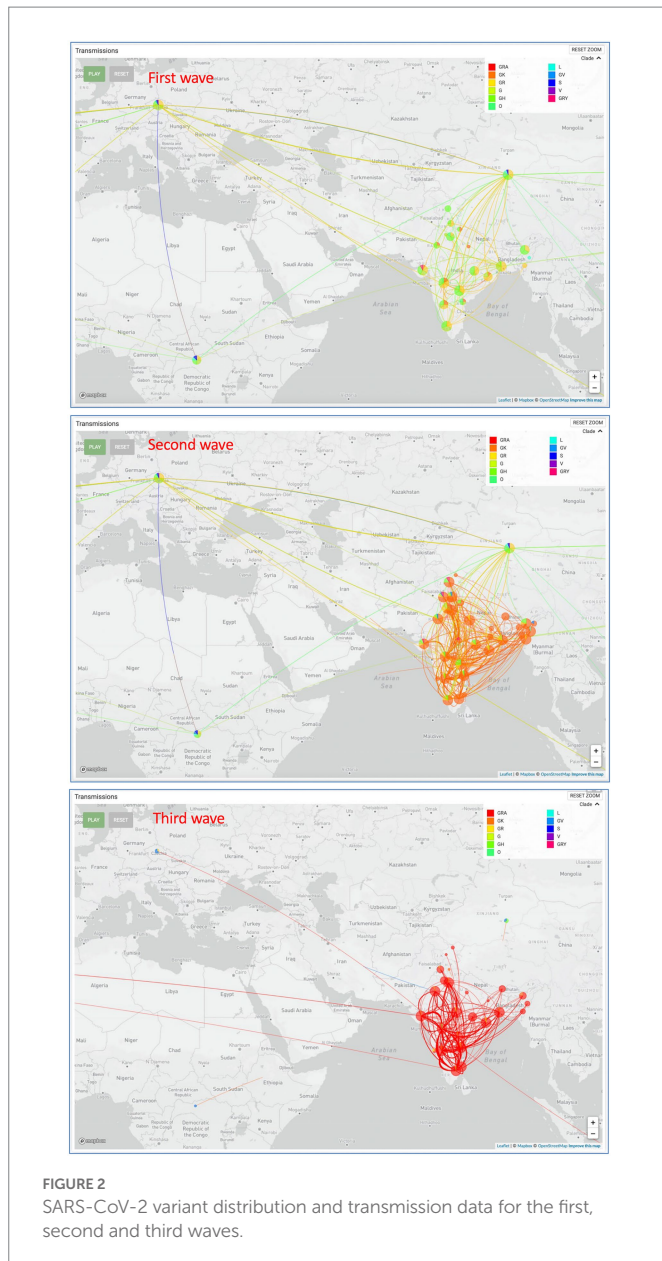
3.2.2. Second wave

Nextclade and GISAID majorly annotated G142D, V143del, Y144del, Y145del, E156G, F157del, R158del, Q173R, A222V, A262S, Q414K, G446V, L452R, R454K, T478K, E484Q, S494P, P499R, D614G, Q677H, P681R, R683W, D950N, S1242I mutations in the Spike glycoprotein of 21A Delta.

A67V, H69del, V70del, T95I, G142D, V143del, Y144del, Y145del, E156G, F157del, R158G, R158del, L452R, T478K, E484Q, P499R, D614G, P681R, D950N, Q954Y, N969K, V1104L mutations were present in the Spike proteins of 21J Delta.

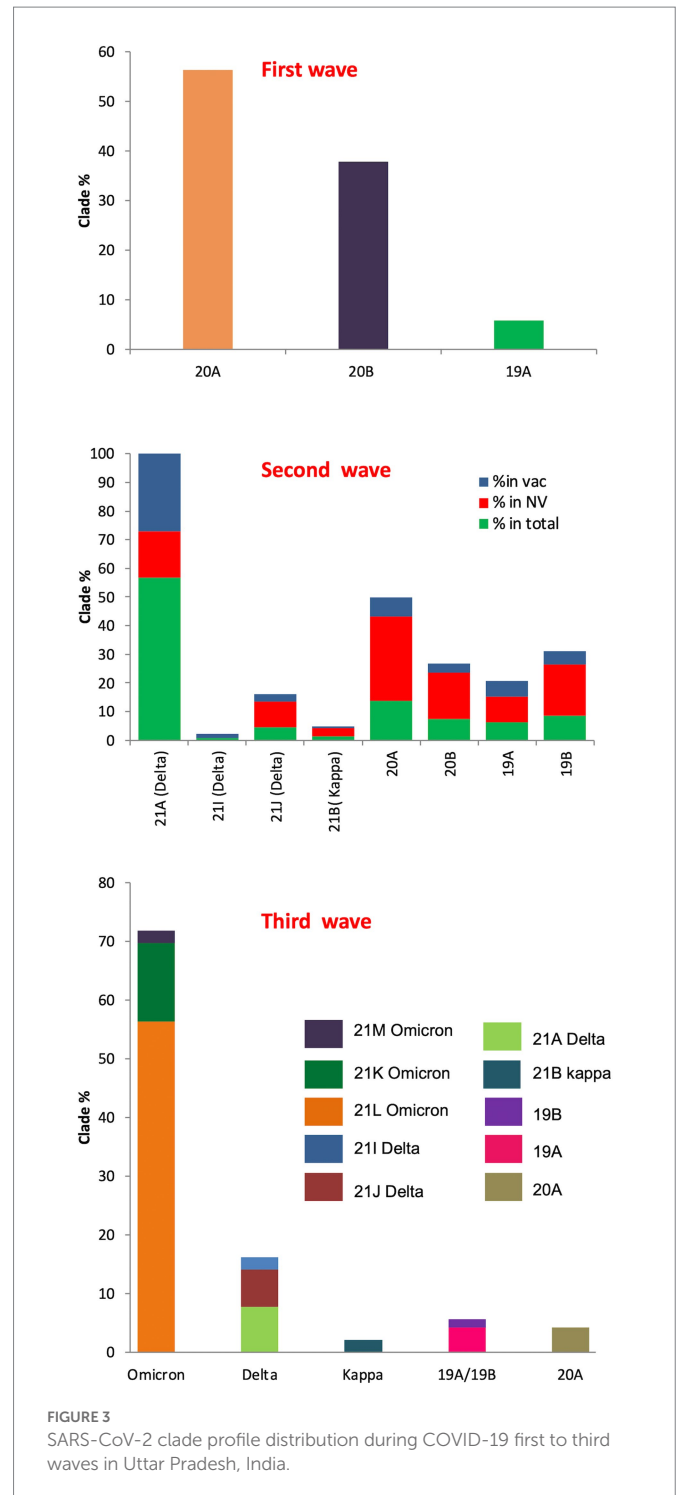
A67V, H69del, V70del, G142D, L452R, T478K, E484Q, D614G, P681R, D950N mutations were present in the Spike protein of 21I Delta variant.

A number of Spike mutations were passed to the Omicron variant without modifications and a few were passed to the Omicron variant with modifications ([Figure 4](#)). Omicron shared at least one Spike mutation (D614G) with Delta and 19A and 20A variants.



3.2.3. Third wave

By the time the third wave arrived (early December 2021 to February 2022), the majority of the population was vaccinated; hence, we could not classify these samples as per their vaccination status. However, a number of spike mutations, such as A67V, H69del, V70del, T95I, G142del, V143del, Y144del, Y145D, G339D, R346K, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F, were found in the Omicron sister lineages 21 K and 21 L (Figure 4). Interestingly, the key determinant mutations for vaccination breach (F157del, R158del, L452R, T478K, D614G, P681R) were present in the Omicron variant as well. Moreover, some mutations, earlier detected in Delta, were found in different forms in Omicron (Q954Y → Q954H and E484Q → E484A, Figure 4). Essentially, Omicron contains profoundly higher number of mutations, yet some mutations were lost in the evolution from Delta to Omicron (R158G, A262S, S494P, S1242I).



3.3. Unique mutation combinations in delta may be responsible for vaccination breach

The frequencies of Spike protein mutations, such as E156G, R158del, L452R, T478K, and D950N were significantly higher in the vaccinated population in comparison to unvaccinated individuals (Table 1).

We also asked if there were specific combinations of mutations that resulted in frequent vaccination breach during the second wave. For this, a matrix analysis of three Spike mutations at a time was undertaken. L452R, T478K, and D614G combination was found to be the most frequent combination, followed by E156G, F157Del and D614G

combination, E156G and R158Del and D614G combination, F157Del, R158Del and D614G combination, F157Del, L452R and D614G combination, and E156G, L452R and D614G combination in the vaccinated people (Figure 5). These combinations highlight E156G, 157Del, L452R and D614G as the most significant mutations for vaccination breach. Though only Spike protein variations have been emphasized, we also observed a combination of NSP mutations with two Spike mutations to be very frequent among vaccinated individuals (Figure 5). LINK Excel.Sheet.12 "E:\CDRI 2021-22\covid19\PAPER\manuscript\THREE_COMBINATIONS_RESULTS_1.xlsx" Sheet1!R3C3:R47C14 \a \f 5 \h * MERGEFORMAT

TABLE 1 The comparison of frequencies of the Spike mutations between vaccinated and unvaccinated COVID-19 patients during the second wave.

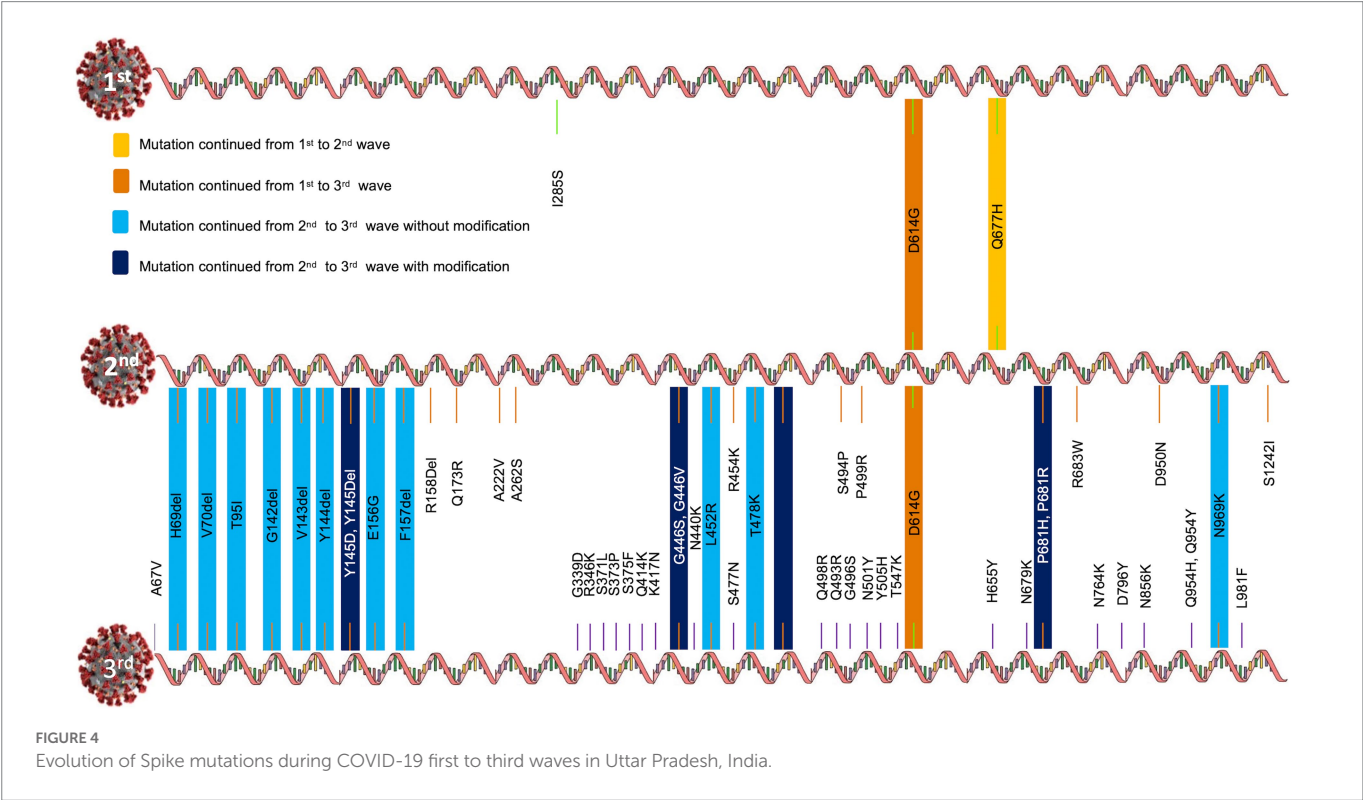
Spike mutation	Unvaccinated (%)	Vaccinated (%)	Fisher exact <i>p</i> value
Spike_R158del	17.86	96.92	6.84e-15
Spike_T478K	13.79	94.32	1.32e-16
Spike_D614G	100	95.23	0.179
Spike_E156G	19.23	78.75	8.03e-8
Spike_D950N	13.33	87.18	6.36e-7
Spike_L452R	12.50	66.30	1.11e-8
Spike_F157del	15.63	9.09	0.328
Spike_P681R	36.36	62.16	0.049
Spike_E484Q	2.78	Not detected	0.281
Spike_H1101D	5.56	Not detected	0.068
Spike_S982A	2.38	Not detected	0.280
Spike_V1104L	Not detected	1.00	1.00

3.4. SARS-CoV-2 binding affinity improved during evolution

The binding affinities and dissociation constants were considered as the key determinants of the pathogenesis and infectivity (Shang et al., 2020; Han et al., 2021; Supplementary Table S1). We evaluated if a change in the binding affinity between spike protein and human angiotensin-converting enzyme 2 (ACE2) receptor was responsible for a sudden shift in infectivity of SARS-CoV-2 in vaccinated cases. The overall configuration of the spike protein provides the ease of binding, as it contains many pores (>15 Å) and tunnels (>25 Å) near the binding cavity, facilitating a smooth entry. The calculation of binding affinity showed much higher affinity in Delta in comparison to the variants observed in the first wave. Similarly, Omicron showed a higher binding affinity and a lower dissociation constant than Delta (Figure 6).

