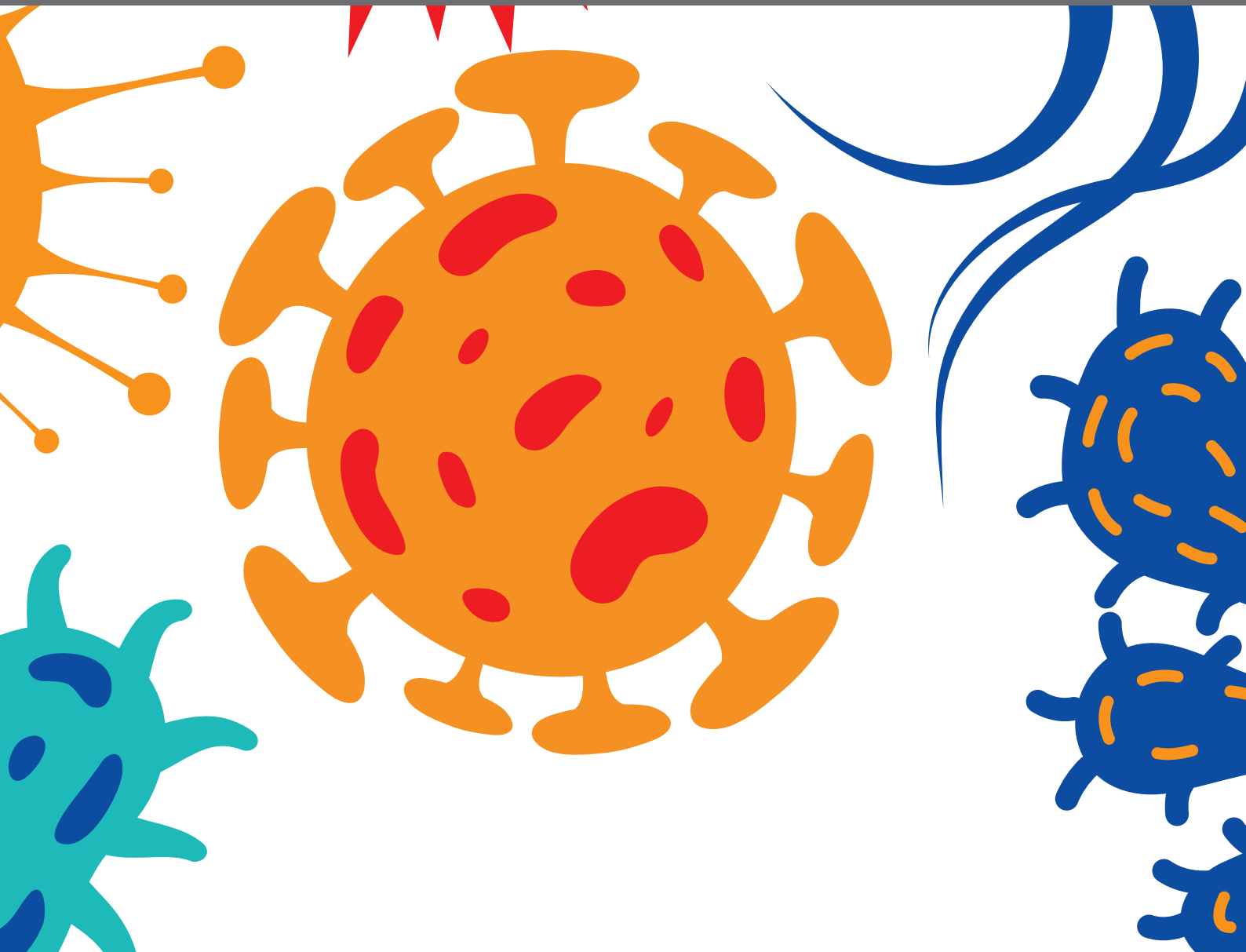


A large, dark blue, spiky virus particle with green dots on its surface, set against a background of a red sun-like shape at the top and a blue shape on the right.

IMPACT OF COVID-19 ON THE CLINICAL MICROBIOLOGY LABORATORY: PREPARING FOR THE NEXT PANDEMIC

EDITED BY: Sherry Dunbar, Esther Babady, Sanchita Das and
Catherine Moore

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IMPACT OF COVID-19 ON THE CLINICAL MICROBIOLOGY LABORATORY: PREPARING FOR THE NEXT PANDEMIC

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Editorial: Impact of COVID-19 on the clinical microbiology laboratory: Preparing for the next pandemic

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COVID-19, SARS-CoV-2, pandemic, clinical laboratory, strategies

Editorial on the Research Topic

Impact of COVID-19 on the clinical microbiology laboratory: Preparing for the next pandemic

Since the first cases of pneumonia caused by the novel coronavirus, SARS-CoV-2, were recognized in December 2019, the world has been consumed by the ongoing COVID-19 pandemic. Early, accurate diagnosis and the ability to differentiate SARS-CoV-2 from other respiratory pathogens remains crucial for rapid clinical intervention and infection control. The clinical diagnostic laboratory is key to these efforts and yet, laboratories and diagnostic manufacturers as well were severely impacted by the rapidly evolving pandemic as never before. In addition to the overwhelming surge in patients with COVID-19, there were few diagnostic tests available at the outset and, with only a fraction of laboratories capable of developing, validating, and running gold standard RT-PCR tests for detection of SARS-CoV-2, the need for commercial tests was quickly apparent. But, as commercial tests became available, laboratories experienced backorders and supply chain issues, not only for SARS-CoV-2 tests, but also for many other routine tests and associated testing supplies, such as swabs, transport media, and extraction reagents. Laboratories experienced staffing shortages as more trained technologists were needed to meet the demand for continuous SARS-CoV-2 testing and staffing was also limited to reduce exposure risk or for quarantine. The laboratory has been required to constantly change and adapt to the evolving pandemic and the need for testing, the type of test needed, and the test demand.

In this Frontiers Research Topic, contributors from diagnostic laboratories in different settings from around the world, as well as diagnostic manufacturers, share their experience with the COVID-19 pandemic, both the common and the unique challenges each faced, as well as mitigation strategies that were implemented to solve

issues and achieve success. It is our hope that the experiences and valuable lessons shared here will help laboratories prepare for the future. Improving laboratory preparedness for the next pandemic is an important proactive step for the future of the clinical microbiology laboratory.

Molecular methods, such as RT-qPCR and isothermal amplification, are the gold standard for SARS-CoV-2 diagnosis, thus it is important to understand how these tests may be utilized in different settings. [Vindeirinho et al.](#) provide a comprehensive review of the nucleic acid amplification tests (NAATs) and methods available for diagnosis of COVID-19 in the clinical laboratory and at the point of care (POC).

While nucleic acid amplification methods have the distinct advantage of exquisite sensitivity, they cannot differentiate viable from dead organisms. Viral RNA may remain detectable in clinical specimens for a prolonged duration after patient recovery but clinical significance and infectivity in these cases remain unclear. [Sung et al.](#) report their research on isolation of SARS-CoV-2 in culture in immunocompromised patients with persistently positive SARS-CoV-2 RT-PCR test results. Of the 20 patients studied, two patients with hematologic malignancies had positive viral cell cultures. More data is needed to determine risk factors for persistent viral shedding and methods to prevent transmission from immunocompromised patients.

Molecular testing is also important for epidemiology and surveillance to monitor the spread and progression of a pandemic. [Morris et al.](#) describe the use of large-scale SARS-CoV-2 molecular testing in combination with whole genome sequencing for genomic surveillance in real-time. The extensive laboratory, clinical, and genomic data provided an important resource to help better understand cases of reinfection versus extended RNA shedding and prolonged infections.

As the demand for rapid and high-throughput detection of viral nucleic acid from clinical samples increased, fast and evident inactivation of SARS-CoV-2 is crucial to ensure operator safety during testing. [Thom et al.](#) determined efficiency of SARS-CoV-2 inactivation using commercially available lysis buffers on 96-well RNA extraction platforms. They found that methods including both chemical and physical methods inactivated the virus at all titers tested.

Nucleic acid POC testing for SARS-CoV-2 emerged quickly during the pandemic and has been widely used in a variety of settings. [Mo et al.](#) reports an expert consensus on SARS-CoV-2 nucleic acid POC testing for China, developed in cooperation by experts in laboratory medicine. Management of the entire process including use cases, biosafety, personnel, verification, quality control, and reporting are described.

Rapid antigen tests are also a common tool for SARS-CoV-2 detection. These tests are relatively fast and inexpensive and have the potential to improve testing capacity in resource-constrained settings. [Morales-Jadán et al.](#) conducted a multi-center evaluation of three commercial SARS-CoV-2 rapid antigen tests compared to RT-qPCR in a community setting in

Ecuador. The authors found that these tests were adequate for surveillance and detection of infectious individuals in this setting.

Concomitant with detection of SARS-CoV-2 is understanding the host immune response to the infection. [Lee et al.](#) evaluated the analytical performance of commercially available surrogate virus neutralization and chemiluminescent assays and determined correlation to SARS-CoV-2 antibody titer by a plaque reduction neutralization test. The chemiluminescent assays had the highest sensitivities and specificities. Measurement of anti-receptor binding domain (RBD) IgG showed the best correlation with the plaque reduction neutralization assay in acute and convalescent phases and correlated to neutralizing activity.

[Rivera-Olivero et al.](#) determined the diagnostic performance for seven commercially available serological assays to assess their utility for seroprevalence population studies in South America. There were no statistically significant differences among the assays for anti-SARS-CoV-2 IgG and the tests were deemed acceptable for seroprevalence screening.

[Chiu et al.](#) studied humoral, cellular, and cytokine immune responses against SARS-CoV-2 variants in COVID-19 patients to assess correlation with disease severity. The delta and omicron variants were significantly resistant to the humoral immune response generated by individuals infected with the alpha variant. There was significant correlation between disease severity, humoral immune response, and cytokine/chemokine levels, but no evident antibody-dependent enhancement (ADE).

Manufacturers of laboratory reagents and diagnostic tests had to deal with many of the same challenges faced by clinical laboratories. As laboratories were depending on these manufacturers, they had to be agile to quickly develop and meet demand for SARS-CoV-2 tests, while also continuing to supply everything needed for all other laboratory testing that had to continue. Four articles in this collection provide the unique point of view from the diagnostic industry and the challenges they had to face and overcome to help laboratories endure during the pandemic.

[Cárdenas and Roger-Dalbert](#) present an industry perspective describing the agility, partnership, and innovation that was critical to respond to the ever-changing needs of caused by the pandemic. The authors point to several issues encountered and recommendations for improvement in the global response to future pandemics.

[Thornberg](#) provides an industry perspective from early in the pandemic within a smaller diagnostic manufacturer. He points out that once the decision is made to develop a test, the information needed is sometimes limited to quickly design a robust test that can be rapidly evaluated and moved into manufacturing. Even with an existing platform and consumable to facilitate new assay development, materials such as oligonucleotides, controls, and clinical samples for testing were in short supply. Guidelines implemented to slow

the spread of COVID-19 made working in person challenging when also trying to scale-up manufacturing capacity. Due to these challenges, there was considerable evolution in company policies. Health precautions for in-person workers were quickly deployed and teams working from home adapted to virtual platforms in an electronic work environment. This report highlights the effort by the diagnostic community to rise to the need and quickly release high quality testing materials, despite these roadblocks. Each function in the organization plays a critical role and by working together, the shared experience will allow a better response to future rapid-onset health emergencies.

Every manufacturer had to deal with these same or similar issues in some capacity. [Das and Dunbar](#) share a perspective describing the challenges faced by the diagnostics industry in general, particularly companies involved in diagnostic assay development and manufacturing. The article presents mitigation strategies employed during the pandemic and provides insights on possible steps to be undertaken to better prepare for future outbreaks.

Challenges faced by the diagnostics industry can be significantly impacted when parts of the operation occur in different locations, different countries, and/or different continents. [Tabb et al.](#) provide an account from a diagnostics manufacturer working with R&D teams in Italy and the U.S., with a U.S.-based manufacturing team. Compounding similar challenges in access to raw materials, control materials, clinical samples, and quarantine requirements, the second global hotspot of the pandemic was in Northern Italy where the company corporate headquarters is located. Assay development had to be accelerated even further to address the urgent situation there. Partnering with laboratories in Italy to assist with testing allowed the assay parameters to be finalized one day before Italy was placed on lockdown. This article describes how the many challenges were overcome and the

entire company worked side-by-side for accelerated delivery of the assay to clinical labs in Europe, the U.S., and Canada.

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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Author SD was employed by Luminex, A DiaSorin Company.

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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Evaluation of the SARS-CoV-2 Inactivation Efficacy Associated With Buffers From Three Kits Used on High-Throughput RNA Extraction Platforms

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Rapid and demonstrable inactivation of SARS-CoV-2 is crucial to ensure operator safety during high-throughput testing of clinical samples. The inactivation efficacy of SARS-CoV-2 was evaluated using commercially available lysis buffers from three viral RNA extraction kits used on two high-throughput (96-well) RNA extraction platforms (Qiagen QIAcube HT and the Thermo Fisher KingFisher Flex) in combination with thermal treatment. Buffer volumes and sample ratios were chosen for their optimised suitability for RNA extraction rather than inactivation efficacy and tested against a representative sample type: SARS-CoV-2 spiked into viral transport medium (VTM). A lysis buffer mix from the MagMAX Pathogen RNA/DNA kit (Thermo Fisher), used on the KingFisher Flex, which included guanidinium isothiocyanate (GITC), a detergent, and isopropanol, demonstrated a minimum inactivation efficacy of 1×10^5 tissue culture infectious dose (TCID)₅₀/ml. Alternative lysis buffer mixes from the MagMAX Viral/Pathogen Nucleic Acid kit (Thermo Fisher) also used on the KingFisher Flex and from the QIAamp 96 Virus QIAcube HT Kit (Qiagen) used on the QIAcube HT (both of which contained GITC and a detergent) reduced titres by 1×10^4 TCID₅₀/ml but did not completely inactivate the virus. Heat treatment alone (15 min, 68°C) did not completely inactivate the virus, demonstrating a reduction of 1×10^3 TCID₅₀/ml. When inactivation methods included both heat treatment and addition of lysis buffer, all methods were shown to completely inactivate SARS-CoV-2 inactivation against the viral titres tested. Results are discussed in the context of the operation of a high-throughput diagnostic laboratory.

Keywords: SARS-CoV-2, high throughput, PCR, biosafety, laboratory-acquired infection, clinical diagnosis

HIGHLIGHTS

- To date, there have been few publications on the inactivation of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in the diagnostic context.
- This publication adds to the published knowledge and helps laboratories that do not have microbiological containment facilities (biosafety level (BSL) 3 or above) and therefore are not able to perform detailed experimental research in this area and assess the safety of their SARS-CoV-2 diagnostic processes.
- The findings of the paper show that a combination of chemical treatments and/or physical methods such as the application of heat are required to inactivate SARS-CoV-2 in nasal swab samples and are in concordance with a similar paper from this group on the inactivation of Ebola virus in diagnostic samples.
- This will support laboratories and reduce the likelihood of laboratory-acquired infections.

INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) belongs to the *Coronaviridae* family and is the causative agent of the respiratory illness, coronavirus disease 2019 (COVID-19) (Gorbalenya et al., 2020). The enveloped positive-sense single-stranded RNA virus was first discovered in early 2020 after a cluster of viral pneumonia cases of unknown cause were reported in the Hubei Province of China (Wu et al., 2020). The virus is highly contagious in humans, and in March 2020, the WHO declared a global pandemic (Chen, 2020).

Diagnostic testing is critical in the fight against the COVID-19 pandemic (Patel et al., 2020), not just for patients displaying symptoms but also for asymptomatic carriers and pre-symptomatic patients (Shental et al., 2020). SARS-CoV-2 has been classified in the United Kingdom as a Hazard Group (HG) 3 pathogen by the Advisory Committee on Dangerous Pathogens (ACDP), meaning that this virus must be handled under Containment Level (CL) 3 conditions [biosafety level (BSL) 3]. However, guidance from the WHO (World Health Organization 2020) and Public Health England, United Kingdom (Public Health England, 2020), has permitted non-propagative diagnostic testing to be carried out at CL 2 with non-inactivated samples being handled within a Class 1 microbiology safety cabinet.

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) is the gold standard test for the detection of SARS-CoV-2 from nasopharyngeal swab samples (Tahamtan and Ardebili, 2020). Inactivation of viral pathogens prior to PCR is typically carried out at the same time as extraction of viral nucleic acids from samples, with chemical or physical methods employed. Typically buffers provided in nucleic acid extraction kits contain chaotropic salts, solvents, and detergents to lyse the virus. Guanidinium salts, such as guanidinium isothiocyanate (GITC), are chaotropic agents found in many lysis buffers, which

in some cases have been demonstrated to inactivate viral pathogens, including alphaviruses, flaviviruses, filoviruses, and a bunyavirus (Blow et al., 2004; Ngo et al., 2017). Other reports suggest that a combination of a GITC containing extraction buffer (such as Qiagen AVL) and a solvent (such as ethanol) is required for the inactivation of viruses such as Ebola virus (Smither et al., 2015) and Middle East respiratory syndrome coronavirus (MERS-CoV) (Kumar et al., 2015). Detergents such as Tween, sodium dodecyl sulphate (SDS), and Triton X-100 have also been shown to disrupt viral envelopes and reduce viral titres (Mayo and Beckwith, 2002; van Kampen et al., 2017; Patterson et al., 2020), with a combination of the GITC-based reagent (Buffer AVL) and Triton X-100 having been reported to inactivate Ebola virus (Burton et al., 2017). Physical processes such as heat can also be incorporated in the nucleic acid extraction workflow and can have an inactivation effect. Some reports suggest that the application of heat alone can inactivate SARS-CoV, MERS-CoV, and SARS-CoV-2 following a heat regimen of 65°C for at least 15 min (Darnell et al., 2004; Leclercq et al., 2014; Kim et al., 2020).

Since the pandemic was declared, United Kingdom's Defence Science and Technology Laboratory (Dstl) and British military clinicians have set up the Defence COVID Laboratory (DCL), which has been awarded an extension to scope (under ISO17025) for the provision of a SARS-CoV-2 PCR test by the United Kingdom Accreditation Service (UKAS). The DCL analyses samples from UK military units and operates two automated high-throughput RNA extraction platforms (Qiagen QIAcube HT and the Thermo Fisher KingFisher Flex). In this study, conducted entirely under CL 3 laboratory conditions (BSL 3), we report the inactivation efficacy of SARS-CoV-2 by buffers from three commercially available kits used on these two platforms. Buffer volumes and ratios were chosen for their suitability for RNA extraction (following manufacturer's instructions) rather than their potential inactivation efficacy; however, in doing so, we have further investigated the inactivation efficacy of combinations of GITC containing buffers, solvents, and/or detergents with and without an additional heat inactivation step. We provide evidence to support protocols for the inactivation of SARS-CoV-2 and the safe use of clinical samples in downstream RT-PCR in high-throughput diagnostic laboratories.

METHODS

Virus Strains, Cell Culture, and Enumeration

All virus manipulations were carried out using the SARS-CoV-2 England 2 strain (GISAID reference EPI_ISL_407073), provided by Public Health England. Virus stock was propagated in Vero C1008 cell, harvested at day 3 and clarified by centrifugation at 350 × g for 15 min (Sigma 3-16K centrifuge). Viral stocks were concentrated by centrifugation at 11,000 × g for 3 h at 4°C to achieve 1 × 10⁸ tissue culture infectious dose (TCID)₅₀/ml and stored at -80°C.

All cell cultures were carried out using confluent monolayers of Vero C1008 cells (European Collection of Authenticated Cell Cultures [ECACC], United Kingdom; catalogue no. 85020206) maintained in Dulbecco's minimal essential medium (DMEM; Sigma, United Kingdom) supplemented with 10% foetal calf serum, 1% L-glutamine, and 1% penicillin-streptomycin (Sigma, United Kingdom) and incubated at 37°C in a 5% CO₂ environment. Prior to virus being added to cell monolayers, 10% DMEM was replaced with Leibovitz's L-15 (to buffer for the lack of CO₂ at CL 3), supplemented as described for DMEM, with the exception of 2% foetal calf serum, and incubated at 37°C.

Viral enumeration (for determining starting concentrations and measuring reductions in concentrations post-inactivation) was carried out by an end-point TCID₅₀ assay (Piercy et al., 2010). In brief, Vero C1008 cells were prepared in 96-well microtitre plates to achieve confluent monolayers on the day of assay. To all wells of column 1 of the plate, 100 µl of test sample was added. From column 1, 20 µl of sample was transferred sequentially across the plate to achieve a 10-fold serial dilution to column 9. Cells in columns 11 and 12 were left in tissue culture medium (TCM) as controls. Plates were incubated in a humidified atmosphere for 3–4 days at 37°C, after which they were scored for cytopathic effect (CPE) by microscopic observation. The TCID₅₀ value was calculated by the method of Reed and Muench (1938). Mean values were calculated as the geometric mean.

Viral Inactivation

Buffers and reagents from three different RNA extraction kits were assessed to determine inactivation of SARS-CoV-2 (Table 1). The composition of these initial reagents and their suitability for extraction of SARS-CoV-2 RNA from clinical

samples was determined based on manufacturers' protocols and after discussions with each manufacturer.

The inactivation efficacy of each lysis buffer was evaluated with and without the inclusion of a heat step. Table 1 summarises the components and volumes used for each lysis buffer preparation. MS2 bacteriophage (10⁶ plaque-forming unit (PFU)/ml) was added to each lysis buffer preparation as an internal control in the DCL and was therefore included in these experiments. Test samples for each experiment were set up in triplicate, and each experiment was performed on at least three separate occasions.

Viral transport medium (VTM; EO Labs, United Kingdom) was inoculated with SARS-CoV-2 to achieve a starting concentration of 5 × 10⁶ TCID₅₀/ml for all experiments. To the lysis buffer preparations, 200 µl of virus in VTM was added; the samples were briefly vortexed and incubated for 10 min at room temperature. For heat-treated samples, the tubes were incubated for 25 min in a heat block (Eppendorf ThermoMixer C heat) set at 75°C. Laboratory tests showed that this was the temperature setting required for this individual heat block to heat and maintain the samples at 68°C for 15 min. Heat steps were carried out after the addition of virus to either lysis buffer reagents or an equivalent volume of TCM, to assess the effect of viability following heat in the presence or absence of reagents. Further controls included sham-inactivated virus, where appropriate volume of TCM replaced the lysis buffer reagents and negative controls consisting of VTM only were added to lysis buffer reagents to assess the effect of the reagents on cell monolayers.

After inactivation (with or without heat treatment), all samples and controls were centrifuged at 6,000 × g for 5 min in a microcentrifuge (Hermle Microlitre Centrifuge Z 160 M), and the supernatant was discarded and replaced with 1 ml of TCM. This

TABLE 1 | Protocols tested for assessing inactivation using lysis buffers.

| Manufacturer, RNA extraction kit, Platform | Reagents (volume/sample) | Active virucidal components* | Reagent: Sample ratio |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------|-----------------------|
| Qiagen, QIAamp 96 Virus QIAcube HT Kit (Cat #: 57731), Qiagen QIAcube HT. (Referred to here as <i>Qiagen protocol</i>) | ACL buffer (190 µl) ATL buffer (100 µl) Proteinase K (20 µl) Carrier RNA (5 µl) MS2 (10 µl) | GITC 30 - <50% 1 - <3% SDS | 1.6: 1 |
| ThermoFisher, MagMax Pathogen RNA/DNA kit (Cat #: 4462359), Kingfisher Flex. (Referred to here as <i>MagMax Protocol 1</i>) | Lysis binding buffer (350 µl) Isopropanol (300 µl) Carrier RNA (2 µl) Water (100 µl) MS2 (10 µl) | GITC 55-80% <0.001% Acrylamide Zwittergent 100% 2-propanol | 3.8: 1 |
| ThermoFisher, MagMax viral/pathogen nucleic acid isolation kit (Cat #: A48310), Kingfisher Flex. (Referred to here as <i>MagMax Protocol 2</i>) | Lysis binding buffer (265 µl) Proteinase K (5 µl) †Water (Magnetic beads) (10 µl) MS2 (10 µl) | GITC 55-80% <0.001% Acrylamide Zwittergent | 1.4: 1 |

*As identified directly from components, manufacturer information, or inferred from the associated MSDS.

†Water was used to replace the magnetic beads as the washing steps described below would not remove the beads and the beads interfered the read-out of the TCID₅₀ assay. GITC, Guanidinium thiocyanate; SDS, Sodium dodecyl sulphate.

step was required to dilute the chemical components that would otherwise cause toxicity in the cell culture-based enumeration assay. Although virus pellets were not visible, this method is known to pellet virus with appropriate efficiency, as demonstrated by virus recovery in positive controls and is similar to methods used successfully in previous studies (Smither et al., 2016). In experiments (data not shown), this step was shown to be required four times for the Qiagen reagents and two times for the KingFisher reagents in order to remove all traces of the inactivation chemicals from the sample and to avoid toxicity during cell culture. After the final wash, the pellets were re-suspended in 1 ml of TCM. Controls in each experiment were washed the same number of times as required by the reagent being evaluated.

Post-Inactivation Viral Viability Assays

To quantify and determine the viability of the virus following inactivation, the samples were enumerated by the TCID₅₀ end-point dilution assay described above; and the remaining sample underwent three rounds of serial passage in tissue culture flasks for a secondary confirmation of viral inactivation. In brief, all of the remaining samples (approx. 180 µl) were added to confluent monolayer of Vero C1008 cells in a 12.5-cm² tissue culture flask. Flasks were incubated in a humidified atmosphere for 3–4 days after which presence or absence of cytopathic effect was recorded. A total of three passages were performed, and CPE was recorded after each round. To control for cross-contamination, a set of un-infected flasks were also prepared, and supernatant was passaged in parallel to the experimental samples. A 10-fold serial dilution of SARS-CoV-2 was also inoculated into a set of flasks starting from 1.7×10^7 TCID₅₀/ml and diluted to 1.1 TCID₅₀/ml to show the limit of detection (LOD) of the flask passage assay and demonstrate a suitable environment for the passage and propagation of the virus.

Statistical Analysis

All data were graphically represented and statistically analysed using GraphPad Prism 8. The Kruskal–Wallis analysis of variance (ANOVA) was performed on data sets with Dunn's multiple comparison *post hoc*.

RESULTS

The inactivation of SARS-CoV-2 was assessed using three different RNA lysis buffers with and without the inclusion of a heat step. The viability of virus was determined quantitatively using the TCID₅₀ assay and qualitatively by serially passaging samples in flask.

Determination of Starting Concentration of Severe Acute Respiratory Syndrome Coronavirus-2

These studies used the highest working concentration of SARS-CoV-2 that was available, and this ranged from 5.9×10^5 to 3.5×10^6 TCID₅₀/ml (Figure 1). Following the inactivation procedure, residual toxic lysis buffer components were removed by way of multiple wash steps. Residual chemical components would otherwise be toxic to the cell-based assays. To determine if the multiple wash steps by centrifugation resulted in a loss of virus, virus was inoculated into TCM without the addition of lysis reagents (as described in the *Methods*) and assayed as described. This highlighted that there was approximately a 1-Log₁₀ drop in titre in each experiment.

Chemical Inactivation of Severe Acute Respiratory Syndrome Coronavirus-2

When virus was added to the Qiagen lysis buffer, there was a statistically significant 5-Log₁₀ drop ($p = 0.002$) in virus titre from 3.3×10^5 TCID₅₀/ml to below the lower limit of quantification (LLOQ) of 32 TCID₅₀/ml. Complete inactivation was not achieved however, as virus was detected below the LLOQ, but this was not quantifiable. However, by extrapolation, it was estimated that the titre was 6.2 TCID₅₀/ml (Figure 1A).

Similar results were observed when virus was inactivated using the MagMAX Protocol 2; complete inactivation was not achieved as virus was detected below the LLOQ and was not quantifiable. The starting titre of virus for these experiments, following washing steps, was 5.8×10^4 TCID₅₀/ml, demonstrating a 4-Log₁₀ drop in viral titre following inactivation ($p < 0.001$) (Figure 1C).

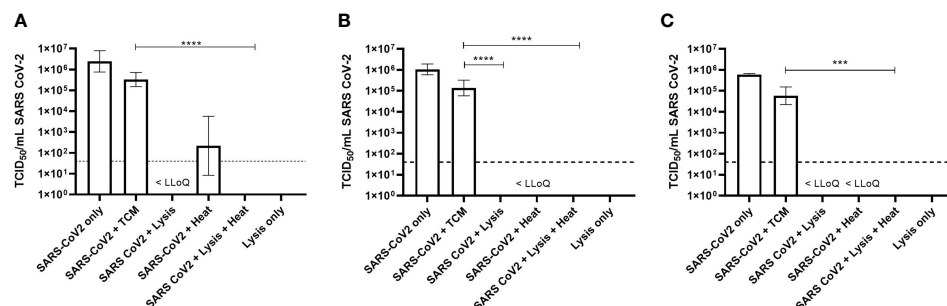


FIGURE 1 | Titre of SARS-CoV-2 by TCID₅₀ assay following inactivation protocols. (A) Qiagen protocol (Virus QIAcube HT Kit). (B) MagMAX Protocol 1 (Pathogen DNA/RNA). (C) MagMAX Protocol 2 (Viral Pathogen Kit). Geometric Mean + Geometric Standard Deviation collated from triplicate results from three separate occasions ($n = 9$). Dashed line = lower limit of quantification (LLOQ < 32 TCID₅₀/ml); tissue culture medium (TCM); <LLOQ on graph indicates viable virus was recovered in some replicates but was below limit of quantification. Kruskal–Wallis ANOVA with Dunn's multiple comparison *post hoc*, where *** $p < 0.001$, **** $p < 0.0001$; statistical analysis excludes virus stock and lysis only data.

Virus inactivation following the MagMAX Protocol 1 resulted in no detectable virus by TCID₅₀ assay. The starting concentration of virus, following washing steps, was calculated to be 1.4×10^5 TCID₅₀/ml, thus demonstrating a 5-Log₁₀ drop in viral titre with this particular protocol ($p < 0.0001$) (Figure 1B).

Heat Inactivation of Severe Acute Respiratory Syndrome Coronavirus-2

Heat alone or in combination with lysis buffer was also investigated as a means to inactivate SARS-CoV-2. For each experiment, virus in TCM was heated at 68°C for 15 min and centrifuged to maintain consistency with samples in lysis buffer. Although not statistically significant, at least a 3-Log₁₀ drop in viral titre was observed following heat treatment alone, though viable virus was observed in replicates across all three heat alone experiments, even when below LLoQ (Figure 1).

When the virus was added to one of the three lysis buffers and subsequently heated, no viable virus was detected following TCID₅₀ assay and an average drop in viral titre of 5-Log₁₀ across all experiments ($p < 0.0001$) (Figures 1A–C).

Confirmation of Inactivation by Viral Propagation

To confirm findings by TCID₅₀ assay, viral samples were propagated in cell culture flasks over a total of three passages to identify potential viral breakthrough. Table 2 shows the results of the presence of CPE after the first passage. The LOD for viral propagation was determined following propagation of serially diluted virus stocks (Table 2 row 1 to 5), and on average, the LOD was 1.3 TCID₅₀/ml.

When virus was added to TCM, CPE was present in all flasks as expected (Table 2 row 6, positive control). No cell toxicity was observed from negative control samples where TCM only was added to lysis buffer and washed as described previously (Table 2 row 10, negative control).

When SARS-CoV-2 was inactivated following the Qiagen protocol, three out of the nine flasks were scored as positive for CPE. Of the flasks where no CPE was observed, no breakthrough of virus was seen as a result of serial passage (Table 2 row 7). These data align with the TCID₅₀ assays, where Qiagen lysis buffer alone did not completely inactivate the virus. Following both MagMAX protocols, zero out of the nine flasks were scored positively for CPE (Table 2 row 7). For the MagMAX Protocol 1, this confirms the TCID₅₀ results, where no viable virus was also observed. For the MagMAX Protocol 2, virus was detected but not quantifiable in the TCID₅₀ assay (below the LLoQ); however, subsequent serial passage did not provide evidence of viability, as all flasks were negative for CPE.

When SARS-CoV-2 was added to TCM and heated for 15 min at 68°C, CPE was observed in all but one flask (Table 2 row 8), confirming the TCID₅₀ results that the heating protocol described here does not completely inactivate the virus.

For all inactivation protocols, when SARS-CoV-2 samples were treated in a two-step manner (lysis buffer and heat), no viable virus was detected in either the quantitative or qualitative assays (Figure 1 and Table 2 row 9). These data provide strong evidence that the lysis buffers described here in combination with the heat protocol can completely inactivate up to 5-Log₁₀ TCID₅₀/ml SARS-CoV-2.

DISCUSSION

Real-time PCR is the gold standard clinical diagnostic method for the detection of SARS-CoV-2 in patients displaying symptoms of COVID-19. There has been a rapid development in RNA extraction and RT-PCR diagnostic methods in order to help prevent further spread of infection through communities. It is crucial that testing is accurate and efficient, both of which must not compromise safety of those processing the samples (Dhamad

TABLE 2 | Summary of results following cell culture passage and TCID₅₀ assay.

| Inactivation protocol | Qiagen protocol (96 Virus QIAcube HT Kit) | | MagMAX Protocol 1 (Pathogen DNA/RNA Kit) | | MagMAX Protocol 2 (Viral Pathogen Kit) | |
|-------------------------------------|----------------------------------------------|--------------------------------|---------------------------------------------|--------------------------------|-------------------------------------------|--------------------------------|
| | Flasks infected/total flasks | TCID ₅₀ /ml (SD) | Flasks infected/total flasks | TCID ₅₀ /ml (SD) | Flasks infected/total flasks | TCID ₅₀ /ml (SD) |
| 1. SARS-CoV-2 starting titre | 3/3 | 1.7×10^7 | 3/3 | 5.9×10^6 | 3/3 | 3.0×10^6 |
| 2. SARS-CoV-2 10^{-4} dilution | 3/3 | $1.7 \times 10^{3*}$ | 3/3 | $5.9 \times 10^{2*}$ | 3/3 | $3.0 \times 10^{2*}$ |
| 3. SARS-CoV-2 10^{-5} dilution | 3/3 | $1.7 \times 10^{2*}$ | 3/3 | 59.4* | 2/3 | 20.0* |
| 4. SARS-CoV-2 10^{-6} dilution | 3/3 | 17* | 1/3 | 2.0* | 1/3 | 0.7* |
| 5. SARS-CoV-2 10^{-7} dilution | 2/3 | 1.1* | 0/3 | ND | 0/3 | ND |
| 6. SARS-CoV-2 + TCM | 9/9 | 3.3×10^5 | 9/9 | 1.4×10^5 | 9/9 | 5.8×10^4 |
| 7. SARS-CoV-2 + lysis buffer | 3/9 | <LLoQ | 0/9 | ND | 0/9 | <LLoQ |
| 8. SARS-CoV-2 + heat | 9/9 | 2.2×10^2 | 9/9 | 29.6 | 8/9 | 20.4 |
| 9. SARS-CoV-2 + lysis buffer + heat | 0/9 | ND | 0/9 | ND | 0/9 | ND |
| 10. TCM + lysis buffer | 0/9 | ND | 0/0 | ND | 0/9 | ND |

Passage results shown are after the third serial. TCID₅₀ titres are geometric mean titre/ml. <LLoQ indicates viable virus was recovered in some replicates but was below limit of quantification.

SARS-2, SARS-CoV-2; TCM, tissue culture medium; LLoQ, lower limit of quantification (10 TCID₅₀/ml); SD, standard deviation; ND, not detected.