In thermodynamic comparison (Supplementary Table S2), Delta consensus Pdb was found to involve 3 salt bridges, 6 hydrogen bonds and 125 non bonded contacts, offering binding free energy (BFE) of -20.2367 ± 1.118 kcal/mol while docking with ACE2. 21 L omicron-spike protein indulged 5 salt bridges, 3 hydrogen bonds and 103 non bonded contacts, contributing BFE of -61.8833 ± 2.254 kcal/mol, confirming a much higher affinity (Supplementary Table S2). Further, the lower dissociation constant (Kd) in 21l Omicron spike-ACE2 complex ($1.04E-07 \pm 1.7E-07$ kcal mol) than 21A Delta spike-ACE2 complex ($7.75E-08 \pm 7.19E-08$ kcal mol) established the reason behind higher level spontaneous and more stable binding of the Omicron 21 L spike protein with the ACE2 receptor (Figure 6).

Later on, when 21 K Omicron variants were subjected to *in silico* docking with the ACE-2 receptor, it showed a higher Gibb's free energy and a higher Kd value than Omicron 21 L as well as Delta, suggesting weaker binding, which may be the reason behind the lower number of 21 K Omicron cases in comparison to 21 L. Although the exact Kd



values differ depending upon the tools and approaches of calculation, our values were quite similar with [Shang et al. \(2020\)](#) and [Buratto et al. \(2021\)](#). However, the gross lowering of dissociation constant as the SARS-CoV-2 evolved from 2020 to 2022 indicated the higher chances of infectivity as it evolved ([Figure 6; Supplementary Table S2](#)).

Moreover, when the receptor and spike complex was checked for configuration stability by Ramachandran plot, both Delta and 21K Omicron showed similar percentages of allowed (98.3%) and disallowed regions (1.7%), whereas 21K presented 2.6% amino acids falling in the disallowed region, which may result in lesser stability of the complex. Additionally, the number of proline residues was higher in the outer region of the spike protein in 21K ([Figure 6](#)).

3.5. SARS-CoV-2 phylogeny and spike consensus homology mapping

MEGA11 was employed to find the molecular evolution from April 2021–June 2021 spread to December 2021–January 2022 spread. The nodes were elaborated further to capture the maximum likelihood homology in detail ([Figure 7](#)).

The tree topology indicated that the Delta lineages in Uttar Pradesh could arise from the ancestral lineage, but Omicron probably did not directly originate from any of the previous variants ([Venkatakrishnan et al., 2021](#)), instead it might have followed a cryptic genomic architecture involving a different recombination history ([Bolze et al., 2022; Ou et al., 2022](#)). The very long branch of the Omicron lineage in the time-calibrated tree might reflect less diversity within the group and a complex evolutionary history.

Proksee was used to find the neighborhood homology of Muscle.2 aligned spike sequences ([Figure 8](#)). The consensus (with the highest coverage) of the most abundant variant from each of the waves was classified by BLAST homology and mapped later in a circular presentation. The gap pattern or non-matched region reflected that although there was a significant homology in 20A Alpha and 21A Delta, suggesting a common parental root, Omicron did not share good homology with others, conferring the plausibility of mystic intervention in the evolutionary history ([Thiruvengadam et al., 2022](#)). The histogram pattern showed significant shifts in the curves due to several mutational changes, which abruptly shifted the GC skewness and made the domain a hot spot for upcoming events.

Mutations	Spike-D614G	Spike-L452R	Spike-P681R	Spike-T478K	Spike-D950N	NSP-P129L	NSP-T77A	NSP-A394V	Spike-F157del	Spike-E156G	Spike-R158del
Spike-D614G+Spike-L452R			39.59	59.068	24.16	6.04	17.44	8.05	40.26	40.93	40.26
Spike-D614G+Spike-P681R				38.92	20.13	6.04	14.09	8.05	26.84	26.84	26.17
Spike-D614G+Spike-T478K					23.48	6.04	17.44	7.38	40.93	41.61	41.61
Spike-D614G+Spike-D950N						4.02	10.73	2.01	19.46	20.13	19.46
Spike-D614G+NSP-P129L							1.34	0	3.35	3.35	3.35
Spike-D614G+NSP-T77A								4.02	16.10	16.10	16.10
Spike-D614G+NSP-A394V									2.68	3.35	2.68
Spike-D614G+Spike-F157del										51	50.33
Spike-D614G+Spike-E156G											51
Spike-L452R+Spike-P681R				40.26	20.80	6.04	14.76	7.38	26.84	26.84	26.17
Spike-L452R+Spike-T478K					25.50	6.71	18.79	7.38	41.61	42.28	42.28
Spike-L452R+Spike-D950N						4.02	11.40	2.01	20.80	21.47	20.80
Spike-L452R+NSP-P129L							1.34	0	3.35	3.35	3.35
Spike-L452R+NSP-T77A								4.02	16.10	16.10	16.10
Spike-L452R+NSP-A394V									2.68	3.35	2.68
Spike-L452R+Spike-F157del										42.28	41.61
Spike-L452R+Spike-E156G											42.28
Spike-P681R+Spike-T478K					20.13	6.04	14.76	6.71	26.17	26.17	26.17
Spike-P681R+Spike-D950N						4.02	11.40	1.34	18.79	18.79	18.12
Spike-P681R+NSP-P129L							1.34	0	3.35	3.35	3.35
Spike-P681R+NSP-T77A								4.02	12.08	12.08	12.08
Spike-P681R+NSP-A394V									2.68	2.68	2.01
Spike-P681R+Spike-F157del										26.84	26.17
Spike-P681R+Spike-E156G											26.17
Spike-T478K+Spike-D950N						4.02	11.40	1.34	20.13	20.80	20.80
Spike-T478K+NSP-P129L							1.34	0	3.35	3.35	3.35
Spike-T478K+NSP-T77A								4.02	16.10	16.10	16.10
Spike-T478K+NSP-A394V									2.01	2.68	2.68
Spike-T478K+Spike-F157del										42.95	42.95
Spike-T478K+Spike-E156G											43.62
Spike-D950N+NSP-P129L							1.34	0	3.35	3.35	3.35
Spike-D950N+NSP-T77A								1.34	10.73	10.73	10.73
Spike-D950N+NSP-A394V									1.34	2.01	1.34
Spike-D950N+Spike-F157del										20.80	20.13
Spike-D950N+Spike-E156G											20.80
NSP-P129L+NSP-T77A									1.34	1.34	1.34
NSP-P129L+NSP-A394V											0
NSP-P129L+Spike-F157del										3.35	3.35
NSP-P129L+Spike-E156G											3.35
NSP-T77A+NSP-A394V									2.01	2.01	2.01
NSP-T77A+Spike-F157del										17.44	17.44
NSP-T77A+Spike-E156G											2.68
NSP-A394V+Spike-F157del										2.01	2.68
NSP-A394V+Spike-E156G											54.36

FIGURE 5

Three mutations combination matrix for identification of mutations facilitating vaccination breach during COVID-19s waveThree mutations combination matrix for the identification of mutations facilitating vaccination breach during COVID-19 second wave. The background colour gradient from white to red indicates increasing frequency of combinations. Green arrows represent high frequency (>40), yellow arrows represent moderate frequency (20–40), and red arrows represent low frequency (<20). The background highlight in the mutation names indicates the most frequent combinations.

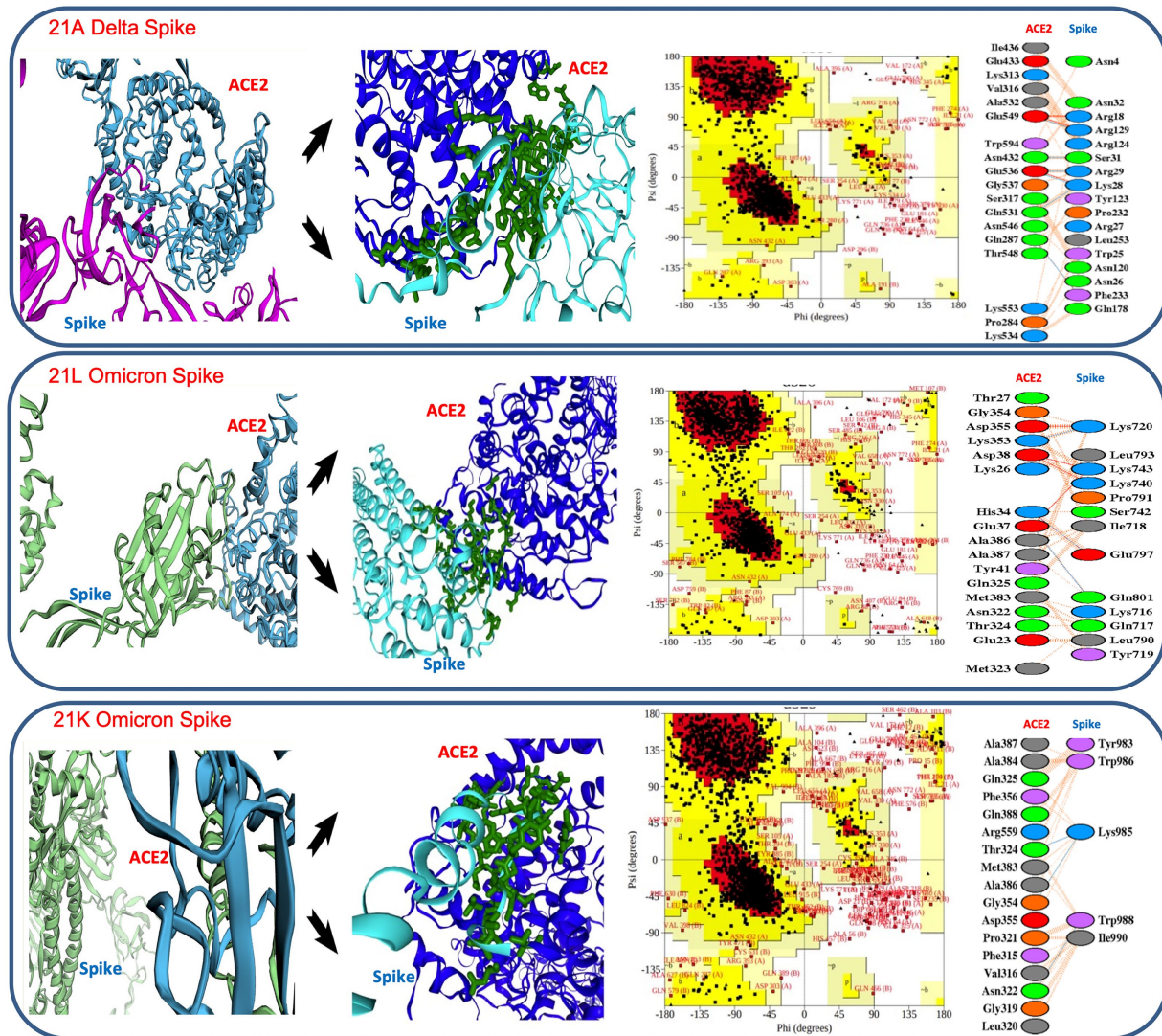


FIGURE 6

Details of *in silico* docking of SARS-CoV-2 Spike protein with host ACE2 receptor (leftmost across all three panels), intrinsic view of docking area (middle of all three panels), Ramachandran plotting indicating the stability of the docked complex (third block across all three panels), details of the amino acids involved in docking (rightmost across all three panels). It shows 21K Omicron Spike fused with ACE2 has the lowest stability as the major amino acids fall in the disallowed or restricted space.

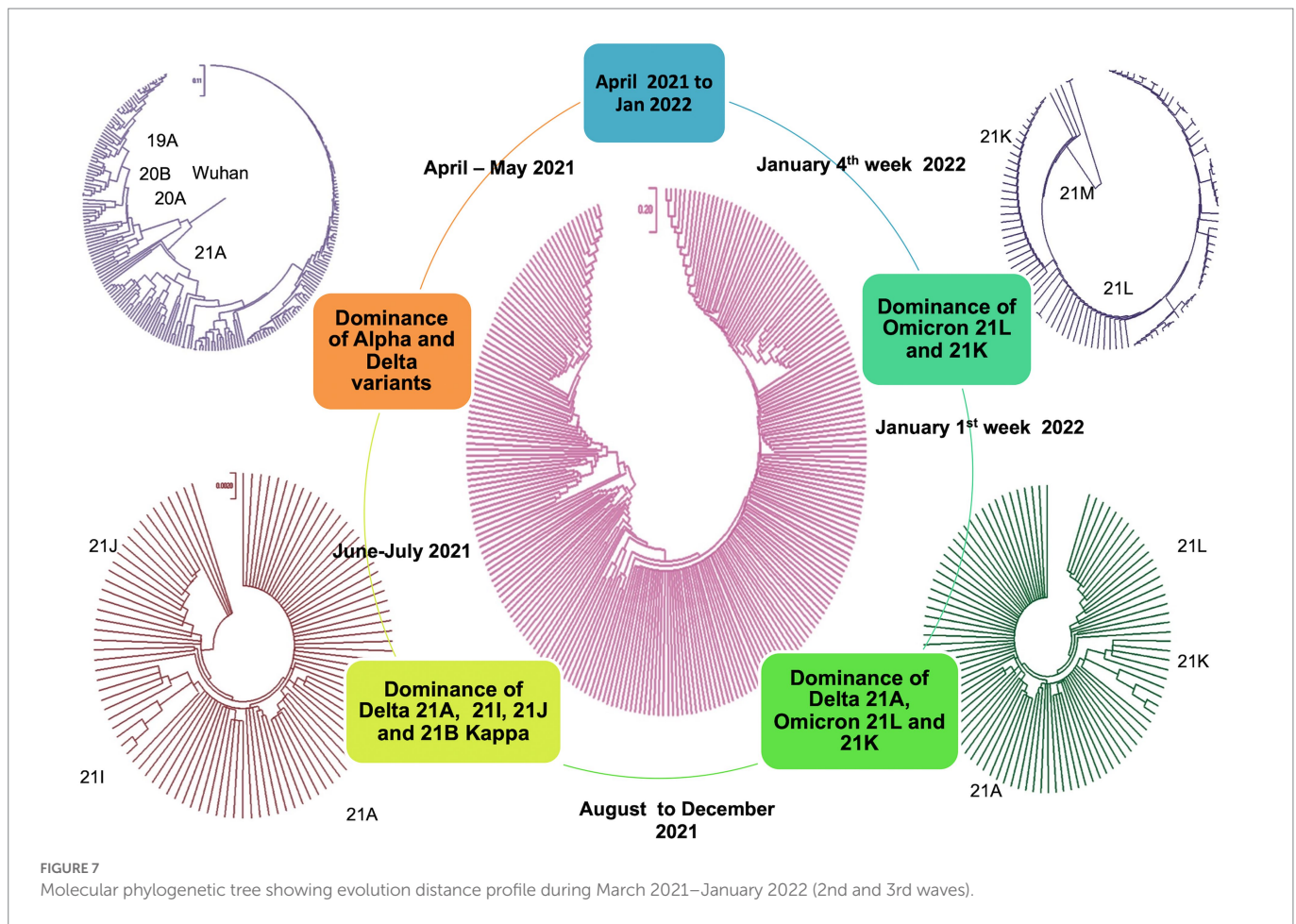
4. Discussion

4.1. SARS-CoV-2 genome evolved to cause breakthrough infections

Interestingly, India stood as an exclusive country where vaccination and the surge of second wave occurred concurrently, raising the selection pressure on the viral genome (Focosi and Maggi, 2022) and simultaneous immunity shift, antigenic drift, which might have triggered the origin and gradual rise of Delta in March 2021 (Kumar et al., 2020; Dhar et al., 2021; Gupta, 2021). COVID-19 vaccination in India started in mid-January, 2021. The vaccine was offered free of cost by the Government of India in phases at various centers across the country. Vaccination was in full swing in the months of June–July, 2021. Only one fourth of the population had received the first dose of vaccine and only 6% of population had received both the doses by July 2021 (Choudhary et al., 2021). The mortality in the first wave was significantly

higher in the elderly population, but the second wave resulted in much higher mortality in less than 45 years age population in comparison to the first wave.