*Indicates the TCID₅₀/ml is extrapolated from performing 10-fold dilutions from a known starting concentration and calculated based on number of flasks infected.

and Abdal Rhida, 2020). Laboratory-acquired infections due to incomplete inactivation or incorrect handling of samples have been reported for SARS-CoV (Lim et al., 2004; Taylor et al., 2005) as well as many other infectious agents (Singh, 2009).

To date, there are only a handful of publications reporting the use of nucleic acid isolation reagents, detergents, and heat to inactivate SARS-CoV-2 (Kim et al., 2020; Pastorino et al., 2020; Welch et al., 2020; Burton et al., 2021); and due to commercial sensitivity, manufacturers of extraction kits are not required to publish the full ingredient list of proprietary buffers [with potential viral inactivating components only inferred if they are listed on associated Material Safety Data Sheets (MSDSs)], and post-treatment viability test methods vary in stringency across studies.

In our study, we investigated the SARS-CoV-2 inactivation efficacy of viral lysis buffers from three commercially available kits developed to allow RNA extraction on high-throughput (96-well) automated platforms. Stringent post-treatment assessments of viral viability were then conducted. For each kit, the initial lysis buffer mix, developed from manufacturer's instructions, included a guanidine-based lysis buffer with additional viral inactivating components such as a solvent and/or a detergent. Each mix was added to 200 μ l of a representative clinical sample (SARS-CoV-2 in VTM). Furthermore, we tested all three protocols with and without the addition of a thermal inactivation step at 68°C for 15 min.

We started with the highest possible titre of SARS-CoV-2 that we had available and first determined the titre of virus following wash steps, which were required to remove any chemical compounds that would be cytotoxic to the cell-based assays. We chose to remove the reagents from the samples by centrifugation and, in doing so, demonstrated a loss of approximately 1-Log₁₀ of virus. Other researchers have used centrifugation columns or filters but again report a similar loss in viral titre (Patterson et al., 2020) or residual toxicity leading to reduced sensitivity of the read-out of the assays (Welch et al., 2020). The wash steps employed here eliminated all residual toxicity, allowing the sensitivity of our assay read-outs to be unaffected.

In our study, the chemicals used to assess the inactivation of SARS-CoV-2 were combinations of GITC, detergent, and solvent. The Qiagen protocol (using reagents from the QIAamp 96 Virus QIAcube HT Kit) and the MagMAX Protocol 2 (using reagents from the MagMAX viral/pathogen nucleic acid isolation kit) both included GITC and a detergent (SDS or Zwittergent, respectively) (**Table 1**). Both of these inactivation buffers significantly reduced viral titres of SARS-CoV-2 by 4-Log₁₀; however, complete inactivation of viable virus was not achieved, as detectable, but not quantifiable, virus was detected in the TCID₅₀ assay (below LLoQ). Subsequent serial passage of viral samples following inactivation using the Qiagen protocol demonstrated virus breakthrough, confirming the results observed in the TCID₅₀ assay. It was also anticipated that serial passage of virus inactivated following MagMAX Protocol 2 would have amplified and enabled virus breakthrough too, but this was not observed. The stated GITC composition of Qiagen Buffer ACL (30%–50%) is lower than that

of the MagMAX Lysis buffer (55%–80%), and thus, the higher GITC composition in the MagMAX buffer may have exerted a greater efficacy of viral inactivation, although we could not demonstrate complete inactivation. As described previously, GITC-based chemicals alone have been reported to inactivate some viruses (Blow et al., 2004; Ngo et al., 2017); but as observed here and by others, this is not always the case (Kumar et al., 2015; Smither et al., 2015; Burton et al., 2017). Studies by Pastorino et al. (2020) have assessed the inactivation of SARS-CoV-2 using the detergent containing Buffer ATL and, in contrast to our findings, reported greater than a 6-Log₁₀ drop in virus titre. The SDS composition of Buffer ATL used by Pastorino et al. (2020) was 1%–10%; however, the SDS composition of ATL buffer in our study was 1% to <3% SDS (**Table 1**). Pastorino et al. (2020) also used a 1:1 ratio of ATL buffer to sample, where as in our protocol we used a reagent-to-sample ratio of 0.5:1. Thus, the work of Pastorino et al. (2020) infers a higher concentration of this detergent, and larger reagent-to-sample ratio would be critical for the inactivation process. This also underlines the potential for different concentrations of components in products that are ostensibly the same. Patterson et al. (2020) and Welch et al. (2020) screened a number of detergents for their inactivation efficacy against SARS-CoV-2. Patterson et al. (2020) reported that 0.5% SDS inactivated SARS-CoV-2 but used a low starting titre of 10² PFU (Patterson et al., 2020), whereas Welch et al. (2020) also reported a drop in virus titre of 6.5-Log₁₀ TCID₅₀/ml, but viable virus was still observed (Welch et al., 2020).

In our study, the only protocol that inactivated virus without an additional heat step was MagMAX Protocol 1 (using reagents from the MagMAX Pathogen RNA/DNA kit), where no CPE was observed from either TCID₅₀ assay or following three rounds of serial passage in tissue culture flasks. The MagMAX Protocol 1 included the MagMAX lysis binding buffer that contained GITC and the detergent Zwittergent. With the addition of 2-propanol within the lysis buffer mix, there were, therefore, three components likely to exert a disruptive effect on the SARS-CoV-2 viral envelope. The reagent-to-sample ratio of 3.8:1 was also higher, with more than double the volume of lysis buffer mix added to each sample, compared with the other two methods assessed (**Table 1**).

Our results suggest that both a high reagent-to-sample ratio and the incorporation of a solvent improved the inactivation efficacy of a chemical only method. The SARS-CoV-2 inactivation efficacy of the GITC-based Buffer AVL (Qiagen) in combination with ethanol has been assessed in two studies. Complete SARS-CoV-2 inactivation was reported by Welch et al. (2020) in contrast to incomplete inactivation by Pastorino et al. (2020). This contradiction in findings could be due to the ratios of reagent, solvent, and sample used. Both studies used 4 volumes of AVL to 1 volume of sample; however, volumes of ethanol used in combination with Buffer AVL may explain the varying results. Welch et al. (2020) used 4 volumes of ethanol in combination with AVL and sample, whereas Pastorino et al. (2020) only added 1 volume of ethanol to the AVL-sample combination. In our studies using the MagMAX Protocol 1, the ratio of lysis buffer and isopropanol was

considerably less with 1.8 volumes of lysis buffer and 1.5 volumes of solvent, but the addition of the detergent Zwittergent (within the MagMAX Lysis Buffer) may have enhanced the inactivation. The addition of the enzyme Proteinase K in both the Qiagen method and MagMAX Protocol 2 (which was absent in MagMAX Protocol 1) did not appear to have enhanced inactivation efficacy.

We also investigated the efficacy of thermal inactivation, by heating the sample to, and then maintaining at, 68°C for 15 min. Heat inactivation alone reduced the viral titre by 3-Log₁₀, although this was not statistically significant compared with the controls and was not as effective as the use of lysis buffers alone. Burton et al. (2021) reported similar findings with incomplete inactivation of SARS-CoV-2 at 56°C and 60°C for up to 60 min. In contrast, some studies have reported the successful use of heat for complete inactivation of SARS-CoV and SARS-CoV-2 (Darnell et al., 2004; Kim et al., 2020). Kim et al. (2020) demonstrated the complete inactivation of SARS-CoV-2 in clinical samples following incubation at 65°C for 30 min, although this work was based on quantitative TCID₅₀ assays alone. Furthermore, Darnell et al. (2004) reported complete inactivation of SARS-CoV after heating at 65°C for 60 min; longer time was required to ensure any viral aggregates were fully exposed and inactivated by the heat treatment.

The use of heat to inactivate virus has been reported to reduce viral RNA stability (Pan et al., 2020; Zou et al., 2020); and depending on the target gene used for RT-PCR, incubation at 65°C for 30 min can significantly reduce the target copy numbers, leading to false-negative results of clinical samples (Kim et al., 2020; Zou et al., 2020). The DCL has an accredited SARS-CoV-2 diagnostic workflow (UKAS, 2020) using the Qiagen and KingFisher (using MagMAX Protocol 1) extraction platforms each with an additional heat inactivation step. Multiple External Quality Assessment panels and reference standards have been tested during DCL set-up and operation. The E-Gene PCR assay (Corman et al., 2020) is used in this laboratory, and in our hands, the heat inactivation regime we employ does not appear to adversely affect PCR results.

In determining the practical relevance of our work, the viral loads in COVID-19 samples likely to be encountered in a high-throughput diagnostic laboratory should be considered. Currently, there is little information on the infectious viral load present on a clinical nasal/throat swab. Most studies only report quantification cycle (C_q) values following RT-PCR (Pan et al., 2020), but one study has estimated that there is a median titre of 10³ TCID₅₀/ml collected from 90 nasopharyngeal or endotracheal clinical samples (Bullard et al., 2020). During DCL validation studies, a precisely defined reference standard dilution series of entire SARS-CoV-2 virions (SARS-CoV-2 Analytical Q Panel; Qnostics Ltd, United Kingdom) was tested (data not shown). Within this series, the highest concentration of material was 6-Log₁₀ digital copies (dC)/ml; and following RNA extraction using the Qiagen method described in this paper, mean E-gene (Corman et al., 2020) quantification cycle (C_q) values of 22.65 were returned from this concentration. During DCL operations, we have commonly tested positive samples with E-gene PCR C_q values in teens, with occasional samples

returning C_q values <13. Although care must be taken in comparing and extrapolating PCR (C_q), TCID₅₀/ml, and dC/ml values, this is consistent with a study reporting similarly low C_q values from COVID patients early in the infection cycle (Jang et al., 2021) and indicates that some swab samples can contain very high viral loads.

We have demonstrated the SARS-CoV-2 inactivation efficacy of the reagents found in lysis buffers of three commercially available kits used on high-throughput extraction platforms. Only when combined with a heat step did all methods show a complete inactivation of SARS-CoV-2 by both TCID₅₀ assay and by sequential passage in tissue culture. Therefore, in the DCL, samples are sequentially mixed with lysis buffer and then followed with heat treatment. This approach also extends the contact time of lysis buffer to sample, which should further enhance the inactivation efficacy of the buffers and mitigates the fact that in this inactivation study we were unable to test samples with a starting concentration greater than 5.8 × 10⁵ TCID₅₀/ml (in view of the likely higher concentrations seen in samples received). In our studies, we also did not include samples that contain potential interfering substances or true samples; however, Pastorino et al. (2020) did include interfering substances and a range of clinical samples, and no obvious impact of these sample types was reported on the efficacy of the viral inactivation process.

Due to the contrasting literature for inactivation of SARS-CoV-2 (and that of viruses generally), a case-by-case assessment of different inactivation protocols is essential to prevent laboratory-acquired infections. To ensure the highest safety standards (and also taking into account the high viral loads of samples tested), in the operational DCL, we employ methods that utilise the inactivation efficacies of the chemical components of lysis buffers found in commercial kits with that of the heat. As a result, the high-throughput RNA extraction platforms are performed on the open bench rather than within a Class 1 microbiological safety cabinet. All laboratories must make the appropriate assessments regarding methods applicable to their unique set of circumstances. The results presented in this study may help laboratories undertake such assessments, especially if they do not have access to high containment facilities to complete in-house inactivation studies.

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

RT: Conceptualisation, data curation, formal analysis, investigation, methodology, validation, writing—original draft, and writing—review and editing. LE: Data curation, investigation, methodology, and writing—review and editing. LO'B: Data curation, investigation, methodology, and writing—review and editing. DU: Data curation,

investigation, methodology, and writing—review and editing. JF: Investigation, methodology, and writing—review and editing. SS: Data curation, formal analysis, investigation, methodology, and writing—review and editing. AP: Data curation, investigation, methodology, and writing—review and editing. HS: Methodology, resources, and writing—review and editing. KH: Conceptualisation, writing—original draft, and writing—review and editing. SW: Conceptualisation, funding acquisition, methodology, project administration, validation, writing—original draft, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Corrigendum: Evaluation of the SARS-CoV-2 Inactivation Efficacy Associated With Buffers From Three Kits Used on High-Throughput RNA Extraction Platforms

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A Corrigendum on

Evaluation of the SARS-CoV-2 Inactivation Efficacy Associated With Buffers From Three Kits Used on High-Throughput RNA Extraction Platforms

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In the original article, there was a mistake in **Table 1: Protocols tested for assessing inactivation using lysis buffers** as published. During the publication process the components for each of the three kits tested in this study (as stated in the 'Reagents' and 'Active virucidal components' columns), were unclearly formatted. The corrected **Table 1: Protocols tested for assessing inactivation using lysis buffers** appears below.

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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TABLE 1 | Protocols tested for assessing inactivation using lysis buffers.

| Manufacturer, RNA extraction kit, Platform | Reagents (volume/sample) | Active virucidal components* | Reagent: Sample ratio |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------|-----------------------|
| Qiagen, QIAamp 96 Virus QIAcube HT Kit (Cat #: 57731), Qiagen QIAcube HT. (Referred to here as <i>Qiagen protocol</i>) | ACL buffer (190 µl) ATL buffer (100 µl) Proteinase K (20 µl) Carrier RNA (5 µl) MS2 (10 µl) | GITC 30 - <50% 1 - <3% SDS | 1.6: 1 |
| ThermoFisher, MagMax Pathogen RNA/DNA kit (Cat #: 4462359), Kingfisher Flex. (Referred to here as <i>MagMax Protocol 1</i>) | Lysis binding buffer (350 µl) Isopropanol (300 µl) Carrier RNA (2 µl) Water (100 µl) MS2 (10 µl) | GITC 55-80% <0.001% Acrylamide Zwittergent 100% 2-propanol | 3.8: 1 |
| ThermoFisher, MagMax viral/pathogen nucleic acid isolation kit (Cat #: A48310), Kingfisher Flex. (Referred to here as <i>MagMax Protocol 2</i>) | Lysis binding buffer (265 µl) Proteinase K (5 µl) †Water (Magnetic beads) (10 µl) MS2 (10 µl) | GITC 55-80% <0.001% Acrylamide Zwittergent | 1.4: 1 |

*As identified directly from components, manufacturer information, or inferred from the associated MSDS.

†Water was used to replace the magnetic beads as the washing steps described below would not remove the beads and the beads interfered the read-out of the TCID₅₀ assay.
GITC, Guanidinium thiocyanate; SDS, Sodium dodecyl sulphate.



Quality Management for Point-Of-Care Testing of Pathogen Nucleic Acids: Chinese Expert Consensus

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COVID-19 continues to circulate globally in 2021, while under the precise policy implementation of China's public health system, the epidemic was quickly controlled, and society and the economy have recovered. During the pandemic response, nucleic acid detection of SARS-CoV-2 has played an indispensable role in the first line of defence. In the cases of emergency operations or patients presenting at fever clinics, nucleic acid detection is required to be performed and reported quickly. Therefore, nucleic acid point-of-care testing (POCT) technology for SARS-CoV-2 identification has emerged, and has been widely carried out at all levels of medical institutions. SARS-CoV-2 POCT has served as a complementary test to conventional polymerase chain reaction (PCR) batch tests, thus forming an experimental diagnosis platform that not only guarantees medical safety but also improves quality services. However, in view of the complexity of molecular diagnosis and the biosafety requirements involved, pathogen nucleic acid POCT is different from traditional blood-based physical and chemical index detection. No guidelines currently exist for POCT quality management, and there have been inconsistencies documented in practical operation. Therefore, Shanghai Society of Molecular Diagnostics, Shanghai Society of Laboratory Medicine, Clinical Microbiology Division of Shanghai Society of Microbiology and Shanghai Center for Clinical Laboratory have cooperated with experts in laboratory medicine to generate the present expert

consensus. Based on the current spectrum of major infectious diseases in China, the whole-process operation management of pathogen POCT, including its application scenarios, biosafety management, personnel qualification, performance verification, quality control, and result reporting, are described here. This expert consensus will aid in promoting the rational application and robust development of this technology in public health defence and hospital infection management.

Keywords: pathogen, nucleic acid, point-of-care testing, expert consensus, whole-process operation management

INTRODUCTION

The development of molecular biology technologies has enabled specific and sensitive detection of pathogen nucleic acids in a sample. Molecular testing has become one of the most important approaches for diagnosing infectious diseases, especially for detecting slow-growing pathogens that are difficult to cultivate under standard laboratory conditions as well as rare or newly emerging pathogens. For example, infection by SARS-CoV-2 is mainly assessed by nucleic acid testing. However, conventional molecular detection methods, such as regular PCR and qPCR testing, require specialized equipment and long batch test cycles and need to be carried out by professionals in a qualified gene amplification laboratory. Overall, aetiological diagnosis of acute or severe infection is often delayed in hospital outpatient and emergency centres and intensive care units (ICUs), thus delaying treatment and increasing the risk of nosocomial infection. In addition, a large number of patients visit fever clinics or emergency centres for observation during epidemics, resulting in serious shortages of consultation space, medical staff, and personal protective equipment. Accordingly, there is currently an urgent need to effectively shorten waiting and diagnosis time to improve medical care for infectious diseases and clinical microbiology.

Point-of-care testing (POCT), also known as “bedside testing” and “proximity testing”, refers to testing performed near or at the patient’s location, the results of which may lead to changes in treatment (ISO 22870, 2016). POCT has been widely used in medical and health institutions at all levels, mainly for the detection of blood biochemical and immunological indicators such as blood glucose and myocardial markers. In the field of infectious disease screening, POCT provides more rapid and sensitive diagnoses than traditional pathogenic detection methods. In general, POCT provided to patients in primary health care institutions and those receiving public health emergency treatment plays an extremely important role in the prevention and control of infectious diseases and hospital infection management. POCT can also help to improve health economic benefits as well as public health management.

Abbreviations: CRE, carbapenem resistance gene; EQA, external quality assessment; GBS, group B *Streptococcus*; HBV, hepatitis B virus; HCV, hepatitis C virus; HIS, hospital information system; HIV, human immunodeficiency virus; HPV, human papilloma virus; ICU, intensive care unit; IQC, internal quality control; IQCP, individualized quality control plan; LIS, laboratory information system; MRSA, methicillin-resistant *Staphylococcus aureus*; PCR, polymerase chain reaction; POCT, point-of-care-testing; TB, *Mycobacterium tuberculosis*.

Moreover, the combination of molecular biology technology and POCT not only integrates sample “extraction-amplification-detection” but also has advantages of portability, easy operation, rapidity, airtightness, and suitability for a variety of scenarios while ensuring high specificity and sensitivity (Kuupiel et al., 2017; Larkin and Garner, 2020).

As a new detection technology with great potential, POCT nucleic acid detection eliminates many steps for specimen processing and large-scale equipment detection and simplifies the tedious process of data processing, and thus can directly and quickly provide reliable results for guiding patient treatment. Therefore, whether it is used in emergency responses to major public health incidents or in the diagnosis, treatment, and management of nosocomial infections, POCT can provide important technical support and guarantee precision treatment as well as scientific prevention and control.

SCENARIOS FOR THE APPLICATION OF POCT

In hospital outpatient and emergency centres and paediatric laboratories, POCT has been used for a long time to detect the antigens of or antibodies against pathogens such as influenza, diarrhoea-causing pathogens, and group A *Streptococcus*. Although such examinations are simple to perform and the rapid results have enabled timely treatment, the detection sensitivity and specificity of these methods are lower than those of molecular detection techniques (Basile et al., 2018; Nelson et al., 2020; Song et al., 2021; Wang et al., 2021). Therefore, examinations based on antigen or antibody detection are prone to missing or misdiagnosing pathogens. In addition, during acute respiratory infection epidemics, false negatives in antigen testing occur due to the high prevalence of the pathogen in the population; as such, pathogen nucleic acid testing should be performed (Harris et al., 2018; Patel and Suh-Lailam, 2019; Nichols et al., 2020).

Pathogen nucleic acid POCT is mainly employed in fever clinics, emergency centres, paediatrics departments, and laboratories in certain wards. As opposed to routine physical and chemical index POCT detection, pathogen nucleic acid POCT first requires assessment of the hazard degree of the biological factor, after which examinations can be carried out under conditions that meet the biosafety requirements. The main applications of different pathogen nucleic acid POCT detection

technologies differ according to the current technical conditions and future technology development trends. Recommendations for scenarios of POCT applications are shown in **Table 1**.

As POCT results are medical test reports, the reports must be accurate and timely, with complete information. Due to the nature of POCT, it can be carried out in the laboratory of a non-clinical testing centre (medical laboratory) by a well-trained person without clinical examination certificates. However, considering the high infectivity and pathogenicity of some pathogens, the requirement for the appropriate laboratory biosafety level and the technologists' qualifications need to follow the regulations of the countries or regions located, and fully consider the actual conditions, including the pathogen risk grade, the waiting time and intensiveness of outpatient and emergency patients, *etc.* Training for POCT-performing technologists includes instrument operation, specimen collection, reagent selection, quality assurance, instrument calibration, maintenance and troubleshooting, biosafety management, and medical waste treatment. Only after the training is completed and exams are passed can technologists carry out POCT examinations and issue reports. Except medical laboratories, whether technologists in one department can perform tests and issue reports for another department should follow the rules of hospital's management committee. The POCT management committee of the hospital authorizes the corresponding work of technologists and conducts management and supervision.

BIOSAFETY MANAGEMENT

Pathogen nucleic acid POCT should be carried out in compliance with national and local biosafety regulations and hospital infection management regulations (Order No. 380 of the State Council of the People's Republic of China, 2011; Health Industry Standard of the People's Republic of China, 2017; Order No. 424 of the State Council of the People's Republic of China, 2018; The State Council of the People's Republic of China, 2020).

The molecular POCT system is an integrated closed system, which means that the nucleic acid extraction, amplification, and detection steps all occur in an enclosed space, which effectively prevents biological samples and genetic materials from being released into the environment. Therefore, nucleic acid POCT poses a relatively small risk to personnel performing the tests. Nevertheless, because of direct contact with patients and samples during sample collection and sample addition to the reagent card or pouch, there is still a risk of pathogen exposure. Therefore, for highly infectious pathogens, such as SARS-CoV-2, the equipment necessary for biosafety level 2 laboratories or above are recommended, including biosafety cabinets and autoclaves, to ensure that pre-treatment of specimens with infection risk (such as liquefaction of sputum specimens and sub-packaging of specimens) is performed safely. To reduce the risk of aerosol formation, spillage, or exposure, patient specimens should be handled in the manner recommended by the product manual.

Fever clinics should meet the regional requirements with three areas (the clean area, the semi-contamination area, and the contamination area) and two aisles (the medical staff aisle and the patient aisle). The clean area mainly include medical and nursing rest areas, which should have independent entrances and exits. The semi-contamination area (also called buffer zone) mainly includes an area for unloading contaminated protective equipment and storage warehouses for disinfection materials. The contamination area mainly includes medical functional areas such as consultation rooms, wards, laboratories, and disposal rooms. The medical functional area should make full use of information technology, such as patient self-service machines for registration, appointment, payment, and printing testing reports, *etc.*, to reduce the waiting time and the risk of cross-infection during the diagnosis and treatment process. In general, pathogen nucleic acid POCT should be carried out in a laboratory in a medical functional area.

Furthermore, the testing area should be kept clean and orderly to prevent cross-contamination. The surface and floor should be disinfected every day as well as immediately after spillage or visible contamination. Technologists should wear

TABLE 1 | Application scenarios of pathogen nucleic acid POCT.

| Laboratory | Purpose | Examples of pathogens detected |
|-------------------------------------|--------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Emergency | Initial diagnosis and treatment | Group A <i>Streptococcus</i> , influenza |
| Fever clinics* | Diagnosis, treatment, and monitoring | Malaria, dengue fever, group A <i>Streptococcus</i> , SARS-CoV-2 and other respiratory viruses, atypical pathogens |
| Paediatric outpatient and emergency | Diagnosis, treatment, and monitoring | Malaria, dengue fever, group A <i>Streptococcus</i> , and pathogens causing respiratory tract infections, rash syndrome, and diarrhoea syndrome |
| Laboratory/third-party laboratory | Diagnosis, treatment, and monitoring | *TB, HIV, HBV, HCV, dengue fever, malaria, group A <i>Streptococcus</i> , central nervous system infection pathogens, bloodstream infection pathogens, respiratory tract infection pathogens, multi-drug resistant bacteria |
| Specific wards* | Treatment and monitoring | Central nervous system infection pathogens, bloodstream infection pathogens, respiratory tract infection pathogens, multi-drug resistant bacteria |

*1. Fever clinics: Pathogen nucleic acid POCT projects carried out in these facilities mainly target fever and respiratory infection pathogens such as SARS-CoV-2, influenza A virus, influenza B virus, and respiratory syncytial virus.

2. Specific wards refer to wards in a hospital with specific prevention and control requirements, such as infectious disease wards and ICUs under closed-loop management of designated hospitals.

3. TB, *Mycobacterium tuberculosis*; HIV, human immunodeficiency virus; HBV, hepatitis B virus; HCV, hepatitis C virus.

TABLE 2 | Common types of clinical specimens and their application.

| Type of infection | Specimen type | Examples of pathogens | Main purpose |
|----------------------------------|--------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------|
| Multi-drug-resistant bacteria | Sputum, stool, anal swab, nasal swab | Carbapenem resistance gene (CRE), Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) | Multi-drug-resistant bacteria control |
| Gastrointestinal tract infection | Stool, anal swab | <i>Clostridium difficile</i> | Detection of <i>Clostridium difficile</i> infection |
| Respiratory tract infection | Throat swab | Group A <i>Streptococcus</i> | Detection and identification of the aetiology of pharyngitis |
| | Nasopharyngeal swab | Acute respiratory viruses (influenza A virus, influenza B virus, respiratory syncytial virus, SARS-CoV-2) | Used for the diagnosis of acute respiratory infections, guiding treatment, and assisting infection control |
| | Sputum/alveolar lavage fluid/tissue | <i>Mycobacterium tuberculosis</i> and rifampicin resistance | Screening and diagnosis of lower respiratory tract infections, guiding medication |
| Genitourinary tract infection | Urine, pus, cervical specimen, swab | Viruses, fungi, atypical pathogens <i>Chlamydia trachomatis</i> , <i>Mycoplasma</i> , <i>Neisseria gonorrhoeae</i> , HPV, GBS* | Screening and diagnosis of sexually transmitted diseases |
| Blood-borne infection | Blood | HIV, HBV, HCV* | Surgery/blood transfusion/haemodialysis/endoscopy preparation |
| Central nervous system infection | Cerebrospinal fluid | EV71, Japanese encephalitis virus, cryptococci, <i>Mycobacterium tuberculosis</i> | Diagnosis of central nervous system infection |

*GBS, group B *Streptococcus*; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papilloma virus.

personal protective equipment correctly in accordance with the requirements of the biosafety laboratory, including disposable gloves, which should be replaced between runs. In addition, test reagents must be stored and handled according to product instructions.

Supervision and verification of POCT safety management measures should be consistent with the current requirements for infectious pathogen gene amplification laboratories, including following guidelines for the preservation, use, and destruction of samples containing suspected infectious pathogens; laboratory biosafety operations; and disinfection and sterilization of laboratory exhaust gas, wastewater, and waste disposal.

SPECIMEN COLLECTION AND PRE-TREATMENT

Specimen collection and pre-treatment for pathogen nucleic acid POCT involve the patient as well as sample collection and testing personnel. Improper handling during one or more of the steps may lead to disqualification of samples before analysis and ultimately lead to incorrect test results. Recommendations for the common types of clinical specimens and their application are shown in **Table 2**.

Selection of the Sample Collection Tube and Preservation Buffer

If one-step nucleic acid extraction is used in pathogen nucleic acid POCT, specific preservation buffer components in the sample collection tube may affect the extraction and amplification efficiency, resulting in a decrease in detection sensitivity. Thus, the sample collection tube and preservation buffer provided by the manufacturer should be used for specimen collection. Additionally, the performance of the sample collection and preservation matching the detection system should be verified before clinical use.

Should the laboratory decide to use a collection tube and buffer not recommended by the manufacturer, it is incumbent on

the lab to determine that the performance of the test is not adversely altered by using that sample collection tube and buffer prior to implementation. Under special circumstances, if a sample collection tube and preservation buffer not provided with the manufacturer's testing reagents are needed, the performance of the collection tube and buffer should be verified before sample collection.

Collection and Pre-Treatment of Specimens

The main types of specimens collected for pathogen nucleic acid POCT are throat swabs, nasal swabs, nasopharyngeal swabs, blood, cervical specimens, sputum, urine, stool, cerebrospinal fluid, puncture fluid, and tissues. The collection methods of various specimens should be consistent with the requirements for conventional molecular biology testing.

Specimen collection method, selection of specimen type, specimen stability, transport temperature and time, acceptance standards, and storage conditions should strictly follow product instructions.

After the specimens are collected, examinations should follow operating instructions. Labelling of the specimens should be kept consistently during the specimen processing procedure when more than one tubes or containers need to be used. Each of these tubes or containers must be labelled with the patient identifying information, including the final testing device, to ensure that the final result is reported for the appropriate patient. Liquefaction of sputum, faeces, tissues, and other specimens must be performed according to manufacturer's instructions. Moreover, special attention should be given to the amount of specimens used for testing, which should specifically follow the manufacturer's instructions. A lesser amount of specimen may give a false negative result due to inadequate target nucleic acids being present. The use of a larger amount of specimen recommended by the manufacturer can also result in a false negative result due to the introduction of impurities and inhibitors of nucleic acid amplification.

PERFORMANCE VERIFICATION OF TESTING PROGRAMMES

Before routine application, the laboratory should conduct independent performance verification of the POCT system to verify that its performance is consistent with declared performance indicators (International Organization for Standardization, 2012a; International Organization for Standardization, 2015; International Organization for Standardization, 2016; International Organization for Standardization, 2020; Verbakel et al., 2020). If any situation that seriously affects the analytical performance of the testing programme occurs, for example, when the main components (such as the fluorescence channel) have been repaired or replaced, performance should be verified before the testing programme is reactivated (International Organization for Standardization, 2012a; International Organization for Standardization, 2016).

Pathogen nucleic acid POCT mainly includes qualitative and quantitative pathogen detection, and different performance parameters are focused for different examinations (International Organization for Standardization, 2010; International Organization for Standardization, 2015; Clinical and Laboratory Standards Institute, 2018). The performance indicators that need to be verified for qualitative testing include, at a minimum, the accuracy, precision (repeatability and reproducibility), and limit of detection (International Organization for Standardization, 2008). The performance indicators that need to be verified for quantitative testing include, at a minimum, the trueness, precision, linearity interval, and limit of quantification (International Organization for Standardization, 2014a). For nucleic acid detection of multiplex pathogens, it is advisable to test for as many common pathogen types and genotypes as possible, especially testing different genotypes and strains of the target pathogens detected by the assay (International Organization for Standardization, 2018).

Performance Verification of Qualitative Detection

Accuracy

To calculate the accuracy, at least five negative samples and generally no less than 10 positive samples (which should include weak positive samples), should be selected. If a sufficient number of positive samples cannot be obtained, a simulated sample can be prepared manually; if a weak positive sample is difficult to obtain, a similar sample can be obtained by appropriately diluting a positive sample. Negative samples should contain nucleic acid sequences that share homology with the nucleic acid sequence of the test subject and are likely to cause the same or similar clinical symptoms. After performing the examination according to the procedure, the results should be compared with known test results to calculate the coincidence rate, and an accuracy $\geq 90\%$ indicates passing verification (International Organization for Standardization, 2008).

Precision (Repeatability and Reproducibility)

To calculate system precision, negative and weak positive samples (fresh or cryopreserved) should be prepared. If a

sufficient number of clinically positive samples cannot be obtained, positive samples can be diluted appropriately, or simulated samples can be manually prepared. In accordance with the testing procedure, the test should be performed three times a day for three consecutive days (if multiple individuals perform the test, a different person should perform it each time). For multi-channel POCT equipment, each test channel should be tested at least once. Test results for negative and weakly positive samples should be exactly the same (International Organization for Standardization, 2008; International Organization for Standardization, 2014b).

Limit of Detection (LoD)

To calculate LoD, a fixed-value reference substance (such as an international reference, national reference, and manufacturer reference) should be diluted to the LoD concentration declared by the manufacturer; the measurement should be repeated 5 times or 20 times in different batches (e.g., measurement performed over 5 days, with 4 samples measured per day). For five repeated tests, 100% of the target nucleic acid must be detected; for 20 tests, the target nucleic acid must be detected at least 19 times (International Organization for Standardization, 2012b; Wang et al., 2020).

Performance Verification of Quantitative Detection

Trueness

One method for measuring trueness is to select at least two concentrations of a reference material (such as certified reference materials, trueness control products, and external quality assessment samples for trueness verification) according to the measurement interval of the POCT system required for verification. The measurement should be repeated three times for the standard substance samples at each concentration, with the mean value compared with the calibrated concentration value to assess bias.

Another method for measuring trueness is to collect at least 20 clinical samples from patients with a clear clinical diagnosis; the pathogen titres in these samples should cover the linear detection range of the POCT system as much as possible. The collected samples can be tested on the same day or within a week. After all experiments are completed, the test results are compared with the original test results, and bias is determined. At least 80% of sample results should have bias less than $\pm 7.5\%$ (International Organization for Standardization, 2014a).

Precision

Fresh or frozen clinical samples can be used to measure precision. When the analyte in the sample is unstable or the sample is difficult to obtain, a sample with a matrix similar to the actual sample (such as a quality control) may be used. Imprecision (coefficient of variation) of at least two samples should be evaluated, and the concentration of the target nucleic acid in the selected sample should be within the measurement interval of the POCT system required for verification. When appropriate, the concentration in at least one sample should be

approximately the medically determined level. Each sample should be tested 3–5 times a day for 5 consecutive days. After all experiments are finished, statistical analysis is performed to calculate intra-run and inter-run precision, which are compared with those in the manufacturer's instructions to judge whether the results are acceptable (International Organization for Standardization, 2014a; International Organization for Standardization, 2014b).

Linear Range (When the Manufacturer's Manual Provides the Performance Index)

To calculate the linear range, a clinical sample in which the concentration of the target nucleic acid is close to the upper limit of the linear range should be selected and diluted to 5~7 concentrations with a normal human negative sample at a tenfold ratio, covering the limit of quantification (lower limit and upper limit). All samples are tested in the same batch, and each concentration is tested at least three times. The mean value of the test results of each sample is calculated separately, and outliers are eliminated. Using the calculated dilution value as the theoretical value, regression analysis is performed on the actual measured and theoretical values for each diluted sample. Performance meeting the linearity requirement stated in the manufacturer's manual is considered acceptable (International Organization for Standardization, 2020b).

Limit of Quantification

To determine the limit of quantification, a standard substance with a known value is diluted to the lower LoD stated in the instructions, and the measurement is repeated at least 20 times. After the experiment is completed, the results of each sample are compared with the reference value of the sample. Bias between the test results (converted to log values) and the reference value should be less than $\pm 7.5\%$; if $n = 20$. It is necessary that ≥ 19 test results meet the above requirements (International Organization for Standardization, 2012b; Wang et al., 2020).

QUALITY ASSURANCE MEASURES

The laboratory should establish and implement standard operating procedures for the entire testing process, including but not limited to specimen collection, transport, storage, specimen reception and pre-treatment, testing operations and re-testing procedures, result reporting and interpretation, instrument and equipment maintenance, performance verification, internal quality control (IQC) and external quality assessment (EQA) (International Organization for Standardization, 2011; International Organization for Standardization, 2012a; International Organization for Standardization, 2012c; International Organization for Standardization, 2016). Qualified laboratories can establish an individualized quality control plan (IQCP) (Centers for Disease Control and Prevention (U.S.), 2015). When the laboratory uses multiple or multiple brands of POCT instruments for routine operation, it is necessary to follow the hospital's POCT management documents and conduct regular inter-

instrument comparisons (International Organization for Standardization, 2016).