The first wave was dominated by 20A Alpha (56.32%) and 20B (37.93%), and 19A (5.74%). The second wave was dominated by 21A Delta (56.88%), with a relatively much higher frequency of 21A Delta (75.84%) in vaccinated people. The unvaccinated pool majorly carried 20A (29.41%), 20B clade (16.17%) and 21A Delta (16.17%) variants. Other delta variants (21J+21I) were more common in unvaccinated (8.83%) in comparison to vaccinated individuals (3.96%; Figure 3; Table 1). The third wave was dominated by Omicron (71.8%), which outcompeted Delta (16.19%) by the end of January (Purohit et al., 2022). Although the ratio of the sister lineages of 21I and 21K of Omicron differed from state to state, 21I Omicron was found to be dominant (78.43%) over 21K sub lineage (18.62%) in Uttar Pradesh. Some of the mutations that originated in the first and second waves were retained by the subsequent variants of the virus, suggesting their contribution to the fitness of the virus (Figure 4).



4.2. Specific mutation combinations facilitated vaccination breach

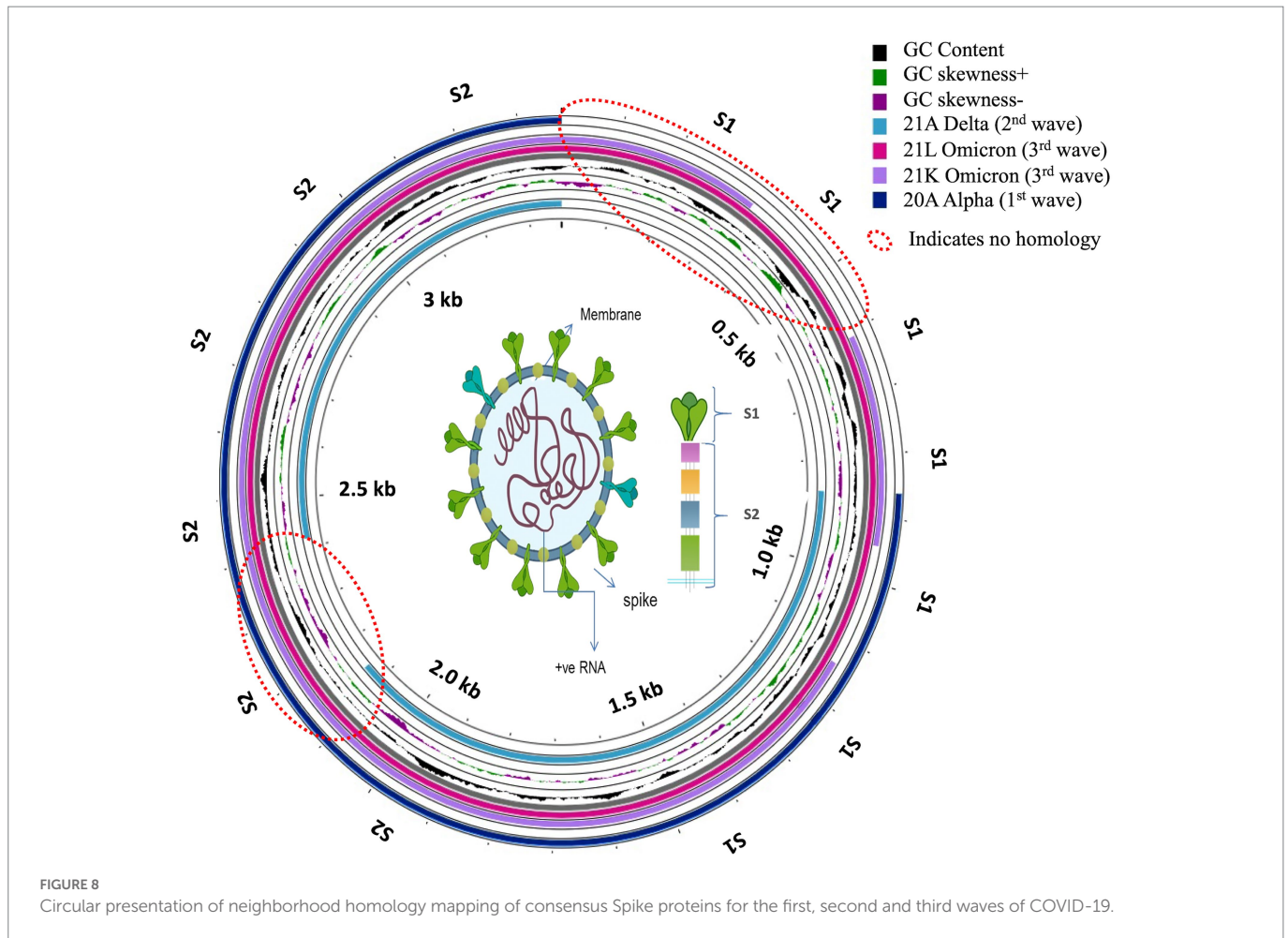
A key mutation noted in the first wave, D614G, significantly increased in frequency as the virus spread from Wuhan to Italy, United Kingdom and India (Mehta et al., 2021). D614G has higher dN/dS ratio (an indicator of selection pressure on coding genes; Volz et al., 2021). The emergence of other combinations of mutations further changed infectivity and virulence; however, D614G remained present and is still present in the Omicron variant as well. We observed that the combinations of spike mutations E156G, F157Del, L452R, T478K, D614G and grouping of NSP-A394V with spike E156G and R158Del evaded the protection provided by vaccination and brought the breakthrough infections in the second wave (Figure 5). Other studies from India also claimed that the emergence of L452R, T478K, E484Q, D614G and P681R mutations in the Spike protein was responsible for dynamic transmissibility and breakthrough of Delta variants in North, West and Mid India (Cherian et al., 2021; Joshi et al., 2021; Singh et al., 2021). The majority of these mutations were found to provide resistance by not being neutralized by convalescent sera and intrinsically enhancing the fusion of ACE-2 receptor with the S1 subunit of the trimeric spike glycoprotein (Kannan et al., 2022). Similarly, Wang et al. (2021), Pondé (2022) and others claimed that specific combinations of K417N, L452R, E484K and would strengthen the infectivity of the emerging SARS-CoV-2 variants. With the rise of Omicron, some of the spike protein mutations R158G, A262S, S494P, S1242I were reversed; however, the overall shift in mutations boosted the viral infectivity and dropped the virulence factor (Bhattacharyya and Hanage, 2022). Spike

R158G was earlier reported to provide fitness to Delta over Alpha variants (Liu et al., 2021) and significantly increased antibody escaping and has been linked with higher infectivity.

Although spike protein constitutes nearly 25% of unique mutations, recent findings have suggested that mutations in the N protein could also alter the function and fitness of SARS-CoV-2 genomes (Rahman et al., 2021; Wu H. et al., 2021). We found that NSP-A394V was associated with nearly 43% breakthrough cases as mentioned earlier, whereas NSP3-P1469S, NSP6-T77A, NSP3-M951I, NS7a-V82A, N-D63G, NS8-E19Stop, N-L139F, NS3-K67N, NSP3-T749A, N-S235F, N-R203K, N-G204R were also annotated in significant frequencies (nearly 5 to 30% cases). Ligand binding, viral oligomerization and packaging, fusion and antibody sensitivity have been reported to be disturbed by these mutations (Arya et al., 2021; Ahamad et al., 2022). Moreover, our findings stand in good agreement with the proposal that in addition to the D614G substitution, mutations in the N protein (R203K/G204R mutations) affect infectivity and virulence of SARS-CoV-2 (Wu S. et al., 2021; Yavarian et al., 2022). Therefore, future vaccine development programs may also focus on regions other than the Spike protein.

4.3. Omicron acquired higher infectivity and replaced delta

Spike protein in 21I had 37 mutations in comparison to 24 mutations in the 21A variant, which significantly changed its binding affinity with the host receptor protein. Binding affinity measured by Gibb's free energy has been used as a key to infectivity. We found that



the binding affinity was similar in 21A Delta and 21L Omicron (Supplementary Table S1), but a lower dissociation value of 21L in Omicron suggested a higher stability of receptor-ligand moiety and subsequent faster spread of the variant (Gupta, 2021; Mlcochova et al., 2021). On the other hand, 21K Omicron has shown relatively higher mean value of $-\Delta G$ and K_d than 21L Omicron and 21A Delta, conferring lower infectivity to the variant. Similarly, Ramachandran plot of the ACE2 receptor -21K ligand complex suggested several amino acids in the disallowed region, confirming lesser stability than Delta and Omicron 21L (Figure 6). Moreover, in 21K, a higher number of proline residues were present in the outer region of generously allowed region, offering lesser permeability and binding with the receptor (Shastri et al., 2021). Further, only five amino acids made significant contact with the receptor motif in comparison with 17 and 18 residues making contact in the cases of 21L and Delta, respectively, making the binding in 21K more fragile. Furthermore, Pymol and PdbSum showed relatively longer distance between 21K-ACE2 in comparison with 21L and Delta. The absence of salt bridges in 21K also delimited the interaction capability (Malladi et al., 2021). Since embedding of the salt bridges in the hydrophobic environment stimulates the virus binding energy due to the lowering of dielectric constant; their absence could significantly reduce the affinity in 21K (Mlcochova et al., 2021). Such a unique combination of mutations in Omicron might have arisen from a recombination between multiple active or dormant variants in the host (Ou et al., 2022). The mutation driven shift in the binding affinity served to confer fitness to Omicron to replace Delta.

5. Limitations

A major limitation of this study was a small overall sample size in general and a very small unvaccinated group in the second wave in particular. For statistical comparisons with high confidence at the population level, we would need a sample size way above the one used in this study. Therefore, the statistical comparisons between the waves, and the vaccinated versus unvaccinated groups must be taken with caution. These findings, though partly replicated by a few concurrent and previous publications, should be subjected to further investigations using a much bigger sample size. The other limitation was the lack of full genome coverage in sequencing, which could mask certain interesting mutations, which might be significant in deciding the course of evolution of the SARS-CoV-2 genome.

6. Conclusion

The present study aimed at addressing the evolutionary dynamics and mutational profile of SARS-CoV-2 in Uttar Pradesh, India during the 2020–2022 period. One of the key mutations during the early spread of SARS-CoV-2 in Uttar Pradesh, India, was D614G, which was critical in providing infectivity to the virus. This mutation has stayed even in the Delta and Omicron variants, suggesting its critical role in infectivity (Hacisuleyman et al., 2021).

In the due course of time, signature combinations of Spike mutations, namely, E156G, F157Del, L452R, T478K, D614G and clustering of NSP-A394V with Spike mutations E156G and R158Del were predominantly associated with vaccination breach infections during the second wave in Uttar Pradesh, India. The key determinants of vaccination breach (F157del, R158del, L452R, T478K, D614G, P681R) were succeeded in the Omicron genome, although some mutations observed in Delta (S1242I, A262S, S494P, R158G) were not seen in the Omicron genome. With this unique selective combination of mutations, Omicron lost virulence and gained infectivity, leading to faster infections but milder effects. Further, the phylogeny tree analysis suggested that the Delta lineage in Uttar Pradesh could arise from the ancestral lineages, but Omicron probably did not directly originate from any of the previously existent single variant, instead it might have arisen from a cryptic genomic architecture involving unusual recombination history. This evolution suggests that new mutations arising in the SARS-CoV-2 genome account for increase in infectivity and reduction in disease severity, eventually leading to the replacement of Delta with Omicron. Significant vaccination breach and wide variations in the infectivity and virulence with molecular changes in the SARS-CoV-2 genome suggest that the emergence of new variants can have significant implications in future pandemics and vaccine efficacy.

Data availability statement

The data presented in the study are deposited in the GISAID repository at <https://gisaid.org> under accession heads Asia/India/Uttar Pradesh/CDRI with multiple accession numbers. The accession numbers can be found in the [Supplementary material](#).

Ethics statement

Ethical review and approval were 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

SP, PM, and AA undertook library preparation and sequencing work. AP, RT, PY, UdG, and UjG undertook sample collection and

RNA isolation. UjG, UdG, RR, TK, and SR planned the study and arranged funding. RP undertook phylogenetic analysis and provided intellectual inputs. SP undertook structure–function and phylogenetic analysis. SP, PM, and SR wrote the article. 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.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2023.986729/full#supplementary-material>

References

- Abraham, P. (2021). Clinical characterization and genomic analysis of samples from COVID-19 breakthrough infections during the second wave among the various states of India. *Viruses* 13:1782. doi: 10.3390/v13091782
- Ahamad, S., Gupta, D., and Kumar, V. (2022). Targeting SARS-CoV-2 nucleocapsid oligomerization: insights from molecular docking and molecular dynamics simulations. *J. Biomol. Struct. Dyn.* 40, 2430–2443. doi: 10.1080/07391102.2020.1839563
- Arya, R., Kumari, S., Pandey, B., Mistry, H., Bihani, S. C., Das, A., et al. (2021). Structural insights into SARS-CoV-2 proteins. *J. Mol. Biol.* 433:166725. doi: 10.1016/j.jmb.2020.11.024
- Banerjee, A., Mossman, K., and Grandvaux, N. (2021). Molecular determinants of SARS-CoV-2 variants. *Trends Microbiol.* 29, 871–873. doi: 10.1016/j.tim.2021.07.002
- Bhattacharyya, R. P., and Hanage, W. P. (2022). Challenges in inferring intrinsic severity of the SARS-CoV-2 omicron variant. *N. Engl. J. Med.* 386:e14. doi: 10.1056/NEJMp2119682
- Bolze, A., White, S., Basler, T., Dei Rossi, A., Roychoudhury, P., Greninger, A. L., et al. (2022). Evidence for SARS-CoV-2 Delta and omicron co-infections and recombination. *medRxiv*. doi: 10.1101/2022.03.09.22272113
- Buratto, D., Saxena, A., Ji, Q., Yang, G., Pantano, S., and Zonta, F. (2021). Rapid assessment of binding affinity of SARS-COV-2 spike protein to the human angiotensin-converting enzyme 2 receptor and to neutralizing biomolecules based on computer simulations. *Front. Immunol.* 12:0099. doi: 10.3389/fimmu.2021.730099
- Chan, Y. A., and Zhan, S. H. (2022). The emergence of the spike furin cleavage site in SARS-CoV-2. *Mol. Biol. Evol.* 39:msab327. doi: 10.1093/molbev/msab327

- Cherian, S., Potdar, V., Jadhav, S., Yadav, P., Gupta, N., Das, M., et al. (2021). SARS-CoV-2 spike mutations, L452R, T478K, E484Q and P681R, in the second wave of COVID-19 in Maharashtra, India. *Microorganisms* 9:1542. doi: 10.3390/microorganisms9071542
- Choudhary, O. P., Choudhary, P., and Singh, I. (2021). India's COVID-19 vaccination drive: key challenges and resolutions. *Lancet Infect. Dis.* 21, 1483–1484. doi: 10.1016/S1473-3099(21)00567-3
- Dhar, M. S., Marwal, R., Vs, R., Ponnusamy, K., Jolly, B., Bhoyar, R. C., et al. (2021). Genomic characterization and epidemiology of an emerging SARS-CoV-2 variant in Delhi, India. *Science* 374, 995–999. doi: 10.1126/science.abj9932
- Dubey, A., Choudhary, S., Kumar, P., and Tomar, S. (2022). Emerging SARS-CoV-2 variants: genetic variability and clinical implications. *Curr. Microbiol.* 79, 1–18. doi: 10.1007/s00284-021-02724-1
- Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with improved accuracy and speed. In *Proceedings 2004 IEEE computational systems bioinformatics conference*, 2004. CSB 2004. (pp. 728–729). IEEE.
- Focosi, D., and Maggi, F. (2022). Recombination in coronaviruses, with a focus on SARS-CoV-2. *Viruses* 14:1239. doi: 10.3390/v14061239
- Grant, J. R., and Stothard, P. (2008). The CGView server: a comparative genomics tool for circular genomes. *Nucleic Acids Res.* 36, W181–W184. doi: 10.1093/nar/gkn179
- Gupta, R. K. (2021). Will SARS-CoV-2 variants of concern affect the promise of vaccines? *Nat. Rev. Immunol.* 21, 340–341. doi: 10.1038/s41577-021-00556-5
- Hacisuleyman, E., Hale, C., Saito, Y., Blachere, N. E., Bergh, M., Conlon, E. G., et al. (2021). Vaccine breakthrough infections with SARS-CoV-2 variants. *N. Engl. J. Med.* 384, 2212–2218. doi: 10.1056/NEJMoa2105000
- Han, Y., Wang, Z., Wei, Z., Schapiro, I., and Li, J. (2021). Binding affinity and mechanisms of SARS-CoV-2 variants. *Comput. Struct. Biotechnol. J.* 19, 4184–4191. doi: 10.1016/j.csbj.2021.07.026
- Jha, N., Hall, D., Kankan, A., Mehta, P., Maurya, R., Mir, Q., et al. (2021). Geographical landscape and transmission dynamics of SARS-CoV-2 variants across India: a longitudinal perspective. *Front. Genet.* 12:648. doi: 10.3389/fgene.2021.753648
- Joshi, M., Puvvar, A., Kumar, D., Ansari, A., Pandya, M., Raval, J., et al. (2021). Genomic variations in SARS-CoV-2 genomes from Gujarat: underlying role of variants in disease epidemiology. *Front. Genet.* 12:586569. doi: 10.3389/fgene.2021.586569
- Kannan, S. R., Spratt, A. N., Sharma, K., Chand, H. S., Byraredy, S. N., and Singh, K. (2022). Omicron SARS-CoV-2 variant: unique features and their impact on pre-existing antibodies. *J. Autoimmun.* 126:102779. doi: 10.1016/j.jaut.2021.102779
- Khare, S., Gurry, C., Freitas, L., B. Schultz, M., Bach, G., Diallo, A., et al. (2021). GISAD's role in pandemic response. *China CDC Weekly* 3, 1049–1051. doi: 10.46234/ccdcw2021.255
- Kumar, P., Pandey, R., Sharma, P., Dhar, M. S., Vivekanand, A., Uppili, B., et al. (2020). Integrated genomic view of SARS-CoV-2 in India. *Wellcome open research* 5:184. doi: 10.12688/wellcomeopenres.16119.1
- Laskowski, R. A., Jablonska, J., Pravda, L., Vařeková, R. S., and Thornton, J. M. (2018). PDBsum: structural summaries of PDB entries. *Protein Sci.* 27, 129–134. doi: 10.1002/pro.3289
- Liu, Z., VanBlargan, L. A., Bloyet, L. M., Rothlauf, P. W., Chen, R. E., Stumpf, S., et al. (2021). Identification of SARS-CoV-2 spike mutations that attenuate monoclonal and serum antibody neutralization. *Cell Host & Microbe* 29, 477–488.
- MacLean, O. A., Lytras, S., Weaver, S., Singer, J. B., Boni, M. F., Lemey, P., et al. (2021). Natural selection in the evolution of SARS-CoV-2 in bats created a generalist virus and highly capable human pathogen. *PLoS Biol.* 19:e3001115. doi: 10.1371/journal.pbio.3001115
- Malladi, S. K., Patel, U. R., Rajamani, R. S., Singh, R., Pandey, S., Kumar, S., et al. (2021). Immunogenicity and protective efficacy of a highly thermotolerant, trimeric SARS-CoV-2 receptor binding domain derivative. *ACS Infectious Diseases* 7, 2546–2564. doi: 10.1021/acsinfectdis.1c00276
- Mehta, P., Sarkar, S., Ghoshal, U., Pandey, A., Singh, R., Singh, D., et al. (2021). Severe acute respiratory syndrome Coronavirus-2 genome sequence variations relate to morbidity and mortality in coronavirus Disease-19. *bioRxiv*. doi: 10.1101/2021.05.24.445374
- Mlcochova, P., Kemp, S. A., Dhar, M. S., Papa, G., Meng, B., Ferreira, I. A., et al. (2021). SARS-CoV-2 B. 1.617. 2 Delta variant replication and immune evasion. *Nature* 599, 114–119. doi: 10.1038/s41586-021-03944-y
- Moreira, I. S., Koukos, P. I., Melo, R., Almeida, J. G., Preto, A. J., Schaarschmidt, J., et al. (2017). SpotOn: high accuracy identification of protein-protein interface hot-spots. *Sci. Rep.* 7, 1–11. doi: 10.1038/s41598-017-08321-2
- Ou, J., Lan, W., Wu, X., Zhao, T., Duan, B., Yang, P., et al. (2022). Tracking SARS-CoV-2 omicron diverse spike gene mutations identifies multiple inter-variant recombination events. *Signal Transduct. Target. Ther.* 7, 1–9. doi: 10.1038/s41392-022-00992-2
- Petersen, E., Ntouni, F., Hui, D. S., Abubakar, A., Kramer, L. D., Obiero, C., et al. (2022). Emergence of new SARS-CoV-2 variant of concern omicron (B. 1.1. 529)-highlights Africa's research capabilities, but exposes major knowledge gaps, inequities of vaccine distribution, inadequacies in global COVID-19 response and control efforts. *Int. J. Infect. Dis.* 114, 268–272. doi: 10.1016/j.ijid.2021.11.040
- Pondé, R. A. A. (2022). Physicochemical effect of the N501Y, E484K/Q, K417N/T, L452R and T478K mutations on the SARS-CoV-2 spike protein RBD and its influence on agent fitness and on attributes developed by emerging variants of concern. *Virology* 572, 44–54. doi: 10.1016/j.virol.2022.05.003
- Purohit, N., Chugh, Y., Bahuguna, P., and Prinja, S. (2022). COVID-19 management: The vaccination drive in India. *Health Policy and Technology* 11:100636. doi: 10.1016/j.hlpt.2022.100636
- Purushotham, J. N., van Doremalen, N., and Munster, V. J. (2021). SARS-CoV-2 vaccines: anamnestic response in previously infected recipients. *Cell Res.* 31, 827–828. doi: 10.1038/s41422-021-00516-7
- Rahman, M. S., Islam, M. R., Alam, A. R. U., Islam, I., Hoque, M. N., Akter, S., et al. (2021). Evolutionary dynamics of SARS-CoV-2 nucleocapsid protein and its consequences. *J. Med. Virol.* 93, 2177–2195. doi: 10.1002/jmv.26626
- Rochman, N. D., Wolf, Y. I., Faure, G., Mutz, P., Zhang, F., and Koonin, E. V. (2021). Ongoing global and regional adaptive evolution of SARS-CoV-2. *Proc. Natl. Acad. Sci.* 118:e2104241118. doi: 10.1073/pnas.2104241118
- Roel-Touris, J., Don, C. G., Honorato R. V., Rodrigues, J. P., and Bonvin, A. M. (2019). Less is more: coarse-grained integrative modeling of large biomolecular assemblies with HADDOCK. *J. Chem. Theory Comput.* 15, 6358–6367. doi: 10.1021/acs.jctc.9b00310
- Shang, J., Ye, G., Shi, K., Wan, Y., Luo, C., Aihara, H., et al. (2020). Structural basis of receptor recognition by SARS-CoV-2. *Nature* 581, 221–224. doi: 10.1038/s41586-020-2179-y
- Shastri, J., Parikh, S., Aggarwal, V., Agrawal, S., Chatterjee, N., Shah, R., et al. (2021). Severe SARS-CoV-2 breakthrough reinfection with delta variant after recovery from breakthrough infection by alpha variant in a fully vaccinated health worker. *Front. Med.* 8:737007. doi: 10.3389/fmed.2021.737007
- Singh, J., Rahman, S. A., Ehtesham, N. Z., Hira, S., and Hasnain, S. E. (2021). SARS-CoV-2 variants of concern are emerging in India. *Nat. Med.* 27, 1131–1133. doi: 10.1038/s41591-021-01397-4
- Singh, D., and Yi, S. V. (2021). On the origin and evolution of SARS-CoV-2. *Exp. Mol. Med.* 53, 537–547. doi: 10.1038/s12276-021-00604-z
- Tamura, K., Stecher, G., and Kumar, S. (2021). MEGA11: molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* 38, 3022–3027. doi: 10.1093/molbev/msab120
- Thiruvengadam, R., Binayke, A., and Awasthi, A. (2022). SARS-CoV-2 delta variant: a persistent threat to the effectiveness of vaccines. *Lancet Infect. Dis.* 22, 301–302. doi: 10.1016/S1473-3099(21)00697-6
- Venkatkrishnan, A. J., Anand, P., Lenehan, P. J., Suratekar, R., Raghunathan, B., Niesen, M. J., et al. (2021). Omicron variant of SARS-CoV-2 harbors a unique insertion mutation of putative viral or human genomic origin.
- Vishvkarma, R., and Rajender, S. (2020). Could SARS-CoV-2 affect male fertility? *Andrologia* 52:e13712. doi: 10.1111/and.13712
- Volz, E., Mishra, S., Chand, M., Barrett, J. C., Johnson, R., Geidelberg, L., et al. (2021). Assessing transmissibility of SARS-CoV-2 lineage B. 1.1. 7 in England. *Nature* 593, 266–269. doi: 10.1038/s41586-021-03470-x
- Wang, R., Chen, J., and Wei, G. W. (2021). Mechanisms of SARS-CoV-2 evolution revealing vaccine-resistant mutations in Europe and America. *J. physical chemistry letters* 12, 11850–11857. doi: 10.1021/acs.jpclett.1c03380
- Wang, S., Sundaram, J. P., and Spiro, D. (2010). VIGOR, an annotation program for small viral genomes. *BMC bioinformatics* 11, 1–10. doi: 10.1186/1471-2105-11-451
- Weng, G., Wang, E., Wang, Z., Liu, H., Zhu, F., Li, D., et al. (2019). HawkDock: a web server to predict and analyze the protein-protein complex based on computational docking and MM/GBSA. *Nucleic Acids Res.* 47, W322–W330. doi: 10.1093/nar/gkz397
- Williams, T. C., and Burgers, W. A. (2021). SARS-CoV-2 evolution and vaccines: cause for concern? *Lancet Respir. Med.* 9, 333–335. doi: 10.1016/S2213-2600(21)00075-8
- Wu, S., Tian, C., Liu, P., Guo, D., Zheng, W., Huang, X., et al. (2021). Effects of SARS-CoV-2 mutations on protein structures and intraviral protein-protein interactions. *J. Med. Virol.* 93, 2132–2140. doi: 10.1002/jmv.26597
- Wu, H., Xing, N., Meng, K., Fu, B., Xue, W., Dong, P., et al. (2021). Nucleocapsid mutations R203K/G204R increase the infectivity, fitness, and virulence of SARS-CoV-2. *Cell Host Microbe* 29, 1788–1801.e6. doi: 10.1016/j.chom.2021.11.005
- Xue, L. C., Rodrigues, J. P., Kastriitis, P. L., Bonvin, A. M., and Vangone, A. (2016). PRODIGY: a web server for predicting the binding affinity of protein-protein complexes. *Bioinformatics* 32, 3676–3678. doi: 10.1093/bioinformatics/btw514
- Yavarian, J., Nejati, A., Salimi, V., Shafiei Jandaghi, N. Z., Sadeghi, K., Abedi, A., et al. (2022). Whole genome sequencing of SARS-CoV2 strains circulating in Iran during five waves of pandemic. *PLoS One* 17:e0267847. doi: 10.1371/journal.pone.0267847



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SARS-CoV-2 evolution among patients with immunosuppression in a nosocomial cluster of a Japanese medical center during the Delta (AY.29 sublineage) surge

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Background: Previous studies have shown that patients with immunosuppression tend to have longer-lasting SARS-CoV-2 infections and a number of mutations were observed during the infection period. However, these studies were, in general, conducted longitudinally. Mutation evolution among groups of patients with immunosuppression have not been well studied, especially among Asian populations.