Internal Quality Control (IQC)

The laboratory should carry out IQC to monitor the stability of the testing process. IQC procedures should be developed, and specific measures for preventing contamination during nucleic acid testing should be included (International Organization for Standardization, 2012a; International Organization for Standardization, 2016).

In POCT, an internal control (internal standard) is usually included for each sample, and an external quality control (should include positive and negative samples) is run as a separate sample. Detection of the internal control indicates that nucleic acids were extracted correctly from patient samples, which is a necessary step to obtain correct results. External quality control products evaluate whether the testing system provides correct results. A complete IQC programme can control the POCT process, evaluate the performance of the testing system, and provide quality assurance.

IQC products should be equipped with negative control products (for monitoring contamination) and weak positive quality control products (to prevent false negatives). When the POCT system is used for the first time, the negative and weak positive quality control products should be tested first, and clinical sample examination should be started after quality control is complete. Thereafter, the negative and weak positive controls should be routinely evaluated according to the local rules or actual situation. For example, in China, the negative and weak positive controls need to be evaluated every 24 hours or 50 samples (depending on the number of specimens processed). For multiple nucleic acid detection systems, it is recommended that as many types of common pathogens as possible be included in evaluation. One or more pathogens can be included in one round of reagent detection, but should rotate all of the target pathogens on repeated quality control testing runs.

Quality control data for qualitative testing items need to match expected negative and positive results; it is recommended that the weak positive quality control results for quantitative testing items be judged using the Westgard multi-rule approach. A report can be issued only when quality control is passed. Moreover, procedures should be developed to analyse the failure of quality control, and corresponding measures should be taken. The impact of quality control failure on the results of previous patient samples should also be checked.

External Quality Assessment (EQA)

Laboratories should participate in EQA activities to monitor the accuracy of the testing process (International Organization for Standardization, 2013; Sciacovelli et al., 2018; Kabugo et al., 2021). The personnel performing nucleic acid POCT examinations should use the same testing system to assess samples for EQA, and there should be regulations prohibiting comparing EQA results with other laboratories. Additionally, the laboratory should analyse unqualified EQA results, take corrective measures, and record them properly. If no EQA program is available for a specific analyte, laboratories should

share clinical samples with another laboratory for testing. The laboratories will then compare the results from both facilities to ensure that they are the same. Such testing should be performed at least twice a year (International Organization for Standardization, 2012a).

ANALYSIS AND REPORTING OF RESULTS

The laboratory should analyse and interpret the results of tests according to manufacturer's instructions. Although POCT equipment provides clear positive, negative, or invalid results, the laboratory still needs to refer to the background data (such as melting peak graphs and amplification curve graphs) when such data are available for comprehensive judgement regarding results.

When the POCT system is running well, the background data for test results (including internal references and pathogens to be tested) are correct and IQC is passed, the result is considered valid and the positive or negative results provided by the equipment can be reported.

Some POCT reagents will produce positive results for highly pathogenic microorganisms, such as *Vibrio cholerae*, SARS-CoV-2, *Mycobacterium tuberculosis*, and *Bordetella pertussis*. The laboratory should report the results to the sample-sending doctor as soon as possible after verifying the background data, and report to public health administration such as CDC for infectious disease if required. Regarding the management and control of major infectious diseases, reporting should be conducted in accordance with national laws, regulations, and management practices. In addition, some POCT reagents will produce positive results for drug resistance genes (such as MRSA, CRE, and rifampicin resistance) or highly virulent strains (such as the highly virulent *C. difficile* strain 027). In these cases, the laboratory needs to indicate the results of drug-resistance genes on the report after confirmation with the corresponding pathogen testing results. The samples should be re-examined if necessary, and the results should be compared with molecular test and microbial culture drug susceptibility results from the central laboratory (Mo et al., 2020; Wen et al., 2021).

The report of POCT results must be clearly marked with the words "POCT" and detection methods; the name of the POCT equipment and reagents used should also be indicated. When the report is released, the testing results should be entered into the laboratory information system (LIS), saved as a backup for inquiries, and connected to the hospital information system (HIS). In addition, the following aspects need to be considered when issuing examination reports:

- i. Since pathogen types are limited by POCT panels, the report should clearly indicate all pathogens tested and indicate whether a certain pathogen is detected or not detected. The report cannot simply state that the sample is "virus not detected" or "bacterium not detected".
- ii. As nucleic acid testing is usually unable to distinguish between viable and non-viable pathogens or between

colonization and active infection, a positive result only indicates that the DNA or RNA of a certain pathogen is currently present in the sample and does not mean that disease symptoms are caused by the pathogen. Because POCT is usually based on specific primers that target a certain pathogenic microorganism, there may be false negatives due to the lack of amplification of the sequence when the sequence of the pathogen in this region is mutated, which does not indicate that the pathogen is not present. Therefore, it is recommended to state the above information on the report sheet, and clinicians also need to make comprehensive judgements based on the actual clinical situation and other examination results.

Re-examination is required when the following situations occur: i) quality control fails, and the result is invalid; ii) background data do not match the POCT result (e.g., the background data have obvious amplification peaks, but POCT reports a negative result because of the threshold or grey area problems); iii) POCT results are inconsistent with routine laboratory examination results (especially for positive results for highly infectious pathogens), and the clinician believes that re-examination is necessary. If the same results are obtained from re-examination, the laboratory should fully communicate with the clinician the existing test results and reasons and then decide whether to collect a new sample for examination.

LIMITATIONS OF POCT NUCLEIC ACID DETECTION

- i. The detection sensitivity of pathogen nucleic acid POCT is higher than that of immunological POCT (Basile et al., 2018; Nelson et al., 2020; Song et al., 2021; Wang et al., 2021), and the use of inexperienced personnel in molecular detection carries the risk of detection failure and environmental cross-contamination (Wiencek and Nichols, 2016). For example, in a clinic or public space where the flu vaccine or SARS-CoV-2 vaccine is administered, contaminated equipment will produce false positives. In view of the simplicity of molecular POCT operation, by following the manufacturer's instructions, the chance of contamination and human error will be minimized.
- ii. Although the POCT instrument provides clear positive, negative, or invalid results, the platform is usually not connected to LIS; hence, the results must be entered manually, and there is a risk of human error in data entry.
- iii. At present, reagents for pathogen nucleic acid POCT are more expensive than those for conventional fluorescent quantitative PCR detection and antigen/antibody-based detection. Although nucleic acid POCT has higher sensitivity and specificity than immunological POCT (Basile et al., 2018; Nelson et al., 2020; Song et al., 2021; Wang et al., 2021), it is still necessary to consider rapid antigen-antibody screening in economically underdeveloped areas where molecular diagnosis is not affordable.

- iv. Although molecular POCT instruments are usually small and portable, detection throughput is usually low. Indeed, some instruments can only run one to two samples at a time. In fever clinics and emergency departments of large hospitals or emergency care clinics, multiple instruments are often needed to effectively meet the requirements for pathogen detection flux.

In general, pathogenic nucleic acid POCT has gradually entered routine operation in the diagnosis, treatment, and management of infectious diseases and has unique advantages. Whether in a general hospital laboratory or in a fever clinic, POCT can greatly reduce the pressures and challenges brought by centralized sample delivery. Therefore, standardizing POCT management is an urgent task that benefits both doctors and patients, and it is necessary to incorporate POCT into the entire experimental detection system. The reliability of results will be increased through systematic quality control and quality assurance measures. Overall, pathogen nucleic acid POCT provides strong support for improving national medical quality and safety and the public health emergency response, standardizing the rational use of antimicrobial drugs, and establishing a hierarchical diagnosis and treatment model for infectious diseases.

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Isolation of SARS-CoV-2 in Viral Cell Culture in Immunocompromised Patients With Persistently Positive RT-PCR Results

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Immunocompromised adults can have prolonged acute respiratory syndrome coronavirus 2 (SARS-CoV-2) positive RT-PCR results, long after the initial diagnosis of coronavirus disease 2019 (COVID-19). This study aimed to determine if SARS-CoV-2 virus can be recovered in viral cell culture from immunocompromised adults with persistently positive SARS-CoV-2 RT-PCR tests. We obtained 20 remnant SARS-CoV-2 PCR positive nasopharyngeal swabs from 20 immunocompromised adults with a positive RT-PCR test ≥ 14 days after the initial positive test. The patients' 2nd test samples underwent SARS-CoV-2 antigen testing, and culture with Vero-hACE2-TMPRSS2 cells. Viral RNA and cultivable virus were recovered from the cultured cells after qRT-PCR and plaque assays. Of 20 patients, 10 (50%) had a solid organ transplant and 5 (25%) had a hematologic malignancy. For most patients, RT-PCR Ct values increased over time. There were 2 patients with positive viral cell cultures; one patient had chronic lymphocytic leukemia treated with venetoclax and obinutuzumab who had a low viral titer of 27 PFU/mL. The second patient had marginal zone lymphoma treated with bendamustine and rituximab who had a high viral titer of 2×10^6 PFU/mL. Most samples collected ≥ 7 days after an initial positive SARS-CoV-2 RT-PCR had negative viral cell cultures. The 2 patients with positive viral cell cultures had hematologic malignancies treated with chemotherapy and B cell depleting therapy. One patient had a high concentration titer of cultivable virus. Further data are needed to determine risk factors for persistent viral shedding and methods to prevent SARS-CoV-2 transmission from immunocompromised hosts.

Keywords: COVID-19, SARS-CoV-2, immunocompromised, rituximab, obinutuzumab, laboratory medicine, viral cell culture

INTRODUCTION

Coronavirus disease 2019 (COVID-19) was declared a public health emergency by the World Health Organization (WHO) in January 2020 and deemed a global pandemic in March 2020. Detection of viral RNA using real time, reverse transcriptase-polymerase chain reaction (RT-PCR) is the gold standard diagnostic test for SARS-CoV-2, the virus that causes COVID-19. Many patients diagnosed with COVID-19 have multiple positive repeat RT-PCR tests for SARS-CoV-2 even after resolution of COVID-19 symptoms (Lu et al., 2020; Owusu et al., 2021). Although replication-competent virus has not been isolated from most immunocompetent patients with mild symptoms after 10 days, there have been reports of persistent shedding of replication-competent, cell culture positive SARS-CoV-2 virus in immunocompromised hosts beyond 20 days (Avanzato et al., 2020; Aydillo et al., 2020; Choi et al., 2020; Kim et al., 2021).

Currently, CDC recommendations acknowledge that severely immunocompromised patients may have persistent viral shedding beyond 20 days, thus recommending consideration of infectious disease consultation and additional testing before removing isolation precautions (“Ending Isolation and Precautions for People with COVID-19: Interim Guidance,” 2021). Whether or not and how long immunocompromised patients shed infectious virus has profound implications for understanding disease transmission and treatment for these patients. This study aimed to determine if SARS-CoV-2 virus could be recovered in viral cell culture from immunocompromised adults with a repeat positive SARS-CoV-2 RT-PCR tests ≥ 14 days after their first positive RT-PCR test.

METHODS

Study Design and Patient Identification

This was a single-center, retrospective, pilot study conducted at Barnes Jewish Hospital (BJH), a large, academic, tertiary referral hospital in Saint Louis, MO.

The BJH medical informatics database was queried to identify adults ≥ 18 years old with ≥ 2 positive SARS-CoV-2 RT-PCR tests between March and December 2020. Patients were considered to have a persistently positive result if they had a repeat positive test ≥ 14 days after their first positive test. Chart review was performed to identify which patients with ≥ 2 positive SARS-CoV-2 RT-PCR tests were immunocompromised (current cancer treatment, bone marrow or solid organ transplantation, immune deficiencies, HIV with low CD4 count or not on treatment, prolonged use of corticosteroids or other immunosuppressive medications). Remnant NP swabs from the patients' 2nd test, which was not necessarily the RT-PCR test that qualified the patient for inclusion in the study, were collected from the BJH clinical microbiology laboratory; patients were excluded if remnant NP swab samples were unavailable. The NP swabs were all originally obtained during routine clinical care and included outpatient, inpatient, and emergency room

clinical settings at various locations across the BJH system. A convenience sample of 20 immunocompromised patients with eligible NP swabs were included in the analyses. These NP swab samples were sent for antigen testing and viral cell culture.

Additional chart review was performed to collect patient demographic data, clinical characteristics including comorbidities, immunosuppressive medications, and duration and severity of COVID-19 clinical illness.

Molecular Detection of SARS-CoV-2

For initial clinical RT-PCR testing, acceptable transport tubes for the NP swabs included Universal Transport Medium (UTM) and ESwab (with Aimes Transport Medium). Molecular testing was performed by the BJH Clinical Laboratory and due to supply chain issues and need for high testing volume, multiple assays under Emergency Use Authorization by the FDA were utilized (Raju et al., 2021). Systems utilized included the: Roche *cobas* SARS-CoV-2 assay on the Roche *cobas* 6800 (Roche Molecular Systems, Branchburg, NJ), Cepheid Xpert Xpress SARS-CoV-2 assay on the GeneXpert (Cepheid, Sunnyvale, CA), BioFire Respiratory Panel 2.1 (BioFire, Salt Lake City, UT), DiaSorin Molecular SimplexaTM on the Liaison[®] MDX (DiaSorin, Saluggia, Italy), and Lyra SARS-CoV-2 Assay (Quidel) on EasyMag(bioMerieux)/RotorGene Q (Qiagen) or KingFisher (ThermoFisher)/Applied Biosystems 7500 Fast Dx (ThermoFisher). PCR cycle time (Ct) thresholds were recorded for all SARS-CoV-2 RT-PCR tests that were performed by the BJH Clinical Laboratory if available. For tests that provided 2 values, the lower value was used if there was a difference of ≤ 3 between the 2 values; if one of the values was 0, the larger number was used. As the Lyra assay does not include the first 10 cycles in the reported Ct value, a correction factor of 10 was added to all Ct values provided by the Lyra assay (Ransom et al., 2020; Potter et al., 2021).

Antigen Testing

The eluate from the NP swab sample (that had been stored frozen at -80°C) from the patients' remnant second positive NP swab sample was sent for antigen testing on the BD Veritor System (BD, Franklin Lakes, NJ). Samples were vortexed for at least 10 seconds and then a swab from the antigen testing kit was placed in the UTM or ESwab solution. The swab was placed in the reaction tube and swirled in the fluid for at least 15 seconds. Analysis was performed according to the manufacturer instructions for use and the assay interpretation was obtained from the BD Veritor analyzer.

Viral Cell Culture Biosafety

For the viral culture, all aspects of this study were approved by the office of Environmental Health and Safety at Washington University School of Medicine prior to the initiation of this study. Work with SARS-CoV-2 was performed in a BSL-3 laboratory by vaccinated personnel equipped with powered air purifying respirators.

Virus Outgrowth Assay

Patient samples were thawed and 250 μ L from each sample was mixed with 2.75 mL of Dulbecco's Modified Eagle Medium (DMEM) containing 5% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 1X Penicillin/Streptomycin, and glutamine and amphotericin-B, then passed through a 0.45 μ m filter. Media-only was used as negative control and media spiked with 1,000, 100, or 10 PFU of the 2019n-CoV/USA_WA1/2019 SARS-CoV-2 isolate were used as positive controls. Filtered samples were inoculated into T-25 flasks containing Vero cells ectopically expressing TMPRSS2 and human ACE2 (provided by Adrian Creanga and Barney Graham, Vaccine Research Center, NIH) in 5 mL of cell culture media (VanBlargan et al., 2021). Cultures were observed daily for 7 days for cytopathic effect (CPE); cultures that displayed CPE that was inconsistent with typical SARS-CoV-2 were passaged forward, and the original samples were re-filtered and re-inoculated using only 10 μ L to reduce cytotoxicity but maximize viral recovery. Upon observation of syncytia formation characteristic of SARS-CoV-2 CPE, culture supernatants were clarified and frozen.

Viral Sequencing

Viral sequencing was performed at the Washington University McDonnell Genome Institute (Saint Louis, MO). RNA was extracted from cell-culture supernatant using the MagMax Viral 96 kit (ABI) on the Flex System (KingFisher). Extracted RNA was subjected to the ARTIC deep-sequencing protocol which was performed on a HiSeq platform (Illumina) (doi:10.17504/protocols.io.bgxjxkn).

Plaque Forming Assay

Vero-TMPRSS2-ACE2 cells (2.5×10^5 cells per well) were added to flat-bottom 12-well tissue culture plates. The following day, media was removed and replaced with 200 μ L of 10-fold serial dilutions of the original patient sample in DMEM supplemented with 2% FBS. One hour later, 1 mL of methylcellulose overlay was added. Plates were incubated for 48 hours, then fixed with 4% paraformaldehyde (final concentration) in PBS for 20 minutes. Plates were stained with 0.05% (w/v) crystal violet in 20% methanol and washed twice with distilled, deionized H₂O.

Statistics

Descriptive statistics were used to describe patient demographics and clinical characteristics. PCR Ct values were plotted against the time since the first positive RT-PCR test. Analyses were performed using SAS 9.4 (SAS Institute, Cary, NC).

RESULTS

Demographics

From 183 patient charts chosen for screening, 36 immunocompromised patients were identified for further chart review until 20 patients with repeat positive tests with available NP swab specimens were identified for inclusion in the study. From April through December 2020, the lab performed 213,940 COVID-19

tests (including antigen and RT-PCR tests) with 18,165 positive tests. In April, November, and December 2020 the positivity rates were around 14-16% and 3-7% in the other months. Demographic and clinical characteristics are presented in **Table 1**. Median age at the date of the first positive RT-PCR test was 64 years. Ten (50%) of the patients had received a SOT (7 with lung transplants); 5 (25%) patients had a hematologic malignancy, including 1 (5%) patient who had received a bone marrow transplant within 6 months prior to their COVID-19 diagnosis; and 3 (15%) patients had another condition treated with immunosuppressive medication including 1 patient with rheumatoid arthritis, 1 patient with polymyositis, and 1 patient on prednisone for COPD.