Methods: Our study targeted a nosocomial cluster of SARS-CoV-2 infection in a Japanese medical center during Delta surge (AY.29 sublineage), involving ward nurses and inpatients. Whole-genome sequencing analyses were performed to examine mutation changes. Haplotype and minor variant analyses were further performed to detect the mutations on the viral genomes in detail. In addition, sequences of the first wild-type strain hCoV-19/Wuhan/WIV04/2019 and AY.29 wild-type strain hCoV-19/Japan/TKYK15779/2021 were used as references to assess the phylogenetical development of this cluster.

Results: A total of 6 nurses and 14 inpatients were identified as a nosocomial cluster from September 14 through 28, 2021. All were Delta variant (AY.29 sublineage) positive. 92.9% of infected patients (13 out of 14) were either cancer patients and/or receiving immunosuppressive or steroid treatments. Compared to AY.29 wild type, a total of 12 mutations were found in the 20 cases. Haplotype analysis found one index group of eight cases with F274F (N) mutation and 10 other haplotypes with one to three additional mutations. Furthermore, we found that cases with more than three minor variants were all cancer patients under immunosuppressive treatments. The phylogenetical tree analysis, including 20 nosocomial cluster-associated viral genomes, the first wild-type strain and the AY.29 wild-type strain as references, indicated the mutation development of the AY.29 virus in this cluster.

Conclusion: Our study of a nosocomial SARS-CoV-2 cluster highlights mutation acquisition during transmission. More importantly, it provided new evidence emphasizing the need to further improve infection control measures to prevent nosocomial infection among immunosuppressed patients.

KEYWORDS

SARS-CoV-2, Delta variant, AY.29, immunosuppression, mutation, genome sequencing, nosocomial cluster

Introduction

SARS-CoV-2, the causative agent of the COVID-19 pandemic, is a positive-sense single-stranded RNA virus, known to acquire new mutations at each cycle of genome replication due to the error-prone nature of the viral RNA-dependent polymerase complex (Drake, 1993; Kim et al., 2020). The mutation rates of RNA viruses are generally higher than DNA viruses, and mutations lead to the selection and evolution of viral genomes (Duffy, 2018; Peck and Lauring, 2018). Since the first report of the Delta variant of SARS-CoV-2 in India in late 2020, this variant quickly became the dominant clade globally until the Omicron variant took over soon after its first report in South Africa in November 2021 (World Health Organization, 2021). Previous studies indicate that the Delta variant has been evolving, supported by evidence of patients positive for Delta harboring newly identified mutations (Baj et al., 2021).

Meanwhile, patients with immunosuppression were reported to be at risk for prolonged infection with SARS-CoV-2, along with a number of identified substitutions and deletions in genome sequences (Choi et al., 2020; Corey et al., 2021; Kemp et al., 2021). Although cases with immunosuppression were traced longitudinally and genome sequences were conducted sporadically during their infection to identify mutations, how mutations evolve among a select group of SARS-CoV-2 infected patients with immunosuppression has not been well studied, especially in Asian populations.

AY.29, a sublineage of the Delta variant, was detected and first reported in April 2021; it quickly became predominant in Japan until the end of the year when Omicron began replacing Delta. Although AY.29 was known to have Y1658Y and V1750A in ORF1ab (NSP3) mutations (EPI_ISL_2723567/EPI_ISL_2723568; Abe and Arita, 2021; Koyama et al., 2022), studies on further mutation acquisition have remained scant. With increased transmission and hospitalization rates compared to previous variants of concern (VOC), nosocomial clusters have been reported worldwide during the Delta surge, including those occurring in hospitals with strict infection control measures (Klompas et al., 2021; Lim et al., 2021). Using whole-genome sequencing analysis of infection cases, nosocomial clusters provide a natural environment for tracing and analyzing mutation emergence. As a referral academic medical center with strict infection control protocols in Japan, Juntendo University Hospital (JUH) experienced a nosocomial cluster in September 2021 during the Delta surge, including ward nurses and inpatients with existing respiratory or rheumatological/autoimmune diseases.

To clinically better understand why this cluster rapidly developed and how mutations emerged among this group of high-risk inpatients, we performed whole-genome sequencing analysis to examine mutation evolution of the infected cases in the nosocomial cluster.

Methods and materials

Description of hospital and baseline infection control measures

Juntendo University Hospital is a 1,051-bed academic medical center in Japan. Since the outbreak of the COVID-19 pandemic, strict infection control measures have been implemented. At the hospital, masks are universally required of all healthcare workers and

patients in all facilities including outpatient clinics and wards (when patients' conditions allow). All inpatients are nasopharyngeal or saliva polymerase chain reaction (PCR) tested at the time of admission. Visitors to wards are prohibited in general; in pediatric wards, one PCR-tested parent may be allowed to stay with the child if needed (from admission day until the discharge day without entry and exit from the ward). For healthcare workers, in addition to universal masking, face shield or eye protection is required when encountering all patients; additionally, use of N95 respirators when caring for patients with suspected or confirmed COVID-19 is mandated. Temperature checks occur daily at the workplace, with COVID-19 symptoms requiring further examination; dining with more than three non-family members outside work hours is discouraged per hospital policy. Close contacts of confirmed cases are PCR-tested and quarantined. Because of these strict infection control measures, there was not a major nosocomial cluster of COVID-19 until September 2021 in this hospital.

Detection of nosocomial cluster

Two nurses who worked in Ward I and reported COVID-19 symptoms on September 13, 2021, were confirmed to be infected by PCR positive tests on September 14. Immediately, all healthcare workers of Ward I, including doctors, nurses and administrative staff, as well as close contacts, were screened by PCR tests and frequently tested thereafter as new cases were identified in the ward. Healthcare workers with confirmed infection were quarantined immediately after detection for a defined period (7 days after symptom onset, or 7 days in total for asymptomatic cases). For the patients of Ward I, PCR tests were conducted on close contacts of confirmed cases or having COVID-19 symptoms. For patients of Ward II on the same floor as Ward I, PCR tests were conducted if patients reported COVID-19 symptoms. All patients of confirmed infection were transferred to COVID-19 wards of the hospital immediately after detection, or into private rooms for temporary stay until COVID-19 rooms became available.

Collection of respiratory specimens and RT-PCR

For diagnosis of SARS-CoV-2 infection, nasopharyngeal and saliva tests (both proved to have high sensitivity and specificity) were performed (Yokota et al., 2021). Nasopharyngeal swabs were performed following a standardized procedure (World Health Organization, 2006). For saliva sampling, The participants collected 1–2 mL of unstimulated saliva into a sterile 50-mL polyethylene tube. Nasopharyngeal swabs and saliva samples were submitted for RT-PCR testing within 3 h after collection (Pandit et al., 2013). RT-PCR was carried out using the 2019 novel coronavirus detection Kit (nCoV-DK; Shimadzu corporation, Kyoto, Japan). The nCoV-DK assay uses the “2019-nCoV_N1” primer and probe sequences as described by the United States CDC’s “2019-novel coronavirus real-time rRT-PCR panel primers and probes” (Centers for Disease Control and Prevention, 2020). This assay also includes internal control oligonucleotides. Specific spike protein variations (L452R, N501Y, E484K, E484Q) were detected with the VirSNIp SARS-CoV-2 mutation assays (Roche diagnostics, Rotkreuz, Switzerland) according to manufacturer

instructions. Real-time PCR analysis was run on a light cycler system (Roche, California, United States).

Next generation sequencing

Purified RNA was reverse-transcribed into cDNA using the SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, United States), and the synthesized cDNA was amplified with the Ion AmpliSeq SARS-CoV-2 Research Panel (Thermo Fisher Scientific, Waltham, MA, United States) on the Ion GeneStudio S5 System according to manufacturer instructions. The Ion AmpliSeq SARS-CoV-2 Research Panel consists of 2 primer pools targeting 237 amplicons tiled across the SARS-CoV-2 genome, with an additional 5 primer pairs targeting human expression controls. The SARS-CoV-2 amplicons range from 125 to 275 bp in length. Amplified samples were then sequenced using Ion 530 chips (Thermo Fisher Scientific) with eight samples per chip on the Ion S5 system. The Torrent Suite 5.14.0 platform and specific plugins were used for Next-Generation Sequencing (NGS) data analysis. The COVID19AnnotateSnpEff (v.1.3.0) plugin was used for annotation of variants. SARS-CoV-2 variants with frequencies of SNP higher than 18% or indel higher than 25% can be reproducibly detected with sequencing depth. All analyzed sequences showed a base accuracy of over 96% and a base coverage over 45×. The pangolin software was used for the assignment of SARS-CoV-2 lineages. Sequencing reads were then submitted as FASTA files and deposited in the EpiCoV database of Global Initiative on Sharing Avian Influenza Data (GISAID) (Shu and McCauley, 2017). Amino acid substitutions in the sequenced viruses were analyzed by GISAID during the registration of the viral genomes, while information was collected from the EpiCoV database. Analysis of PANGO lineage was performed based on v.3.1.15.

Samples were processed, sequenced and analyzed according to the following schedule: Case 1 to Case 16: September 24–29, 2021; Case 17 to Case 20: October 1–6, 2021.

Mutation analysis

Because this cluster occurred during the Delta (AY.29 sublineage) surge in the Tokyo metropolitan area, mutations of the nosocomial cluster were identified using an AY.29 strain as reference. Sequence hCoV-19/Japan/TKYK15779/2021, which was registered in April 2021 when AY.29 was first detected and reported in Japan (Koyama et al., 2022), was used. A table of mutations was prepared, with conserved mutations in all samples identified as the index type, and variables shown for other cases.

Phylogenetic tree and haplotype network analysis

To clarify the relationship of each cluster-related virus and its relationship with AY.29, phylogenetic tree analysis was performed by using the 20 samples from the studied cluster, and the wild type SARS-CoV-2 hCoV-19/Wuhan/WIV04/2019 and the AY.29 strain hCoV-19/Japan/TKYK15779/2021 as references. These sequences were aligned with the MAFFT v7.490. Poorly aligned regions in 5' and 3' ends were trimmed, and the core regions were determined to

be from the 55-to 29,856-nt position in the reference sequence. A Maximum Likelihood phylogenetic tree with ultrafast bootstrap support values (calculated from 1,000 replicates) was constructed by IQ-TREE 2.1.2 under the TIM2 + F nucleotide substitution model, which was selected by the ModelFinder software. The haplotype data were generated in DnaSP v6.12.03 (Rozas et al., 2017), and a median-joining network was constructed by PopART v1.7 (Leigh and Bryant, 2015).

Detection of minor variants

Variant callers were performed with the parameters: minimum allele frequency was set to indel=0.05; snp=0.05; mnp=0.05; gen_min_alt_allele_freq=0.025; and gen_min_indel_alt_allele_freq=0.025. Variations were annotated to the reference genome SARS-CoV-2 strain Wuhan-Hu-1 (accession number: NC_045512.1) using SARS CoV-2 annotate SnpEff. The resulting alignments were visualized to examine false positive with the Integrated Genomics Viewer (IGV) v2.15.4 (Robinson et al., 2011). Identified mutations in the cluster cases were compared to the mutations in AY.29 wild type against Wuhan-Hu-1. The different nucleotide and amino acid sequences between cluster-associated viruses and AY.29 wild type were summarized.

Results

From Sep 14 through Sep 28, 2021, a total of 20 nurses and patients in Ward I were confirmed to be infected with SARS-CoV-2. All were Delta variant positive. Characteristics of the 20 cases are shown in Table 1. The case numbers were assigned chronologically by the PCR confirmation date. Among them, 6 were nurses (age range: 23 to 50) working in Ward I, including 5 in Team A and 1 in Team B; 5 were fully vaccinated with COVID-19 mRNA vaccines (two doses) and 1 partially vaccinated (1 dose). None needed medical care; 2 were asymptomatic while 4 had light symptoms such as runny nose, fatigue, cough, joint pain, or sore throat.

Regarding the 14 infected patients (age range: 18 to 77), 7 had rheumatology/autoimmune diseases; 7 had respiratory diseases. 92.9% of these infected patients (13 out of 14) were either cancer patients and/or receiving immunosuppressive or steroid treatments. Specifically, 6 out of 7 patients with rheumatology/autoimmune diseases were under immunosuppressive and/or steroid treatment; for the 7 patients with respiratory disease, all had cancer and 5 were either under immunosuppressive and/or steroid treatment. Only 4 (out of 14) infected patients had been fully vaccinated; 1 was partially vaccinated; 9 were either unvaccinated or without available vaccination record. A total of 3 senior patients, all aged more than 70 and with severe existing conditions, died after identification of the Delta variant infection.

Ward I of this medical center consists of 7 rooms with 4 beds each, 14 regular private rooms, and 3 private rooms adjacent to the nurse station for patients who may need immediate attention. Layout of these rooms is shown in Figure 1. Except for case 18, all patients were found to be in shared rooms A, B, C, D, E, or F at symptom onset of SARS-CoV-2 infection. These patients were then transferred to either COVID-19 wards directly or temporarily to the private rooms inside the ward before moving to the COVID-19 wards.