SARS-CoV-2 Molecular Testing, Antigen Testing, and Viral Cell Culture

The median number of RT-PCR tests after the initial positive test was 2 (range 1-7). The median time between the first and second positive RT-PCR tests was 21 days (range 7-62). PCR Ct values for 15 of the 20 patients are shown in **Figure 1**; 5 patients had RT-PCR tests performed on the BioFire assay, which does not provide a Ct value, or had missing Ct values and were therefore not included in the figure. There were 8 patients with increasing Ct values over time, 3 patients with decreasing Ct values, and in 4 patients, the Ct value remained stable or only had one value recorded. However, one patient (#1) had Ct values that initially increased before significantly decreasing on subsequent tests.

Of the 20 samples tested, antigen testing was positive in 6 (30%). There were 2 samples that yielded positive viral cell cultures (**Table 2**). Both patients had hematologic malignancies and were being actively treated with chemotherapy and an anti-CD20 monoclonal antibody with associated hypogammaglobulinemia.

Patient Characteristics

Patient #1 was a 60 year-old man with chronic lymphocytic leukemia (CLL) undergoing treatment with venetoclax chemotherapy and obinutuzumab (an anti-CD20 monoclonal antibody). He started this treatment regimen about 5 months prior to his COVID-19 diagnosis, with the last dose of obinutuzumab received 19 days prior to his first positive SARS-CoV-2 RT-PCR test. He was initially hospitalized for management of diarrhea and tested positive for COVID-19 at admission. He developed severe COVID-19 complicated by septic shock, respiratory failure requiring mechanical ventilation, acute on chronic kidney injury with initiation of dialysis in the intensive care unit, and ultimately died from COVID-19 at 18 weeks after diagnosis. Over a span of 4 months, he had 7 positive SARS-CoV-2 RT-PCR tests. Between the 3rd test (day 45 from first positive test) and 4th test (day 66), the Ct values increased. However, the Ct values subsequently decreased. Viral cell culture from the day 23 sample was positive at 27 plaque forming units (PFU)/mL; he also had a positive antigen test at this time.

Patient #2 was a 75 year-old man with marginal zone lymphoma undergoing treatment with bendamustine chemotherapy and rituximab (an anti-CD20 monoclonal

TABLE 1 | Patient demographics.

| Variable | All patients (N=20) N (%) or Median (range) |
|---------------------------------------------------------------|---------------------------------------------------|
| Sex | |
| Male | 11 (55) |
| Race^a | |
| White | 14 (70) |
| Black | 6 (30) |
| BMI | 27.2 (20.1 – 52.0) |
| Age at date of first positive PCR | 64 (20 – 79) |
| Time between first and second positive PCRs (days) | 21 (7 – 62) |
| Number of positive PCR tests after the initial positive test | 2 (1-7) |
| Immunosuppressive condition^b | |
| Solid organ transplant | 10 (50) |
| Hematologic malignancy | 5 (25) |
| Bone marrow transplant 6 months before first positive PCR | 1 (5) |
| Other | 3 (15) |
| Solid organ malignancy ^b | 1 (5) |
| Immunosuppressive medication^c | |
| Receiving high dose steroids at time of positive PCR test | 5 (25) |
| Receiving biologic medication in prior 30 days | 2 (10) |
| Receiving other immunosuppressive medication in prior 30 days | 11 (55) |
| Other comorbidities | |
| Hypertension | 13 (65) |
| Heart disease ^d | 12 (60) |
| Chronic kidney disease | 10 (50) |
| Dialysis | 3 (15) |
| Chronic lung disease | 7 (35) |
| Chronic obstructive pulmonary disease | 4 (20) |
| Diabetes | 6 (30) |
| Obesity | 6 (30) |
| Current smoker | 2 (10) |
| Chronic liver disease | 1 (5) |

^aAll patients were non-Hispanic.

^bThe patient qualified for the study as they were on dexamethasone for >30 days prior to the first positive RT-PCR test.

^cPrednisone status unknown for 1 patient; autoimmune diseases status unknown for one patient.

^dHeart failure, coronary artery disease, congenital heart disease, cardiomyopathies, pulmonary hypertension.

antibody). He started this treatment regimen about 3 months prior to COVID-19 diagnosis, with the last dose of rituximab received 11 days prior to his first positive RT-PCR test. He was hospitalized for 4 days and discharged on home oxygen therapy with daily telephone follow-up. He reported persistent fever and shortness of breath, and his symptoms resolved around 8 weeks after his initial COVID-19 diagnosis. Over about 3 months, he had 8 positive RT-PCR tests. On initial repeat testing, the Ct values increased and subsequently stabilized. The viral cell culture from the day 22 sample was positive at 2×10^6 PFU/mL. Notably, he had a negative antigen test at this time.

There were 2 patients with negative SARS-CoV-2 viral cell cultures who were also being treated with rituximab around the time of their first positive SARS-CoV-2 RT-PCR test. One patient was a 38 year-old man with polymyositis on high dose steroids and rituximab who presented with fevers and shortness of breath; he last received rituximab 4 months prior to his first positive RT-PCR test. He underwent a 3rd RT-PCR test 4 weeks after the second positive test due to persistent respiratory symptoms; his symptoms and progressive fibrotic pulmonary changes were ultimately attributed to pulmonary complications of polymyositis. The other patient was a 77 year-old woman with CLL on venetoclax and rituximab; she received rituximab 8 days before her first positive RT-PCR test. She initially presented with fevers, shortness of breath with activity, and fatigue. Her shortness of breath lasted for 6 weeks and she had a relatively mild clinical course. She continued to have fatigue afterwards but did not have any additional repeat RT-PCR tests after the second test.

DISCUSSION

In this pilot study of 20 immunocompromised adults with persistently positive SARS-CoV-2 RT-PCR tests, only 2 patients had positive viral cell cultures detected from their 2nd positive test. Antigen testing of the repeat sample was positive for 6 patients.

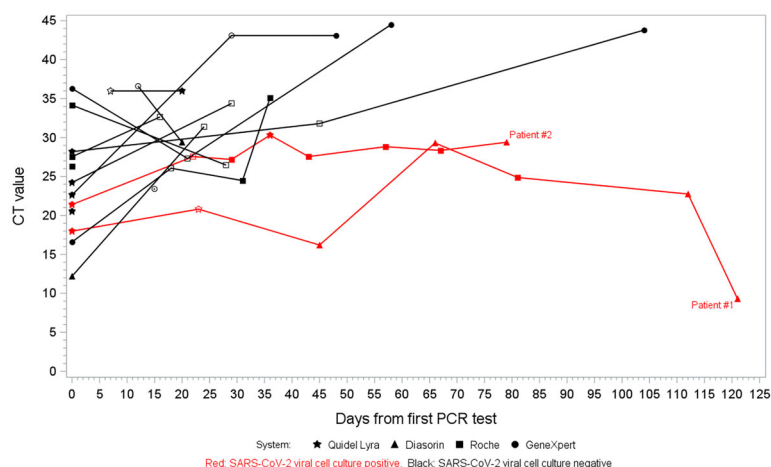
**FIGURE 1 |** Open symbol indicates sample was cultured.

TABLE 2 | Characteristics of patients with a positive SARS-CoV-2 cell culture.

| Variable | Patient #1 | Patient #2 |
|---------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|
| History at time of first positive SARS-CoV-2 RT-PCR | 60 year old male with chronic lymphocytic leukemia who presented with cough and diarrhea. | 75 year old male with marginal zone lymphoma who presented with 2 weeks of cough. |
| Other medical conditions | Fibromyalgia Hyperlipidemia | Hyperlipidemia Deep vein thrombosis Acute hemolytic anemia |
| Positive SARS-CoV-2 RT-PCR tests (days after first positive test Day 0) (study samples in bold) | Day 0 Day 81 Day 23 Day 111 Day 45 Day 120 Day 66 | Day 0 Day 57 Day 22 Day 67 Day 29 Day 79 Day 36 Day 43 |
| Malignancy treatment (last dose prior to positive test) | Obinutuzumab and venetoclax (Day -19) | Bendamustine and rituximab (Day -11) |
| SARS-CoV-2 antigen test | Positive | Negative |
| Cause of death | COVID-19 | Alive as of 16 months after COVID-19 diagnosis |
| SARS-CoV-2 cell culture results from the repeat test (plaque forming units/mL) | 27 PFU/mL | 2×10^6 PFU/mL |
| Spike protein mutations from the repeat test | D614G | D614G, S98F, S813I |

Many case reports of immunocompromised patients with persistent COVID-19 have suggested that patients with hematologic malignancy and treatment with B-cell depleting therapy complicated by hypogammaglobulinemia are at particular risk for persistent viral shedding and severe COVID-19 disease (Avanzato et al., 2020; Choi et al., 2020; Fürstenau et al., 2020). Consistent with this, the 2 patients with positive viral cell cultures in our cohort also had hematologic malignancies and were undergoing treatment with chemotherapy and either rituximab or obinutuzumab, both of which are monoclonal antibodies against CD20 which deplete B cells. There were also 2 patients in this study who, despite being on rituximab, had negative viral cell cultures. This is important to note as it has potential implications for viral transmissibility, management, and infection prevention measures. However, despite this need to understand which immunocompromised patients are at risk for continued viral shedding, there are no routinely available tests for use in a real-time, clinical setting to confirm whether a patient has persistent shedding of infectious virus.

SARS-CoV-2 RT-PCR tests are reported as positive or negative, and Ct values are not reported in routine clinical practice. Higher Ct values have been associated with lower viral RNA concentration in a sample load, although the use of the Ct thresholds as a clinical tool has been somewhat controversial. Previous data from outpatient settings demonstrated that Ct values were similar among symptomatic and asymptomatic patients with COVID-19 (Lee et al., 2020), but in hospitalized patients, high viral loads based on lower Ct values at admission were associated with increased mortality (Magleby et al., 2020; Westblade et al., 2020). An association between Ct value and culture positivity has also been previously observed, with Ct values of 13-15 reported from the EZ1 Virus Mini Kit 2.0, which targets the E-gene, being highly correlated

with positive viral cell cultures and a Ct value ≥ 34 associated with negative viral cell cultures (La Scola et al., 2020). Although Ct values have been shown to correlate with severity of SARS-CoV-2 infection and have been used as a surrogate marker for viral load for research purposes, there are many different RT-PCR assays with different gene targets, Ct values differ between testing platforms, and there are no standardized cutoff thresholds for clinical interpretation (Potter et al., 2021; Rhoads et al., 2021).

The use of Ct values as a tool for COVID-19 infection prevention purposes has been investigated previously (Bullard et al., 2020; He et al., 2020). This is of particular interest in immunocompromised patients as SARS-CoV-2 viral load and transmission are likely to be more unpredictable than in immunocompetent patients, and this may have implications with regards to isolation precautions. Recognizing that caution must be exercised in interpretation of Ct values to make clinical inferences, we observed that, for the most part, Ct values increased with subsequent tests, which is what would be expected during the clinical course of COVID-19 and resolution. However, this was not the case for the 2 patients with positive viral cell cultures weeks after their initial positive RT-PCR test.

Patient #1 ultimately developed severe COVID-19 and had multiple RT-PCR tests from the same testing platform showing a significant decrease in Ct values (greater viral RNA levels) while he was clinically decompensating. His viral cell culture had a low titer of 27 PFU/mL, but the sample was collected fairly early in his clinical course at 23 days after his initial COVID-19 diagnosis, and we did not test the subsequent samples to assess the trend in viral load after this time. Antigen testing was positive in this patient from the same sample collected at 23 days after the initial positive RT-PCR test. In contrast, patient #2 had more moderate disease but unexpectedly had a very high viral titer of 2×10^6 PFU/mL from a sample obtained 22 days after his initial COVID-19 diagnosis with a negative antigen test. Although he initially

required a short hospitalization of 4 days, he was primarily managed as an outpatient on home oxygen for most of his prolonged 2-month clinical course. He had an initial increase in Ct values as expected, but the Ct values subsequently remained stable until testing was stopped after his first negative test at 93 days after his first positive RT-PCR test.

In this study, only 6 out of 20 patients had a positive antigen test on the repeat sample. Notably, the two patients with positive viral cell cultures both had negative antigen tests. Although the antigen tests have very good specificity near 95–100%, they have lower analytical sensitivity compared to PCR based assays (Schoy et al., 2020; Liu & Rusling, 2021). The BD Veritor system used herein reports a limit of detection of 1.4×10^2 TCID₅₀/mL, an estimated clinical sensitivity of 84% with diminished sensitivity after the first five days of symptom onset, and that negative results should be confirmed with a molecular assay (https://www.bd.com/documents/guides/directions-for-use/IDS_BD-Veritor-Plus-SARS-CoV-2-500048916_DF_EN.pdf). Therefore, it is difficult to draw any inferences about the significance of the negative antigen tests from the patients with positive viral cell cultures.

The findings of this study support prior reports showing that immunocompromised patients can have positive SARS-CoV-2 cell cultures beyond 20 days and that patients with hematologic malignancies on B cell depleting therapy seem to be at particularly increased risk (Avanzato et al., 2020; Aydillo et al., 2020; Choi et al., 2020; Fürstenau et al., 2020). We have also shown that the 2 patients we identified with persistent, active COVID-19 with positive viral cell cultures did not have the expected increase in Ct values over multiple repeat RT-PCR tests. Although the use of Ct values is not routine in the clinical management of patients with COVID-19, Ct values may be useful on a case-by-case basis in high-risk patients to make clinical decisions regarding treatment and isolation precautions.

Notably, there were also 2 patients in our cohort treated with rituximab prior to their first positive SARS-CoV-2 RT-PCR test who had negative viral cell cultures from the second positive NP swab sample. Despite having prolonged symptoms, medical records indicate that the treating clinicians thought that these were secondary to the patient's underlying medical conditions rather than persistent COVID-19 disease and they were not subject to repeat RT-PCR testing because of this.

There were 2 patients who qualified for the study because they had a positive RT-PCR test ≥ 14 days after the first positive test. However, their 2nd positive tests were at days 7 and 12 respectively and these were the samples that were sent for viral cell culture. Both of these patients had negative viral cell cultures even though this repeat sample was collected < 14 days after their first positive test.

When this study was conceived, there were primarily case reports of persistent, cultivable SARS-CoV-2 virus isolated from samples derived from immunocompromised patients. This study used a more systematic approach to assess the question of whether immunocompromised patients might have persistent shedding of potentially infectious virus and included a balanced distribution of patients with immunocompromising conditions of interest (50% with a SOT, 25% with a hematologic

malignancy) and thus provides additional clinical insight. There are several limitations to our study. This was a retrospective pilot study with a small sample size utilizing convenience samples. Because we only queried our institutional database for information, we would have missed any additional COVID-19 tests performed by facilities other than the BJH clinical laboratory. Due to supply chain constraints and high volume of testing needed, different SARS-CoV-2 PCR assays were used for diagnosing COVID-19 in the clinical laboratory. Therefore, patients in this study had results reported from different assays, which complicates comparison of Ct values across instruments and genes targeted in the assay (Rhoads et al., 2021). We performed viral cell cultures at a single point and therefore lack longitudinal data about viral load and how it correlated with clinical status and Ct values; this is an important topic for future investigations. This study was performed on samples collected earlier in the pandemic and no known variants of concern were identified; the results may differ now that the predominant strains in the U.S. have changed.

CONCLUSION

Our findings indicate that patients with hematologic malignancies on B-cell depleting therapy who develop COVID-19 are at particular risk of having prolonged SARS-CoV-2 viral cell culture positivity. Additional studies should be performed in immunocompromised patients with COVID-19 to further clarify the risk factors and features associated with persistent shedding of potentially infectious SARS-CoV-2. Further study is also needed to determine the best management and infection prevention strategies for these patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA780421>.

ETHICS STATEMENT

This study was approved by the Washington University in St. Louis Human Research Protection Office, IRB #202006011. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Study conception and design: JK, HB, and VF. Data acquisition, analysis, and interpretation: AB, HS, DM, MW, KP, AS, JK, HB, C-AB, KR, and CM. Drafting the article: AS, KR, JK, and HB.A.S.

wrote the manuscript, with contributions and comments from all authors. All authors contributed to the article and approved the submitted version.

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Learning From Agility, Partnership and Innovation During the Covid-19 Pandemic: A Perspective From Industry

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Two years after the COVID-19 pandemic started, the world continues to adapt to the profound effects that Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has had on our lives. As the global crisis took hold, many looked to the medical technology/device industry for guidance and solutions. All while the industry itself, was disrupting its own processes and activities. In order to evolve and deliver accelerated innovation the industry had to be agile, resilient and collaborative with the broader healthcare community and technology partners. Now comes a time when we will start to see what changes were temporary and which ones will become part of the new process, but one thing is certain, we will not be going back to where we were pre-pandemic.

Keywords: COVID-19, SARS-CoV-2, medtech industry, healthcare industry, diagnostics

AGILITY

As the COVID-19 pandemic disrupted labor markets, the medical technology (medtech) industry had to adapt its practices. Accommodations needed to be made for associates working from home and new considerations were necessary in order to support associates, mainly in manufacturing and research and development, who needed to be at work physically. Adapting meant implementing worksite restrictions to reduce the spread of the novel coronavirus (United States Department of Labor and Occupational Safety and Health Administration (OSHA), 2021), as well as personnel screening, testing, and vaccination to reduce both the individual risk and community risk and limit viral spread. Associates required to be on-site to provide customer service were those most severely impacted. Consequently, it was necessary for the industry to adapt and find new ways to support customers remotely. In some instances our instruments offer remote monitoring; allowing the extraction of de-identified databases to support timelier offsite troubleshooting and providing near real-time analytics such as positivity rates or trends in cycle threshold (Ct) values. This is a practice

that we should certainly continue to push but that is not always easy to implement because of data security and protection surrounding health information. In addition, we developed tools to replace in-person trainings of customers and associates with virtual offerings. One such example was the creation of virtual environments where customers interact with the solution and associates can train remotely. This agility was especially beneficial when borders closed and travel to customer locations was almost impossible.