Figure 2 illustrates the dates of PCR detection and any accompanying symptoms. The first two nurses were PCR-confirmed on September 14,

TABLE 1 Characteristics of infected nurses and patients in the nosocomial cluster, September 14 through September 28, 2021.

	Haplotype ^a	Sex	Age	PCR-confirmed date	SARS-CoV-2 variant	CT value of PCR test	COVID-19 symptoms at confirmation of infection	Vaccination	Nurse	Patient				
									Team A/B	Room type at symptom onset of infection ^b	Diagnosis at admission	Cancer (Yes/No)	Under immunosuppressive treatment (Yes/No)	Under steroid treatment (Yes/No)
Case 1	Type 2 (index type)	F	30	2021/9/14	Delta (AY.29)	21.62	Runny nose; joint pain	Fully (2 doses)	Team A	--	--	--	--	--
Case 2	Type 2	F	25	2021/9/14	AY.29	25.37	Fatigue	Partially (1 dose)	Team A	--	--	--	--	--
Case 3	Type 11	F	50	2021/9/15	AY.29	17.31	Runny nose	Fully	Team A (Post breast cancer surgery)	--	--	--	--	--
Case 4	Type 2	F	73	2021/9/15	AY.29	11.20	Fever	Not yet	--	4-bed room E	Polymyalgia rheumatica	No	No	Yes (prednisolone, 40 mg)
Case 5	Type 4	M	60	2021/9/16	AY.29	30.48	Fever	N/A	--	4-bed room F	Small cell lung cancer	Yes	Yes (carboplatin, etoposide)	Yes (dexamethasone, 6.6 mg)
Case 6	Type 9	F	31	2021/9/16	AY.29	20.43	Fever; coughs	Not yet	--	4-bed room E	Behçet's disease	No	No	No
Case 7	Type 7	M	52	2021/9/16	AY.29	24.35	Asymptomatic	Not yet	--	4-bed room D	Polymyositis	No	Yes (Neoral 300 mg)	Yes (prednisolone, 5 mg)
Case 8	Type 7	F	28	2021/9/17	AY.29	29.63	Asymptomatic	Fully	Team A	--	--	--	--	--
Case 9	Type 2	M	73	2021/9/17	AY.29	18.39	Asymptomatic	Fully	--	4-bed room D	Non-small cell lung cancer	Yes	No	No
Case 10	Type 2	F	67	2021/9/17	AY.29	20.94	Asymptomatic	Fully	--	4-bed room B	Systemic sclerosis	No	No	Yes (methylprednisolone, 6.5 mg)
Case 11	Type 1	F	77	2021/9/17	AY.29	22.84	Asymptomatic	Fully	--	4-bed room B	Lung adenocarcinoma	Yes	Yes (methotrexate, 4 mg)	Yes (prednisolone, 5 mg)
Case 12	Type 5	F	23	2021/9/18	AY.29	25.91	Asymptomatic	Fully	Team B	--	--	--	--	--
Case 13	Type 3	M	49	2021/9/18	AY.29	30.79	Fever; coughs	Not yet	--	4-bed room D	Adult Still's disease	No	No	Yes (prednisolone, 60 mg)
Case 14	Type 10	F	31	2021/9/19	AY.29	27.93	Fever	Not yet	--	4-bed room A	Mixed connective tissue disease	No	Yes (Tacrolimus, 2.4 ng/ml)	Yes (prednisolone, 30 mg)

(Continued)

TABLE 1 (Continued)

	Haplotype ^a	Sex	Age	PCR-confirmed date	SARS-CoV-2 variant	CT value PCR test	COVID-19 symptoms at confirmation of infection	Vaccination	Nurse	Patient	Room type at symptom onset of infection ^b	Diagnosis at admission	Cancer (Yes/No)	Under immunosuppressive treatment (Yes/No)	Under steroid treatment (Yes/No)
Case 15	Type 8	F	46	2021/9/21	AY.29	13.33	Coughs	Not yet	--		4-bed room B	Systemic lupus erythematosus	No	No	Yes (prednisolone, 40 mg)
Case 16	Type 2	F	28	2021/9/21	AY.29	22.45	Sore throat	Fully	Team A		--	--	--	--	--
Case 17	Type 2	F	68	2021/9/23	AY.29	29.87	Fever; coughs	Partially	--		4-bed room E	Lung adenocarcinoma	Yes	No	Yes (prednisolone, 2.5 mg)
Case 18	Type 6	M	68	2021/9/24	AY.29	21.55	Fever	Fully	--		Private room 7	Lung adenocarcinoma	Yes	No	No
Case 19	Type 2	M	70	2021/9/25	AY.29	28.63	Fever	N/A	--		4-bed room C	Infiltrative thymoma	Yes	Yes (tegafur/gimeracil/oteracil)	No
Case 20	Type 6	M	18	2021/9/28	AY.29	25.30	Fever; coughs; runny nose	Not yet	--		Ward II of the same floor	Mediastinal germ cell tumor	Yes	Yes (bleomycin)	No

^aTypes were defined based on haplotype-based analysis. ^bExcept for case 20, room types here indicate rooms in Ward I.

2021. After PCR testing for all healthcare workers of the ward and any inpatients identified as close contacts, 6 cases were then detected asymptotically, including Case 11 who was identified as a close contact. Case 11 was a 77-year-old female patient of lung adenocarcinoma who left Ward I for rehabilitation during her stay, was discharged, but then asked to return for PCR testing and readmitted after positive detection. Case 20 was identified lastly on September 28, after he was discharged from Ward II (the same floor as Ward I), developed COVID-19 symptoms at home and returned to be PCR tested.

The complete SARS-CoV-2 genomes showed the signatures of the Delta variant (AY.29 sublineage), which was the clade primarily circulating in Tokyo's metropolitan area (Tani-Sassa et al., 2021; Tsuchiya et al., 2022). Phylogenetic tree analysis included 20 samples from the studied cluster, wild type SARS-CoV-2 and AY.29 (hCoV-19/Japan/TKYK15779/2021) as references. The consensus tree, generated from 1,000 replicates, is shown in Figure 3. The log-likelihood of this tree was -40836.33. The cluster-associated viruses were shown to be very similar but some of them had different nucleotide sequences, which indicated the mutation development of the AY.29 virus in this cluster.

Mutation analysis of these 20 sequences revealed 12 mutations compared to AY.29 (hCoV-19/Japan/TKYK15779/2021). In addition, haplotype analysis found that 8 cases shared a common mutation of F274F (N) among the samples, therefore defined as the index type. However, case 11 was found lacking G142D in S compared to the AY.29 wild type. All other cases had acquired one to three additional mutations, either non-synonymous or synonymous. Most of the genetic changes identified were located in the ORF1ab gene, followed by the S gene (Table 2; Figure 4).

Table 2 presents these additional mutations, in order of the cases' genetic distance to the index type. Specifically, case 13 obtained a mutation G618G in ORF1ab (NSP2) compared with the index type; case 5 had S3099L in ORF1ab (NSP4); case 12 obtained a mutation I2501T in ORF1ab (NSP3). Case 18 and 20 acquired a synonymous mutation L3935L in ORF1ab (NSP7); case 7 and 8 acquired an amino acid substitution A65V in ORF8. A mutation found in case 15 was a synonymous H2659H in ORF1ab (NSP3). While case 6 had a synonymous mutation Y489Y in the S gene, case 14 additionally had L140L in ORF1ab (NSP1). Case 3 possessed I3944T in ORF1ab (NSP8), A4577T in ORF1ab (NSP12), and T1006I in Spike.

Minor variants in the cluster-related viruses compared to the AY.29 wild-type reference sequence are shown in Table 3, with nine minor variants found in Case 17, three in Case 19, and four in Case 20. These three cases were all cancer patients under immunosuppressive treatment. Regarding the transmission of the viruses with minor variants, each minor variant was found only in patients or nurses individually, but not transmitted to another host.

Discussion

In this study, we showed that SARS-CoV-2 infection spread rapidly, mostly in a single ward, having affected a total of 20 nurses and patients within 2 weeks during the Delta surge (Delta sublineage) in a Japanese medical center. The ward, with mixed inpatients of respiratory diseases and rheumatology/autoimmune diseases, shared strict infection control measures with other wards in the medical center, including a universal mask policy for medical staff and inpatients, PCR testing before admission and a no-visitor policy for all inpatients. By combining the affected cases' epidemiological

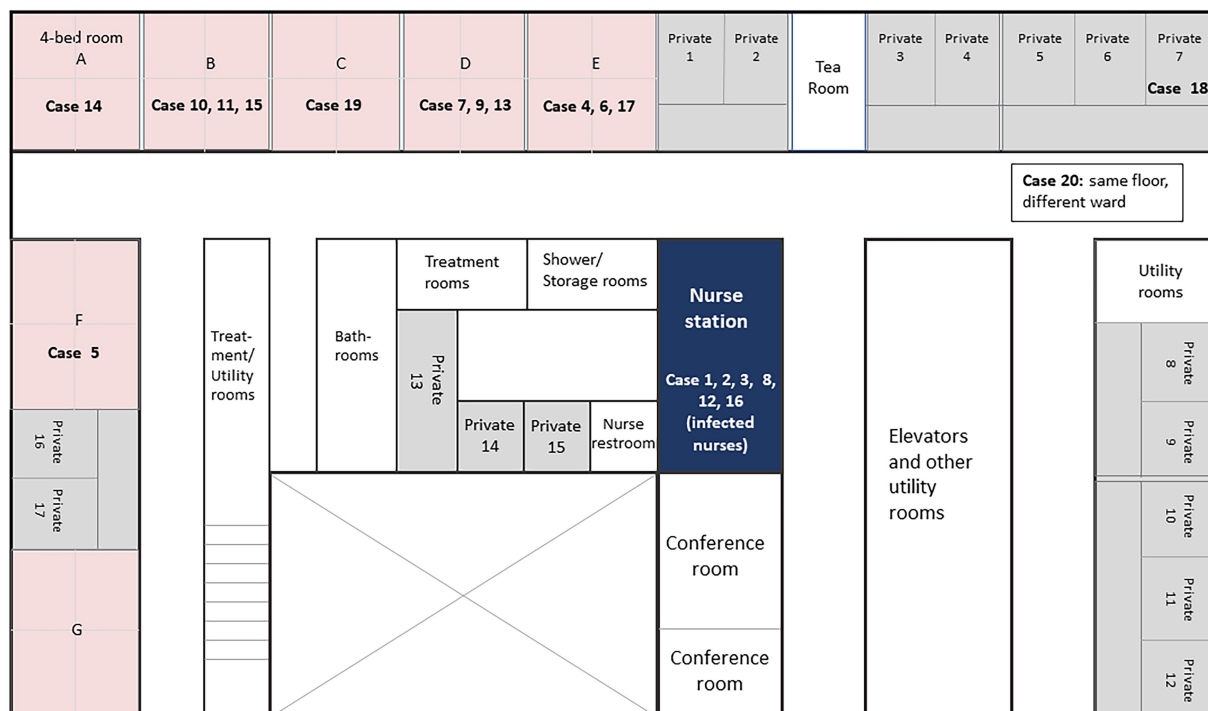


FIGURE 1

Floor map of Ward I in Juntendo University Hospital with cases at the onset of SARS-CoV-2 infection. Room numbers indicate patient location at the onset of SARS-CoV-2 infection (showing various symptoms including fever, cough, and/or running nose). After diagnosis of SARS-CoV-2, patients were transferred either to COVID-19 wards directly or temporarily to the private rooms within the same ward before transferring to COVID-19 wards.

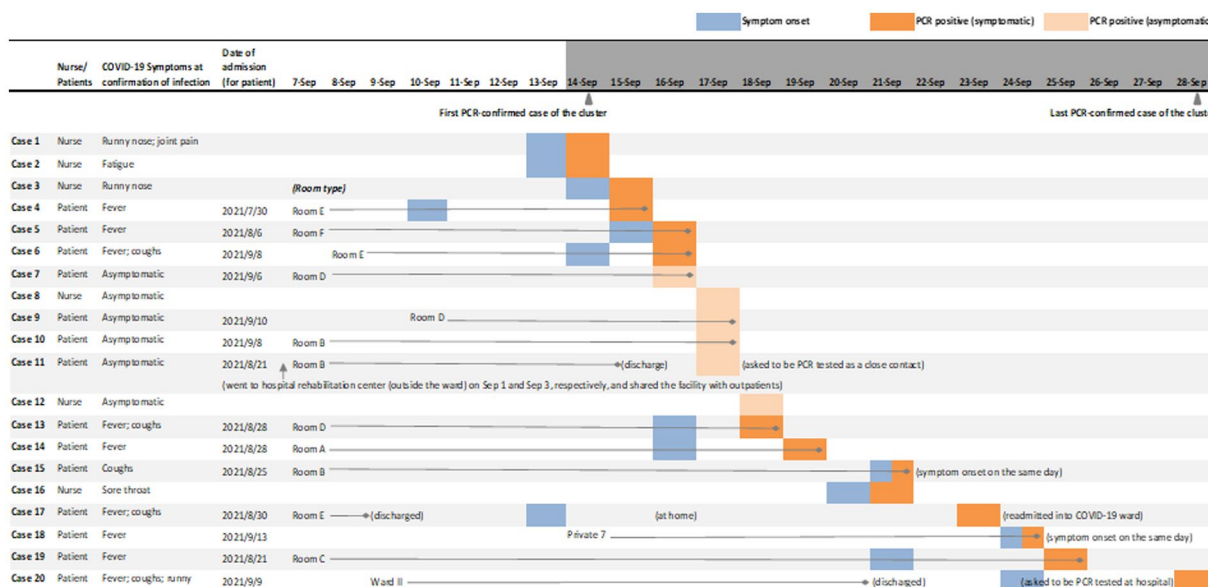


FIGURE 2

Symptoms and PCR-confirmed detection of SARS-CoV-2 infection cases in the nosocomial cluster. This figure shows the PCR-confirmed infected patients' room type (for inpatients at the timepoint of 1 week prior to the first PCR-confirmed case as September 7, 2021, or their admission date if admitted thereafter), the date of symptom onset and the date of PCR positive test; for infected nurses, date of symptom onset and the date of PCR positive test are shown. Type Room A to F are four-bed shared rooms; along with Private 7, these rooms are all located in Ward I. Case 20 stayed in Ward II of the same floor until discharged on September 21, reported symptoms on September 24, and tested positive on September 28. No other patients in Ward II were detected during the period of the nosocomial cluster. Patients might be transferred during their stay in Ward I due to reasons such as care requirements (for instance, increased proximity to the nurse station); these internal transfers are not shown in this figure. After PCR-confirmed detection, patients were transferred either to COVID-19 wards directly, or to private rooms inside Ward I for temporary stay until transferring to COVID-19 wards; all infected nurses were quarantined immediately after a PCR positive test. Immediately after the first two cases were detected on September 14, 2021, all healthcare workers of Ward I, including doctors, nurses and administrative staff, as well as close contacts, were screened by PCR tests and frequently tested thereafter as new cases were identified in the ward.