Early on during the pandemic, the need to switch gears to rapidly develop and deploy assays to diagnose infection with SARS-CoV-2 led to a large mobilization of the workforce towards creation of point-of-care and lab-based antigen, antibody and molecular diagnostic assays. In order to support this demand, not only did the labor force have to shift priorities but there was also an increased demand to hire the right talent and expand teams in a short amount of time. Conversely, as fluctuations in hospital procedure volumes and deferral of elective procedures affected other segments of the medtech industry, balancing prevention of layoffs with increasing demand on manufacturing pushed the industry to become more nimble. While there was unpredictable fluctuation in demand and production volumes, the industry had to not only face a shortage in workforce, but also a workforce affected by quarantine measures. Companies took different approaches towards hiring practices and incentives and implemented more flexible work hours to adjust the manufacturing output to the demand. Other functions also stepped up to provide support to the manufacturing function in order to keep up with the increased demand.

Unpredictable fluctuations in manufacturing, supply chains, raw materials and transport logistics of products across the globe led the medtech industry to look for ways of minimizing risk. By diversifying the supply base and validating multiple in-country and external suppliers we can lessen the dependence on high-risk sources and by opting for vertical integration of the supply chain, we can streamline our operations. We have also taken advantage of process innovations such as integration of upstream manufacturing steps or materials for molding of parts. Process improvements and incorporation of new technologies will need to be revisited alongside the trade-off between product variety and capacity flexibility to maintain agility.

PARTNERSHIP

In order to accommodate the needs of patient care during the pandemic, key partnerships emerged. Industry partners, manufacturers, and suppliers came together in addition to the public health sector, clinical laboratories, regulatory agencies, non-profit organizations and governments worldwide. The enormous redeployment of resources to ensure rapid regulatory authorization allowed us to develop and launch COVID-19 diagnostic assays in 90 days, compared to what would have normally taken more than 3 years. Institutions fostered unprecedented collaboration between diverse and

interdisciplinary groups, allowing for an open exchange of information and rapid learning from one another. The sharing of risk mitigation strategies, efficiencies gained, and innovations may not have happened without the pandemic, and should continue for the long term. If we have learned anything, it is the importance of cross-segment dialog and strong collaboration.

Early on in the pandemic, collection devices were in very short supply. Companies including Copan, Roche, Thermo Fisher, Abbott and BD worked together to evaluate the possibility of validating alternate collection devices as a group, agnostic to the platform used. The group also worked with the United States Food and Drug Administration (FDA) to assess potential options along these lines. We also created partnerships with new swab or collection device suppliers to augment supply and validated the compatibility of their swabs with various molecular or antigen assays. Since different regions of the world had access to different swab suppliers, we tried to ensure availability of solutions across the globe. There was also a lot of lobbying and support from non-profit organizations, like the Bill and Melinda Gates Foundation, to create a network of startup and established companies to foster collaborations and bring to market new diagnostics tools, new technologies or help with manufacturing of new products.

The laser sharp focus on combating a public health emergency negatively affected the approval process of non-COVID products as well, as review processes took longer than anticipated (United States Food and Drug Administration (FDA), 2021). Leaner but still quality driven practices to facilitate approval of medtech products is one of the lessons learned that we hope is here to stay. Based on learnings from COVID-19, new product development best practices that give access to new technologies faster will require collaboration with regulatory bodies as well as post-launch efforts/activities for the medtech industry. Better coordination to prioritize access and sharing of viral genome sequence information, clinical specimens and data, in addition to having commutable international standards and reference materials, would also accelerate the development and validation of diagnostic tools worldwide.

Increased harmonization in the requirements and regulations surrounding the validation and distribution of diagnostic tests would be beneficial. A complex reality is that different countries have different requirements when it comes to study design validations, acceptance criteria and clinical specimen types required. In order to answer specific needs during the COVID-19 pandemic, we found ourselves having to perform additional internal validation studies that added time, effort and increased pressure on an already overloaded workforce. One such example was obtaining claims for saliva testing for France with specific clinical specimen collection for this intended use while claims elsewhere were for nasal swabs. In other instances we collaborated with clinical or public health laboratories or institutions who may have self-validated a given application or published studies utilizing our products. This way we could leverage real-world evidence in order to build the regulatory filing requirements needed to meet local regulations.

INNOVATION

Deployment of different kinds of tests that did not need to rely on the already overburdened healthcare systems such as point-of-care testing, and more recently at-home testing, drove the development of added functionality and digital connectivity. Not only have we seen the creation of platforms that allow for remote data capture and instant access to testing results, but also technological innovations facilitating activities that previously required in-person contact. A recent example of this digital innovation is the development of our at-home digital test kit that combines the use of the Veritor rapid test with the use of a smartphone to interpret, deliver and display results without relying on human interpretation.

The at-home test portfolio expansion will continue, driven more by a better understanding of what tests have value to be done in an at-home setting and less on the specific technologies used. Prescribed infectious disease diagnostic tests have significant benefits to public health as they help reduce exposure of staff members and patients and decrease large volumes of individuals seeking care at health care facilities. COVID-19 drove a significant number of hospitals to implement hospital-at-home programs and widespread adoption and successful implementation still hold promise (Balatbat et al., 2021). At-home test portfolio expansion could help lessen the load for overburdened clinical and public health laboratories that struggle with additional testing volumes and staffing shortages. For infectious disease diagnostics, at-home molecular platforms may take hold as they provide increased clinical sensitivity, but antigen assays are less expensive, more available, and continue to be used in instances when obtaining rapid results (15 min or less) has an actionable impact on human behavior, such as when planning a gathering with friends and family.

Both medtech and healthcare industries are at an inflection point where the way health care gets delivered is changing. The use of telecommunications and digital information technologies to access and facilitate health care services remotely, otherwise known as telehealth, is expanding at a rapid rate (Koonin et al., 2020). We have witnessed digital developments when using QR codes readily available for restaurant menus, event platforms to facilitate educational activities, fitness and activity tracking devices and remote health-related patient monitoring. The next generation of technologies need to improve the quality of health care by increasing access to care and helping the clinician be more efficient. For example, expansion of home hospitalizations will move diagnostic testing closer to the at-home patient and transform the healthcare system by affecting clinical sample collection and testing as well as data transmission and clinician utilization of information. This creation of new care settings will foster innovation in the medtech industry. In addition, when looking at at-home testing for chronic diseases, protein quantitation and collection of other specimen sources, such as blood, will be required. Therefore, new technologies allowing for simpler sample collection and transportation while still ensuring sample integrity will expand. Here once again, the need for more efficient and adaptable manufacturing stands out.

DISCUSSION

The majority of our response to the SARS-CoV-2 pandemic was reactive, and therefore overwhelmed resources all at once. We developed and deployed a plethora of diagnostic assays in an impressively short period, implemented surveillance systems and other technologies to increase connectivity, yet data access remained slow. We still need better mechanisms to track infection, react quicker, and continue to work closely with public agencies so we can reach near real-time data for ongoing and new infections. We will need to continue to close the gap to get information between individual test results and healthcare providers for follow-up and surveillance testing. There needs to be improved coordination between regulatory and development aspects of diagnostic tests. We have significantly increased the speed at which we can roll out tests but still lack the centralized ability to do so, especially in the United States, at a larger scale. The two phase response of 1) having lab developed tests and home brewed diagnostic assays followed by 2) emergency use authorization of low-cost, high-quality commercial assays took longer than what we needed it to be.

We have achieved adequate and reliable direct-to-consumer and decentralized testing without compromising test accuracy or quality and yet there is still a lot of confusion about what types of diagnostic tests to use when. Further partnerships between academia, public health and industry can provide clarity on getting the right test, at the right place, at the right time. Especially when endemic COVID-19 is the likely reality. As we rely more on telehealth, adequate self-collection and guided diagnostic therapy, there needs to be better interactions with healthcare workers and care outside of the hospital setting. In addition, it is necessary to leverage digital technology to promote real-time surveillance and early warning systems if new infections start to emerge in hot spots or specific areas.

A byproduct of this pandemic was a significantly reduced 2020-2021 influenza season. The use of community control tools (masks, social distancing, hand hygiene) to reduce the spread of other pathogens, especially during winter viral season, proved effective. It remains to be seen if we can incorporate behavioral changes that decrease mortality caused by other viral pathogens. Another area that needs attention is how to shift the focus towards prevention and apply lessons learned from quick identification and treatments strategies. For example, an area where we need to leverage surveillance and prevention is towards antimicrobial resistance.

The medtech industry responded to the ever-changing needs posed by the SARS-CoV-2 pandemic and continued to deliver healthcare solutions from bedside care to at-home testing. Recent digital innovations will improve the industry's ability to continue to support the healthcare community and public health sector in the future. This environment of stronger collaboration and partnerships will facilitate the deployment of new approaches to personalized medicine and diagnostic testing innovations to provide better holistic solutions throughout the patient care continuum.

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.

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AUTHOR CONTRIBUTIONS

AMC: conceptualization, writing, and review and editing; CR-D: conceptualization, writing, and review and editing. All authors contributed to the article and approved the submitted version.

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Diagnostic Performance of Seven Commercial COVID-19 Serology Tests Available in South America

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Background: Although RT-qPCR remains the gold-standard for COVID-19 diagnosis, anti-SARS-CoV-2 serology-based assays have been widely used during 2020 as an alternative for individual and mass testing, and are currently used for seroprevalence studies.

Objective: To study the clinical performance of seven commercial serological tests for COVID-19 diagnosis available in South America.

Methods: We conducted a blind evaluation of five lateral-flow immunoassays (LFIA) and two enzyme-linked immunosorbent assays (ELISAs) for detecting anti-SARS-CoV-2 antibodies.

Results: We found no statistically significant differences among ELISA kits and LFIAs for anti-SARS-CoV-2 IgG sensitivity (values ranging from 76.4% to 83.5%) and specificity (100% for the seven serological assays). For anti-SARS-CoV-2 IgM, the five LFIAs have a significantly higher sensitivity for samples collected 15 days after the first time RT-qPCR positive test, with values ranging from 47.1% to 88.2%; moreover, the specificity varied from 85% to 100%, but the only LFIA brand with a 100% specificity had the lowest sensitivity.

Conclusion: The diagnostic performance of the seven serological tests was acceptable for the seven brands tested for anti-SARS-CoV-2 IgG detection for seroprevalence screening purposes. On the other hand, our results show the lack of accuracy of anti-SARS-CoV-2 IgM detection in LFIAs as a tool for SARS-CoV-2 acute-phase infection diagnosis.

Keywords: SARS-CoV-2, COVID-19, serological test, ELISA, rapid test, diagnosis, Ecuador

INTRODUCTION

The detection of the novel coronavirus SARS-CoV-2 in the Chinese province of Hubei in December 2019 led to the Coronavirus Disease 2019 (COVID-19) outbreak that resulted in the World Health Organization (WHO) declaring a pandemic in March 11th 2020 (Gorbalenya et al., 2020; Zhou et al., 2020). By the end of September 2021, more than 230 million cases and 4.7 million deaths have been reported worldwide (<https://coronavirus.jhu.edu/map.html>). The Americas is one of the most affected regions with millions of reported cases and deaths, and considering only the numbers for

USA and Brazil, more than 63 million cases and 1.2 million of deaths have been reported (<https://coronavirus.jhu.edu/map.html>). In Ecuador, more than 500,000 cases and 32,000 deaths were reported by the end of September 2021 (<https://www.salud.gob.ec/actualizacion-de-casos-de-coronavirus-en-ecuador/>).

The insufficient SARS-CoV-2 testing capacity even at high-income countries during the first months of the COVID-19 pandemic has been suggested as one of the reasons for the dramatic scenario created by COVID-19 pandemic (Pullano et al., 2021). Control and prevention of SARS-CoV-2 transmission are the aims of any containment strategy, based in a testing and tracking approach as recommended by the World Health Organization. However, current numbers of cases and deaths related to the COVID-19 pandemic worldwide would suggest that these control and prevention strategies have been hampered by a lack of massive testing in several regions of world, particularly at low- and middle-income countries (Torres and Sacoto, 2020; Henriquez-Trujillo et al., 2021; Pullano et al., 2021). During the first semester of COVID-19 pandemic, SARS-CoV-2 genomic material detection by RT-qPCR was the main gold standard method available for COVID-19 diagnosis worldwide. This technique has significant logistic and capacity limitations like the need for sophisticated and expensive equipment, such as real time thermal cyclers, trained personnel, or permanent supply of expensive reagents. Thus, RT-qPCR-based SARS-CoV-2 testing capacity was limited even in high income countries during the first wave of the COVID-19 pandemic (Pullano et al., 2021). However, the Emergency Use Authorization of SARS-CoV-2 antigen tests since the end of 2020 and the worldwide improved capacity of reagents supply and RT-qPCR testing have partially overcome this problem.

These point-of-care rapid antigen tests became increasingly available, endorsed by regulatory agencies such as the Federal Drug Administration of the USA and have successfully replaced IgM serological testing as a rapid diagnostic tool for active SARS-CoV-2 infection detection (Cubas-Atienzar et al., 2021). However, serology is still a useful tool for epidemiological studies to determine the prevalence of infection in the general population or for screening of individuals who had a contact with SARS-CoV-2 infected people, but did not receive a confirmatory molecular test, to assist on vaccination policies (Watson et al., 2020).

In this context, numerous anti-SARS-CoV-2 serology-based assays, based on detection of antibodies and including point-of-care rapid diagnostic tests or conventional platforms, have recently become available and approved for clinical use worldwide, aiming to provide information about the individual seroprotection status but with a reduced sensitivity and specificity (WHO). These tests that detect anti-SARS-CoV-2 antibodies are typically based on lateral-flow immunoassays (LFIA) or enzyme-linked immunosorbent assays (ELISA). As an additional advantage, serological tests require less technical expertise and equipment, and have a much lower cost-per-patient diagnosis than RT-qPCR assays. Additionally, since the sample to be processed is whole blood collected in tubes or taken from fingerstick, they present a lower risk to the healthcare staff

than collecting potentially infectious respiratory specimens for RT-qPCR. These advantages made serological tests widely used during 2020 even at middle- and low-income countries not only to detect previous infection (IgG seropositivity), but usually as a rapid diagnostic tool for ongoing SARS-CoV-2 infection (IgM seropositivity). However, the main disadvantage of serological test is related to lack of specificity due to cross reactivity with other pathogens, particularly for IgM detection, so the serodiagnostic power of antibodies against SARS-CoV-2 remains a topic of further research (Cota et al., 2020; Hou et al., 2020; Zhao et al., 2020).

Although clinical performance studies for COVID-19 diagnostic tests have become increasingly available, reports related to COVID-19 tests commercially available at low- or middle-income countries are still scarce (Cota et al., 2020; Deeks et al., 2020; Lisboa Bastos et al., 2020; Freire-Paspuel and Garcia-Bereguian, 2021a; Freire-Paspuel and Garcia-Bereguian, 2021b). In top of that, the high percentage of false-positive results of these tests, compromising their specificity, has been described for middle and low-income countries associated to higher prevalence of certain infectious diseases (Echeverría et al., 2021; Tso et al., 2021), and also at tropical latitudes associated to endemic infections caused by arboviruses (Faccini-Martínez et al., 2020). For these reasons, locally assessed clinical performance studies are necessary, especially for regions like South America where there is a single study of this kind to the best of our knowledge (Cota et al., 2020). The aim of this work was to evaluate the clinical performance of seven COVID-19 serology available in South American countries including Ecuador.

METHODS

Study Design

In the present panel-based study, two panels of specimens were used. A “COVID-19 positive panel” formed by 127 serum samples collected 15 and/or 30 days following positive SARS-CoV-2 detection by RT-qPCR, performed at the diagnostic laboratory of “Universidad de Las Américas”, as previously reported (Freire-Paspuel and Garcia-Bereguian, 2021a; Freire-Paspuel and Garcia-Bereguian, 2021b; Freire-Paspuel et al., 2021; Freire-Paspuel B and Garcia-Bereguian MA, 2021). A “COVID-19 negative panel” including 40 sera samples collected in the pre-pandemic period prior to June 2019. This samples were randomly selected from a sera bank from asymptomatic individuals included in previous seroprevalence studies. Only one sample per individual was included in each of the panels, and all the samples included in the study were from individuals living in Ecuador.

Serological Assays

Two groups of serological assays were included in this study:

- Lateral Flow Immunoassays (LFIAs). Five commercially available LFIAs for SARS-CoV-2 IgM/IgG detection were evaluated. At the time of testing, for each tested IgM/IgG one cartridge per sample were labeled by a randomized sample number. The appropriate sample volume was transferred from

the tube to the indicated sample port, followed immediately by provided diluent, following manufacturer's instructions. The lateral flow cartridges were incubated for the recommended time at room temperature before readings. Cartridges were read for test line intensity by two independent readers blinded to specimen status, according to manufacturer's instructions. Briefly, the tests tested, volumes of sample, and the time to read the results were as follows: LFIA 1: for Artron Laboratories Inc. (Burnaby, British Columbia, Canada), 10 ul of serum sample were applied on sample well and IgG/IgM responses were read after 15-20 minutes, but no later than 30 min. LFIA 2: for Biohit Healthcare Co.Ltd (Hefei, Anhui Province, China), 10 ul of serum sample were added in the sample hole and results were read within 15 minutes. LFIA 3: for Camtech Diagnostics Pte Ltd (Henderson, Singapore), 10 ul of serum sample were added in each sample well (1 sample well for IgG/1 sample well for IgM, 2 sample well per cassette) and results were read after 10 minutes, but no later than 18 minutes. LFIA 4: for INNOVITA (TANGSHAN) Biological Technology Co.,Ltd (Hebei, China), 10 ul of serum sample were added on each sample well (1 sample well for IgG/1 sample well for IgM, 2 sample well per cassette) and results were read within 15 minutes. LFIA 5: for Zybion Inc (Dadukou District, Chongqing, China), 5 ul of serum sample were added to the sample well and results were read within 15 minutes.