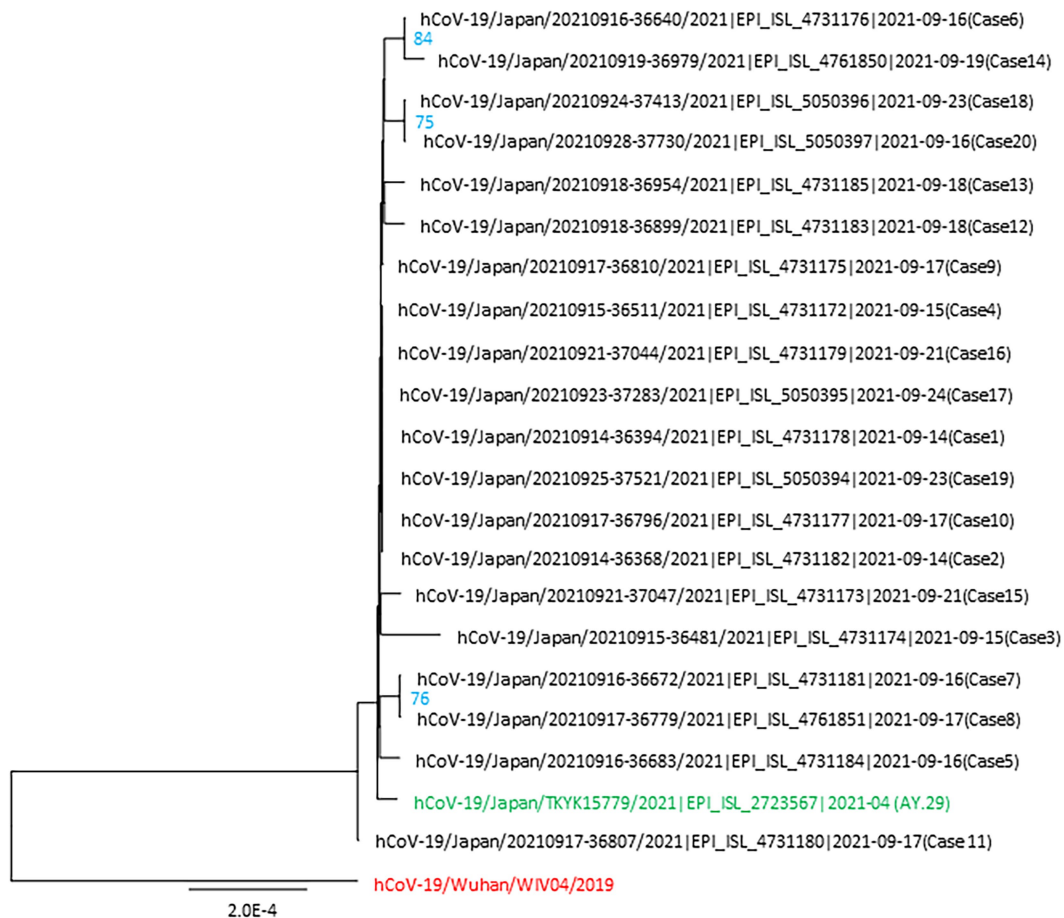


FIGURE 3

Phylogenetic tree of the SARS-CoV-2 genomes identified in the nosocomial cluster. This tree includes 20 viral genomes associated with the cluster, the first wild-type strain hCoV-19/Wuhan/WIV04/2019 and AY.29 wild-type strain hCoV-19/Japan/TKYK15779/2021 was used as the references. Each genome from the cluster is indicated with the virus name, accession ID of GISAID, and the case number. The ultrafast bootstrap support values of more than 70% were shown in the constructed tree.

information which includes symptom onset, PCR positive tests, and records of leaving and entering the ward, along with the complete genome sequencing analysis of nasopharyngeal or saliva samples, our study suggests that this cluster possibly started with a patient (case 11; asymptomatic). Case 11 left and re-entered the ward during her stay to use the hospital's rehabilitation facility, where she shared rooms with outpatients. The cluster might have started from this asymptomatic case and spread *via* attending nurses and patient roommates.

In addition, our study demonstrated that the SARS-CoV-2 genome can acquire one to three additional mutations within 2 weeks during the Delta surge (AY.29 sublineage). It has been shown that viruses mutate within their hosts where they develop into variants, and the number of within-host variants tends to increase over time (Jombart et al., 2011, 2014; Tonkin-Hill et al., 2021). Regarding the rapid spread of infection and acquisition of mutations in this nosocomial cluster, we offer the following two considerations.

First, droplets/aerosol transmission in shared and confined rooms, possibly due to patient conditions which physically do not permit continuous face masking, may be a risk factor for the rapid nosocomial spread of SARS-CoV-2 infections. Except for one case,

all infected patients in this nosocomial cluster of Ward I were found to be in one of the 4-bed rooms at symptom onset of their infection. Previous studies have revealed that aerosol (micrometer droplets) may be a risk factor for causing and spreading COVID-19 infections, particularly with prolonged exposure in confined spaces (Yu et al., 2004; Greenhalgh et al., 2021; Lim et al., 2021). As demonstrated by supercomputing systems, small droplets can stay airborne for hours, spreading far beyond standard social distance limits, suggesting that ventilation is as important as wearing masks (Ando et al., 2022). Our hospital has been implementing a universal masking policy, with exceptions only to those with severe conditions and ordered by doctors to not mask. Some of the patients in this cluster, especially these with severe respiratory diseases, are likely unable to continually mask. Thus, droplets/aerosol transmission is considered a risk factor for the spread of infections among inpatients in these shared rooms, suggesting the importance of ventilation, including not only air-conditioning but also air purifiers, especially in dated facilities (Morawska and Milton, 2020).

Second, cancer patients and/or patients receiving immunosuppressive or steroid treatment, are higher risk for COVID-19, and may become the hosts for rapid mutation evolution. Previous studies revealed that cancer patients are more vulnerable

TABLE 2 Additional mutations compared to the AY.29 wild-type reference sequence.^a

Haplotype 1	Haplotype 2 (index type)	Haplotype 3	Haplotype 4	Haplotype 5	Haplotype 6	Haplotype 7	Haplotype 8	Haplotype 9	Haplotype 10	Haplotype 11
Case 11	Case 1; 2; 4; 9; 10; 16; 17; 19	Case 13	Case 5	Case 12	Case 18; 20	Case 7; 8	Case 15	Case 6	Case 14	Case 3
F274F (N)	F274F (N)	F274F (N)	F274F (N)	F274F (N)	F274F (N)	F274F (N)	F274F (N)	F274F (N)	F274F (N)	F274F (N)
(without G142D (S) compared to AY.29 wild type reference)		G618G (ORF1ab)	S3099L (ORF1ab)	I2501T (ORF1ab)	L3935L (ORF1ab)	A65V (ORF8)	H2659H (ORF1ab)	Y489Y (S)	Y489Y (S)	I3944T (ORF1ab)
									L140L (ORF1ab)	A4577T (ORF1ab)
										T1006I (S)

^aAY.29 was first reported in Japan in April 2021, and the sequence of hCoV-19/Japan/TKYK15779/2021|EPI_ISL_2723567|2021-04 was selected as the reference of the AY.29 wild type.

to SARS-CoV-2 with higher rates of hospitalization and death (Dai et al., 2020; Kuderer et al., 2020; Luo et al., 2020; Nayak et al., 2021). In addition, a previous study with 585 cancer patients found that first-time infected persons with solid tumors developed lower neutralizing antibodies against the Delta variant (Fendler et al., 2021; Mahase, 2021). Another study of 152 double-vaccinated patients hospitalized due to COVID-19 found that 40% were immunosuppressed, including those under chronic corticosteroid treatment, chemotherapy/antimetabolite treatment and anti-CD20 treatment (Brosh-Nissimov et al., 2021). In addition, the rapid evolutionary rate in immunocompromised patients has been reported previously (Choudhary et al., 2021). In this nosocomial study, involving 6 nurses and 14 patients (13 out of 14 were either cancer patients and/or receiving immunosuppressive or steroid treatments), we found that the case with the most mutations (three additional mutations compared to the index type) was a nurse of post-breast cancer surgery (case 3); furthermore, we found that cases with more than three minor variants were all cancer patients under immunosuppressive treatments. Although with a limited number of samples, our study demonstrated that cancer patients/survivors and/or patients under immunosuppressive treatments can become hosts for fast SARS-CoV-2 virus spread and evolution.

Limitations

There are number of limitations worth addressing. First, this is a single nosocomial cluster study during Delta (AY.29 sublineage) surge in Tokyo with a limited number of samples, without intention to fully elucidate the mechanism of Delta variant's evolution. Second, the small sample size of the nosocomial cases did not allow us to identify a significant association between vaccination and the difference in mutation acquisition frequencies, although it has been reported that the vaccination is inversely correlated to the mutation frequency of SARS-CoV-2 Delta variants (Yeh and Contreras, 2021).

Conclusion

Our analysis of emerging mutations in a nosocomial COVID-19 cluster highlights mutation acquisition during transmission, demonstrating rapid mutations of the Delta variant (AY.29 sublineage) within 2 weeks, especially among patients with rheumatology/autoimmune diseases, lung cancer and other respiratory diseases. More importantly, because these patients are at higher risk for becoming hosts for rapid mutations, our study provides new evidence emphasizing the need to further improve infection control measures to prevent nosocomial clusters among patients with immunosuppression, even in hospitals with already strict protocols.

Data availability statement

The whole genome sequenced in this study can be found in online repositories. The name of the repository and accession numbers can be found at: <http://getentry.ddbj.nig.ac.jp/>, with accession numbers LC752087–LC752106 for the studied nosocomial cases 1–20.

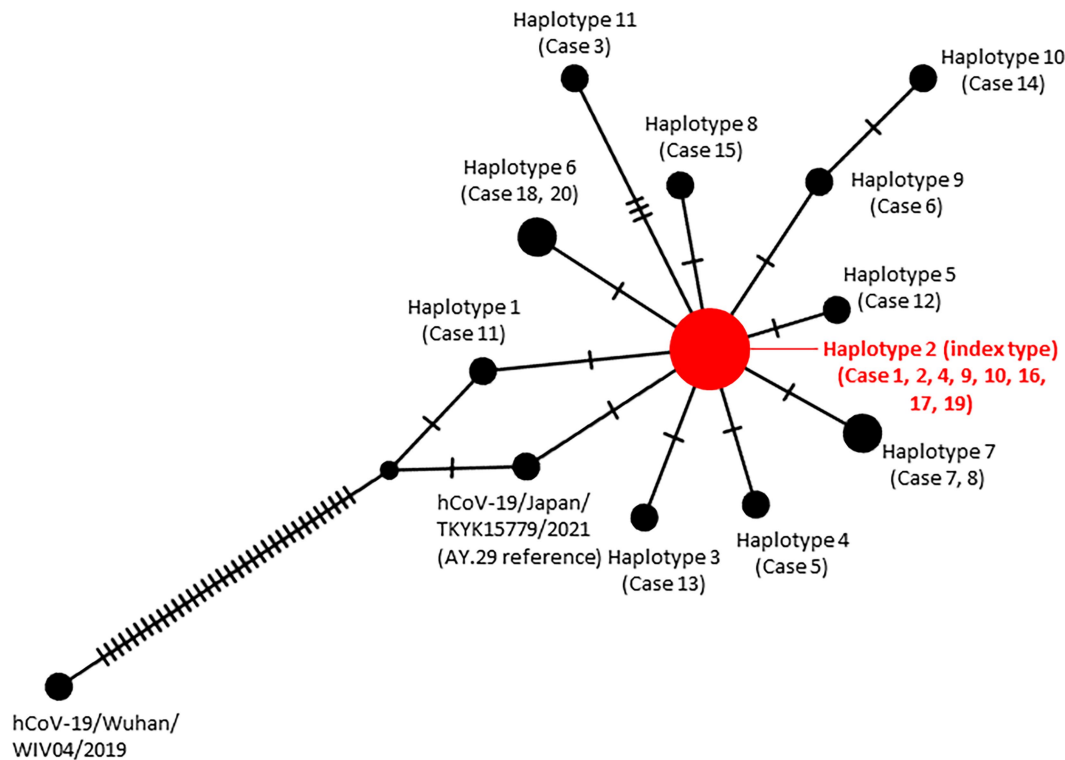


FIGURE 4

Haplotype network analysis of the SARS-CoV-2 genomes identified in the nosocomial cluster. The size of each node is proportional to the number of samples that belong to that haplotype.

Ethics statement

This study complied with all relevant national regulations and institutional policies and was conducted in accordance with the tenets of the Declaration of Helsinki. It was approved by the Institutional Review Board (IRB) at Juntendo University Hospital, Japan (IRB #20–036). Informed consent from individual patients was waived because all samples were de-identified in line with the Declaration of Helsinki.

Author contributions

TN: full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. YH, NY, YT, RO, KT, and TN: acquisition, analysis, or interpretation of data. YY, NY, and YT: draft of the manuscript. YH, SN, and YY: statistical analysis. YT, TN, and RO: administrative, technical, or material support. SH and KT: supervision. All authors: concept and design, critical revision of the manuscript for important intellectual content, and meet the ICMJE authorship criteria.

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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.

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TABLE 3 Minor variants in the cluster-related viruses compared to the AY.29 wild-type reference sequence.^a

Case No.	Position (Wuhan-Hu-1 numbering)	Ref	Alt	Read depth	Allele frequency	Variant type	Gene	Amino acid modification
Case 1	N/A							
Case 2	21,646	C	T	15,776	0.11500	Synonymous variant	S	Y28Y
Case 3	N/A							
Case 4	N/A							
Case 5	17,589	T	C	5,223	0.12250	Synonymous variant	ORF1ab	T5775T
	29,554	G	T	713	0.0858586	Upstream gene variant	ORF10	
Case 6	5,945	C	T	10,220	0.09500	Non-synonymous variant	ORF1ab	P1894S
Case 7	N/A							
Case 8	N/A							
Case 9	N/A							
Case 10	10,029	CCT	TCG	18,495	0.14070	Non-synonymous variant	ORF1ab	TS3255IA
Case 11	N/A							
Case 12	N/A							
Case 13	N/A							
Case 14	N/A							
Case 15	N/A							
Case 16	N/A							
Case 17	801	G	A	3,795	0.07750	Non-synonymous variant	ORF1ab	G179E
	5,313	T	C	2,921	0.10250	Non-synonymous variant	ORF1ab	L1683P
	11,201	A	TG	10,515	0.13819	Frameshift_variant	ORF1ab	T3646fs
	18,268	GA	G	4,050	0.08500	Frameshift_variant	ORF1ab	E6003fs
	19,036	G	A	2,593	0.07000	Non-synonymous variant	ORF1ab	G6258S
	22,776	A	C	3,991	0.07500	Non-synonymous variant	S	D405A
	24,458	CTTAGCTCCAAT	C	2,462	0.10579	Frameshift variant	S	S967fs
	26,111	C	T	11,006	0.15750	Non-synonymous variant	ORF3a	P240L
	26,885	C	T	5,202	0.15000	Synonymous variant	M	N121N
Case 18	11,074	CT	C	28,910	0.06650	Frameshift variant	ORF1ab	L3606fs
Case 19	23,255	T	G	11,328	0.13000	Non-synonymous variant	S	F565V
	26,681	C	T	23,345	0.14250	Synonymous variant	M	F53F
	27,945	C	T	31,822	0.08500	Stop gained	ORF8	Q18stop
Case 20	1,115	A	T	8,563	0.07500	Non-synonymous variant	ORF1ab	I284F
	6,539	C	T	13,520	0.06750	Non-synonymous variant	ORF1ab	H2092Y
	20,080	T	C	5,930	0.07500	Non-synonymous variant	ORF1ab	S6606P
	28,898	AGAATGGCTGGCA ATGGCGGTGATGCT GCTCTTGCTT	A	11,782	0.08549	Disruptive inframe deletion	N	R209-L221delinsMet

^aSARS-CoV-2 strain Wuhan-Hu-1 (accession number: NC_045512.1) was used as the reference genome for mapping reads. Identified mutations in the cluster cases were compared to the mutations in AY.29 wild type against Wuhan-Hu-1. The different nucleotide and amino acid sequences between cluster-associated viruses and AY.29 wild type are summarized in this table.

References

- Abe, T., and Arita, M. (2021). Genomic surveillance in Japan of AY.29—a new sub-lineage of Sars-Cov-2 delta variant with C5239T and T5514C mutations. *medRxiv*. doi: 10.1101/2021.09.20.21263869
- Ando, K., Bale, R., Li, C. G., Matsuoka, S., Onishi, K., and Tsubokura, M. (2022). Digital transformation of droplet/aerosol infection risk assessment realized on “Fugaku” for the fight against COVID-19. *Int. J. High Perform. Comput. Appl.* 36, 568–586. doi: 10.1177/10943420221116056
- Baj, A., Novazzi, F., Ferrante, F. D., Nononi, A., Tettamanzi, E., Catanoso, G., et al. (2021). Spike protein evolution in the SARS-CoV-2 delta variant of concern: a case series from northern Lombardy. *Emerg. Microbes Infect.* 10, 2010–2015. doi: 10.1080/22221751.2021.1994356
- Brosh-Nissimov, T., Orenbuch-Harroch, E., Chowders, M., Elbaz, M., Neshet, L., Stein, M., et al. (2021). BNT162b2 vaccine breakthrough: clinical characteristics of 152 fully-vaccinated hospitalized COVID-19 patients in Israel. *Clin. Microbiol. Infect.* 27, 1652–1657. doi: 10.1016/j.cmi.2021.06.036
- Centers for Disease Control and Prevention. (2020) *Research use only 2019-novel coronavirus (2019-nCoV) real-time RT-PCR primers and probes*. Available at: <https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html>
- Choi, B., Choudhary, M. C., Regan, J., Sparks, J. A., Padera, R., Qiu, X., et al. (2020). Persistence and evolution of SARS-CoV-2 in an immunocompromised host. *N. Engl. J. Med.* 383, 2291–2293. doi: 10.1056/NEJMc2031364
- Choudhary, M. C., Crain, C. R., Qiu, X., Hanage, W., and Li, J. Z. (2021). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) sequence characteristics of coronavirus disease 2019 (COVID-19) persistence and reinfection. *Clin. Infect. Dis.* 74, 237–245. doi: 10.1093/cid/ciab380
- Corey, L., Beyer, C., Cohen, M. S., Michael, N. L., Bedford, T., and Rolland, M. (2021). SARS-CoV-2 variants in patients with immunosuppression. *N. Engl. J. Med.* 385, 562–566. doi: 10.1056/NEJMs2104756
- Dai, M., Liu, D., Liu, M., Zhou, F., Li, G., Chen, Z., et al. (2020). Patients with cancer appear more vulnerable to SARS-CoV-2: a multicenter study during the COVID-19 outbreak. *Cancer Discov.* 10, 783–791. doi: 10.1158/2159-8290.CD-20-0422
- Drake, J. W. (1993). Rates of spontaneous mutation among RNA viruses. *Proc. Natl. Acad. Sci. U. S. A.* 90, 4171–4175. doi: 10.1073/pnas.90.9.4171
- Duffy, S. (2018). Why are RNA virus mutation rates so damn high? *PLoS Biol.* 16:e3000003. doi: 10.1371/journal.pbio.3000003
- Eckerle, L. D., Lu, X., Sperry, S. M., Choi, L., and Denison, M. R. (2007). High fidelity of murine hepatitis virus replication is decreased in nsp14 exoribonuclease mutants. *J. Virol.* 81, 12135–12144. doi: 10.1128/JVI.01296-07
- Fendler, A., Shepherd, S. T. C., Au, L., Wilkinson, K. A., Wu, M., Byrne, F., et al. (2021). Adaptive immunity and neutralizing antibodies against SARS-CoV-2 variants of concern following vaccination in patients with cancer: the capture study. *Nat. Cancer* 2, 1321–1337. doi: 10.1038/s43018-021-00274-w
- Greenhalgh, T., Jimenez, J. L., Prather, K. A., Tufekci, Z., Fisman, D., and Schooley, R. (2021). Ten scientific reasons in support of airborne transmission of SARS-CoV-2. *Lancet* 397, 1603–1605. doi: 10.1016/S0140-6736(21)00869-2
- Jombart, T., Cori, A., Didelot, X., Cauchemez, S., Fraser, C., and Ferguson, N. (2014). Bayesian reconstruction of disease outbreaks by combining epidemiologic and genomic data. *PLoS Comput. Biol.* 10:e1003457. doi: 10.1371/journal.pcbi.1003457
- Jombart, T., Eggo, R. M., Dodd, P. J., and Balloux, F. (2011). Reconstructing disease outbreaks from genetic data: a graph approach. *Heredity* 106, 383–390. doi: 10.1038/hdy.2010.78
- Kemp, S. A., Collier, D. A., Datir, R. P., Ferreira, I. A. T. M., Gayed, S., Jahun, A., et al. (2021). SARS-CoV-2 evolution during treatment of chronic infection. *Nature* 592, 277–282. doi: 10.1038/s41586-021-03291-y
- Kim, D., Lee, J. Y., Yang, J. S., Kim, J. W., Kim, V. N., and Chang, H. (2020). The architecture of SARS-CoV-2 transcriptome. *Cells* 181, 914–921.e10. doi: 10.1016/j.cell.2020.04.011
- Klompas, M., Baker, M. A., Rhee, C., Tucker, R., Fiumara, K., Griesbach, D., et al. (2021). A SARS-CoV-2 cluster in an acute care hospital. *Ann. Intern. Med.* 174, 794–802. doi: 10.7326/M20-7567
- Koyama, T., Tokumasu, R., Katayama, K., Saito, A., Kudo, M., and Imoto, S. (2022). Cross-border transmissions of the Delta substrain AY.29 during Tokyo Olympic and Paralympic games. *Front. Microbiol.* 13:883849. doi: 10.3389/fmicb.2022.883849
- Kudriner, N. M., Choueiri, T. K., Shah, D. P., Shyr, Y., Rubinstein, S. M., Rivera, D. R., et al. (2020). Clinical impact of COVID-19 on patients with cancer (CCC19): a cohort study. *Lancet* 395, 1907–1918. doi: 10.1016/S0140-6736(20)31187-9
- Leigh, J. W., and Bryant, D. (2015). PopART: full-feature software for haplotype network construction. *Methods Ecol. Evol.* 6, 1110–1116. doi: 10.1111/2041-210X.12410
- Lim, W. Y., Tan, G. S. E., Htun, H. T., Phua, H. P., Kyaw, W. M., Guo, H., et al. (2021). The first nosocomial cluster of COVID-19 due to the Delta variant in a major acute-care hospital in Singapore—investigations and outbreak response. *J. Hosp. Infect.* 122, 27–34. doi: 10.1016/j.jhin.2021.12.011
- Luo, J., Rizvi, H., Preeshagul, I. R., Egger, J. V., Hoyos, D., Bandlamudi, C., et al. (2020). COVID-19 in patients with lung cancer. *Ann. Oncol.* 31, 1386–1396. doi: 10.1016/j.annonc.2020.06.007
- Mahase, E. (2021). Covid-19: just a third of blood cancer patients had antibodies against delta variant after two vaccine doses, study finds. *BMJ* 375:n2623. doi: 10.1136/bmj.n2623
- Morawska, L., and Milton, D. K. (2020). It is time to address airborne transmission of coronavirus disease 2019 (COVID-19). *Clin. Infect. Dis.* 71, 2311–2313. doi: 10.1093/cid/ciaa939
- Nayak, B., Lal, G., Kumar, S., Das, C. J., Saraya, A., and Shalimar, A. (2021). Host response to SARS-CoV-2 and emerging variants in pre-existing liver and gastrointestinal diseases. *Front. Cell. Infect. Microbiol.* 11:753249. doi: 10.3389/fcimb.2021.753249
- Pandit, P., Cooper-White, J., and Punyadeera, C. (2013). High-yield RNA-extraction method for saliva. *Clin. Chem.* 59, 1118–1122. doi: 10.1373/clinchem.2012.197863
- Peck, K. M., and Luring, A. S. (2018). Complexities of viral mutation rates. *J. Virol.* 92, e01031–e01017. doi: 10.1128/JVI.01031-17
- Robinson, J., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E., Getz, G., et al. (2011). Integrative genomics viewer. *Nat. Biotechnol.* 29, 24–26. doi: 10.1038/nbt.1754
- Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., et al. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Mol. Biol. Evol.* 34, 3299–3302. doi: 10.1093/molbev/msx248
- Shu, Y., and McCauley, J. (2017). GISAID: global initiative on sharing all influenza data – from vision to reality. *Euro Surveill.* 22:30494. doi: 10.2807/1560-7917.ES.2017.22.13.30494
- Tani-Sassa, C., Iwasaki, Y., Ichimura, N., Nagano, K., Takatsuki, Y., Yuasa, S., et al. (2021). Viral loads and profile of the patients infected with SARS-CoV-2 Delta, alpha, or R.1 variants in Tokyo. *J. Med. Virol.* 94, 1707–1710. doi: 10.1002/jmv.27479
- Tonkin-Hill, G., Martincorena, I., Amato, R., Lawson, A. R. J., Gerstung, M., Johnston, I., et al. (2021). Patterns of within-host genetic diversity in SARS-CoV-2. *eLife* 10:e66857. doi: 10.7554/eLife.66857
- Tsuchiya, K., Yamamoto, N., Hosaka, Y., Wakita, M., Hiki, M., Matsushita, Y., et al. (2022). Molecular characterization of SARS-CoV-2 detected in Tokyo, Japan during five waves: identification of the amino acid substitutions associated with transmissibility and severity. *Front. Microbiol.* 13:912061. doi: 10.3389/fmicb.2022.912061
- World Health Organization. (2006). Collecting, preserving and shipping specimens for the diagnosis of avian influenza A(H5N1) virus infection: guide for field operations. World Health Organization. Available at: <https://apps.who.int/iris/handle/10665/69392>
- World Health Organization. (2021). Classification of Omicron (B.1.1.529): SARS-CoV-2 Variant of Concern. Available at: [https://www.who.int/news/item/26-11-2021-classification-of-omicron-\(b.1.1.529\)-sars-cov-2-variant-of-concern](https://www.who.int/news/item/26-11-2021-classification-of-omicron-(b.1.1.529)-sars-cov-2-variant-of-concern)
- Yeh, T. Y., and Contreras, G. P. (2021). Full vaccination against COVID-19 suppresses SARS-CoV-2 delta variant and spike gene mutation frequencies and generates purifying selection pressure. [Preprint]. Available at: <https://www.medrxiv.org/content/10.1101/2021.08.08.21261768v3>
- Yokota, I., Shane, P., Okada, K., Unoki, Y., Yang, Y., Inao, T., et al. (2021). Mass screening of asymptomatic persons for severe acute respiratory syndrome coronavirus 2 using saliva. *Clin. Infect. Dis.* 73, e559–e565. doi: 10.1093/cid/ciaa1388
- Yu, I. T. S., Li, Y., Wong, T. W., Tam, W., Chan, A. T., Lee, J. H. W., et al. (2004). Evidence of airborne transmission of the severe acute respiratory syndrome virus. *N. Engl. J. Med.* 350, 1731–1739. doi: 10.1056/NEJMoa032867

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