- ELISA Tests. Two different commercially available ELISA kits were included in the study. ELISA Kit 1: COVID-19 IgG Enzyme ImmunoAssay manufactured by Dia Pro Diagnostic Bioprobes S.r.l. (Sesto San Giovanni, Milan, Italy) for the determination of IgG antibodies against the SARS-CoV-2-specific nucleocapsid (core) and spike antigens. The test was performed as per manufacturer's instructions. The internal controls (Negative control, Positive control, and blank well) were tested every time the kit was used to verify whether their OD values matched the manufacturer's requirements. If OD values were within the expected range, the test results were calculated by means of a cut-off value; after that test results were interpreted as a ratio of sample OD/Cut-off OD. A positive result was assigned to ratios >1.1. A negative result is assigned to ratio values < 0.9. An undetermined result was assigned to ratio values within the range 0.9-1.1. ELISA Kit 2: ID Screen®.

SARS-CoV-2 -N IgG Indirect manufactured by IDVet (Grabels, France) for the specific detection of IgG antibodies against the nucleocapsid of SARS-CoV-2. The test was performed as per manufacturer's instructions. A ratio sample OD (S)/positive control OD (P) was calculated for each sample. The results are analyzed as follows: positive S/P $\geq 40\%$; negative S/P $\leq 30\%$ Negative; undetermined $30\% < S/P < 40\%$.

Statistical Analysis

IC intervals for 95% probability values for sensitivity and specificity were calculated individually for the sensitivity and specificity values using Jamovi software.

RESULTS

An evaluation of the clinical performance of 7 commercial serological test for COVID-19 diagnosis was carried out using 167 sera, including 127 sera from SARS-CoV-2 RT-qPCR positive individuals (positive panel) and 40 sera sampled before 2020 (negative panel). For the positive panel, a stratification of the results was carried out in terms of the time between first RT-qPCR positive result and sera sampling. Two groups were defined at 15 days and 30 or more days post-detection of SARS-CoV-2 infection. **Tables 1** and **2** summarize the performance of 7 serological kits tested, including 2 ELISA kits for the detection of anti-SARS-CoV-2 IgG and five LFIAs for anti-SARS-CoV-2 IgM and IgG detection.

Clinical Performance of Lateral Flow Immunoassays (LFIAs)

The results of the evaluation of the five LFIAs are detailed in **Table 1**. For anti-SARS-Cov-2 IgG detection, the overall specificity of the five brands was 100%, while the overall sensitivity ranged from 76.4% (68-85.5 IC 95%) to 80.3% (72.3-86.8 IC 95%), although no statistically significant differences were found among the five LFIAs brands. Four of the five LFIAs brands did not show statistically significant differences for the sensitivity values for SARS-CoV-2 positive samples between 15 days samples or 30 or more days samples since RT-qPCR positivity. However, for the "Zybion" brand, there

TABLE 1 | Diagnostic performance of SARS-CoV-2 IgG/IgM lateral flow immunoassays.

| Performance parameter | Brand | | | | |
|---------------------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| | Artron™ | BioHit™ | Camtech™ | Innovita™ | Zybion™ |
| IgG antibodies detection | | | | | |
| Overall sensitivity - % (IC95%) | 76.4 (68 - 83.5) | 76.4 (68 - 83.5) | 80.2 (72.1 - 86.7) | 79.5 (71.5 - 86.2) | 80.3 (72.3 - 86.8) |
| Sensitivity at 15 days | 76.5 (58.8 - 89.3) | 82.4 (65.5 - 93.2) | 79.4 (62.1 - 91.3) | 85.3 (68.9 - 95.1) | 50.0 (32.4 - 67.6) |
| Sensitivity post-infection at 30 days | 76.3 (66.4 - 84.5) | 74.2 (64.1 - 82.7) | 79.6 (70 - 87.2) | 77.4 (67.6 - 85.5) | 72.3 (62.2 - 81.1) |
| Overall specificity - % (IC95%) | 100.0 (84.6 - 100.0) | 100.0 (91.2 - 100.0) | 100.0 (91.2 - 100.0) | 100.0 (91.2 - 100.0) | 100.0 (91.2 - 100.0) |
| IgM antibodies detection | | | | | |
| Overall sensitivity - % (IC95%) | 59.8 (50.8 - 68.4) | 63.8 (54.8 - 72.1) | 46.8 (37.9 - 55.9) | 79.5 (71.5 - 86.2) | 40.9 (32.3 - 50) |
| Sensitivity at 15 days | 70.6 (52.5 - 84.9) | 88.2 (72.6 - 96.7) | 67.6 (49.5 - 82.6) | 76.5 (58.8 - 89.3) | 47.1 (29.8 - 64.9) |
| Sensitivity post-infection at 30 days | 55.9 (45.2 - 66.2) | 54.8 (44.2 - 65.2) | 38.7 (28.8 - 49.4) | 38.7 (28.8 - 49.4) | 25.8 (17.3 - 35.9) |
| Overall specificity - % (IC95%) | 95.5 (77.2 - 99.9) | 85.0 (70.2 - 94.3) | 97.5 (86.8 - 99.9) | 92.5 (79.6 - 98.4) | 100.0 (91.2 - 100.0) |

TABLE 2 | Diagnostic performance of SARS-CoV-2 IgG ELISA tests.

| Performance parameter | Brand | |
|---------------------------------|----------------------|----------------------|
| | DiaPro™ | IDVet™ |
| Overall sensitivity - % (IC95%) | 82.7 (74.9 - 88.8) | 83.5 (75.8 - 89.5) |
| sensitivity at 15 days | 82.4 (65.5 - 93.23) | 88.2 (72.6 - 96.7) |
| sensitivity at 30 days | 83.9 (74.8 - 90.7) | 82.8 (73.6 - 89.8) |
| Overall specificity - % (IC95%) | 100.0 (91.2 - 100.0) | 100.0 (91.2 - 100.0) |

was a significant increase ($p < 0.05$) in sensitivity from 50% (32.4-67.6 IC 95%) at 15 days to 72.3% (62.2-81.8 IC95%) at 30 or more days after a RT-qPCR positive result.

For anti-SARS-CoV-2 IgM detection, the overall specificity of four of the five LFIA brands was over 92.5%, while this value for “Zybio” brand was 85.5% (70.2-94.3 IC95%), although those differences were not statistically significant. The sensitivity for positive samples collected 15 days after the RT-qPCR test ranged from 47.1% (29.8-64.9 IC95%) to 88.2% (72.6-96.7 IC95%). Furthermore, there was a statistically significant ($p < 0.05$) reduction in the sensitivity values for the five LFIA brands for samples collected 30 or more days after the RT-qPCR positive result.

Clinical Performance of ELISA Tests

The results of the evaluation of the different tests are detailed in **Table 2**. For anti-SARS-CoV-2 IgG detection, the overall sensitivity of the two brands was neither statistically significant between them nor compared to LFIAs. For both ELISA kits, there were no statistically significant differences of the sensitivity values among samples collected 15 days or 30 or more days after the RT-qPCR test. The overall sensitivity was 82.7% (74.9-88.8 IC 95%) and 83.5% (75.8-89.5 IC 95%) for “DiaPro” and “IDVet” brands, respectively. Moreover, both ELISA kits had a specificity of 100%.

Figure 1 includes the ROC curves for the five LFIAs and two ELISA kits tested for IgG detection, showing that the ELISA kits had a slightly higher sensitivity, although it was not found to be statistically significant.

DISCUSSION

To our knowledge, this is the first report addressing the clinical performance of serological tests for COVID-19 diagnosis commercially available in Ecuador and other South American countries like Colombia and Peru. Although there are some reviews already published on the subject (Cota et al., 2020; Deeks et al., 2020), there is only one similar study carried out in South America, specifically in Brazil, including a different set of serological test brands (Lisboa Bastos et al., 2020). Local accuracy data based on real scenarios are essential given the marked regional differences reported for the performance of the tests. This issue is specially relevant in tropical regions and/or middle- and low-income countries where a higher prevalence of certain infectious diseases is expected, potentially compromising the specificity of the serological tests (Cota et al., 2020; Faccini-Martínez et al., 2020;

Echeverría et al., 2021; Tso et al., 2021). For instance, lack of specificity due to cross reactivity with Zika and Dengue positive sera samples have been described, ranging from 2% to 26% for IgG and IgM depending on the commercial brands (Cota et al., 2020; Faccini-Martínez et al., 2020). Additionally, local clinical evaluations are also required for COVID-19 related tests in South America, since several RT-qPCR kits and serological tests either did not receive or had their clinical use authorization revoked at their countries of production (Cota et al., 2020; Freire-Paspuel and Garcia-Bereguain, 2021a; Freire-Paspuel and Garcia-Bereguain, 2021b).

In our study, we did not report a lack of specificity for the seven serological tests analyzed for anti-SARS-CoV-2 IgG detection. However, for anti-SARS-CoV-2 IgM detection, the specificity was lower than 100% for four of the five LFIA brands evaluated. Moreover, only one of the brands evaluated maintained a 100% specificity for IgM detection, although in that case the reduction in sensitivity for IgM detection was over 50%. On the other hand, although we did not find statistically

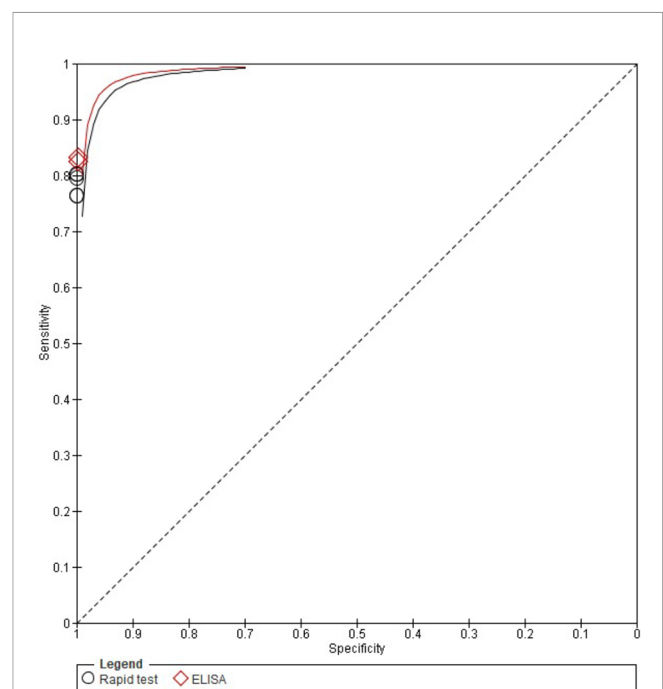


FIGURE 1 | ROC curves for anti-SARS-CoV-2 IgG detection of the seven commercial COVID-19 serology test available included in this study. ELISA kits are shown in red. LFIAs are shown in black.

significant differences among ELISA and LFIAs kits for anti-SARS-CoV-2 IgG sensitivity, the values obtained (ranging from 76.4% to 83.5%) were clearly below the high sensitivity values (over 90%) reported by manufacturers. However, the sensitivity values for the serological tests included in this study, are higher than the values reported for some serological kits used in Brazil (Cota et al., 2020). The sensitivity values were lower for anti-SARS-CoV-2 IgM detection, with only two LFIAs presenting a sensitivity $\geq 75\%$ even for samples collected 15 days after a positive RT-qPCR test. However, these brands were found to have a strong reduction of specificity, with values of 92.5 and 85%, respectively.

Overall, the clinical performance of ELISA kits and LFIAs was quite similar, with a slight increase in sensitivity for anti-SARS-CoV-2 IgG detection by ELISA. So far, regarding the choice between ELISA kits or LFIAs, logistical issues and cost evaluation should be considered. For instance, although this study did not evaluate the direct point-of-care use with finger peripheral blood for LFIAs, this is something recommended by the manufacturers. If the sensitivity of LFIAs is maintained for this alternative type of use, their cost-effectiveness would definitely compensate their lower sensitivity compared to the ELISA kits.

Regarding the potential use of these serological tests in the current scenario of availability of highly specific and cheap SARS-CoV-2 antigen test (Cubas-Atienzar et al., 2021), our results clearly endorse the inadequacy of the use of anti-SARS-CoV-2 IgM antibodies as markers of active SARS-CoV-2 infection, as it has also been suggested by other reports (Cota et al., 2020). On the other hand, the high specificity and the acceptable sensitivity values obtained for anti-SARS-CoV-2 IgG, considering that antibodies release is not the only immune response to COVID-19 infection (GeurtsvanKessel et al., 2020; Turner et al., 2021), suggest that the serological COVID-19 tests included in our study can be useful tools for seroprevalence studies. Estimating the percentage of the population that has already been infected in the community is essential for understanding the spread of the pandemic, and will also assist vaccination program decisions in middle- and low-income countries (Santander-Gordon et al., 2021).

In conclusion, our results reveal no significant differences in terms of sensitivity and specificity for anti-SARS-CoV-2 IgG detection among ELISA kits and LFIAs. The overall clinical performance obtained for the seven serological tests included in the study was worse than promised by manufacturers. However, with an overall specificity of 100% and sensitivity values over 75% for anti-SARS-CoV-2 IgG detection, these tests are an affordable and useful tool for seroprevalence studies in the context of middle- and low-income countries like Ecuador.

UDLA COVID-19 TEAM

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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.

ETHICS STATEMENT

This study was approved by the IRB from the Dirección Nacional de Inteligencia de la Salud (Ministerio de Salud Pública, Ecuador) under the code 008-2020. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IR-O and MG-B wrote the manuscript. All the authors have contributed to the experimental design, data collection and analysis, and also reviewing the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SARS-CoV-2 Diagnostics Based on Nucleic Acids Amplification: From Fundamental Concepts to Applications and Beyond

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COVID-19 pandemic ignited the development of countless molecular methods for the diagnosis of SARS-CoV-2 based either on nucleic acid, or protein analysis, with the first establishing as the most used for routine diagnosis. The methods trusted for day to day analysis of nucleic acids rely on amplification, in order to enable specific SARS-CoV-2 RNA detection. This review aims to compile the state-of-the-art in the field of nucleic acid amplification tests (NAATs) used for SARS-CoV-2 detection, either at the clinic level, or at the Point-Of-Care (POC), thus focusing on isothermal and non-isothermal amplification-based diagnostics, while looking carefully at the concerning virology aspects, steps and instruments a test can involve. Following a theme contextualization in introduction, topics about fundamental knowledge on underlying virology aspects, collection and processing of clinical samples pave the way for a detailed assessment of the amplification and detection technologies. In order to address such themes, nucleic acid amplification methods, the different types of molecular reactions used for DNA detection, as well as the instruments requested for executing such routes of analysis are discussed in the subsequent sections. The benchmark of paradigmatic commercial tests further contributes toward discussion, building on technical aspects addressed in the previous sections and other additional information supplied in that part. The last lines are reserved for looking ahead to the future of NAATs and its importance in tackling this pandemic and other identical upcoming challenges.

Keywords: SARS-CoV-2, PCR, diagnostics, isothermal amplification, molecular detection, viral sample processing, NAATs, POCTs

INTRODUCTION

SARS-CoV-2 is classified as part of the family *Coronaviridae* and the genus *Betacoronavirus*, which includes two other well-known human pathogens, SARS-CoV and MERS-CoV; moreover it belongs to the subgenus *Sarbecovirus* together with SARS-CoV (Gorbalenya et al., 2020). The disease directly provoked by SARS-CoV-2 would become known as COVID-19 and was rapidly confirmed

to be originated from a new strain of severe acute respiratory syndrome-related coronavirus (Gorbalenya et al., 2020). Following more than two years since the beginning of the pandemic, the combat against the virus is mainly supported by widespread testing and mass vaccination (To et al., 2021). SARS-CoV-2 has been subject to mutational events that reinforced the progression of the virus, contributing for increased transmissibility and high morbidity (Becerra-Flores and Cardozo, 2020; Challen et al., 2021). Furthermore, the growing number of vaccinated people can lead to a precocious relaxation in the adoption of preventive measures, like social distancing and frequent sanitation of people and spaces (To et al., 2021). For all these reasons, testing and efficient isolation of suspected and confirmed cases continues to be of paramount importance for tackling the disease.

The diagnostics industry has reached a certain level of maturity that is reflected on the wide range of testing options available, following the continuous development of new solutions since the first days of the health crisis. This effort has been strongly supported by academy and industry, as well as by assessment and certification institutions, which have accelerated the marketing of new *in vitro* diagnostics (IVDs), only turned possible due to exceptional solutions like the dispatch of Emergency Use Authorizations (EUAs), famously provided by Food and Drug Administration (FDA) (Mitchell et al., 2020), in USA. The landscape of SARS-CoV-2 detection comprises molecular analysis tools directed for protein detection like antigen tests (Dinnes et al., 2020) and mass spectrometry-based techniques (Cardozo et al., 2020; Deulofeu et al., 2021), or nucleic acid tests (NATs). NATs can be further divided in nucleic acid amplification tests (NAATs) and other non-NAAT approaches involving the detection of nucleic acids (Figure 1). The main application of non-NAAT methods has been focused on sequencing (Harilal et al., 2020; Wang M. et al., 2020; Lu et al., 2020b); moreover, innovative assays for immediate detection of RNA, without an amplification step have also been reported (Moitra et al., 2020; Farzin et al., 2021; Fozouni et al., 2021). While protein antigen tests have an important role in point-of-care testing (Dinnes et al., 2020), the routine diagnosis for the purpose of virus detection is mostly assured by NAATs. The main route for NAAT-based SARS-CoV-2 diagnosis continues to be reverse transcription-real time-polymerase chain reaction (RT-qPCR), which is performed in well-equipped and more and more automated clinical settings (Barra et al., 2020; Nörz et al., 2020). RT-qPCR joins very high sensitivity, good specificity and was successfully adapted to the screening of large numbers of samples, what contributed for the implementation of the technique as the gold-standard for SARS-CoV-2 detection and COVID-19 management (Nörz et al., 2020). Isothermal nucleic acid amplification has been intensively explored for point-of-care tests (POCTs) (Bektaş et al., 2021), although being also used in clinical settings, but continues to be less appealing than RT-qPCR (Silva et al., 2021).

This work aims to describe the landscape of NAATs, addressing in the first place associated SARS-CoV-2 virology aspects, such as the rise of concerning mutations and its eventual

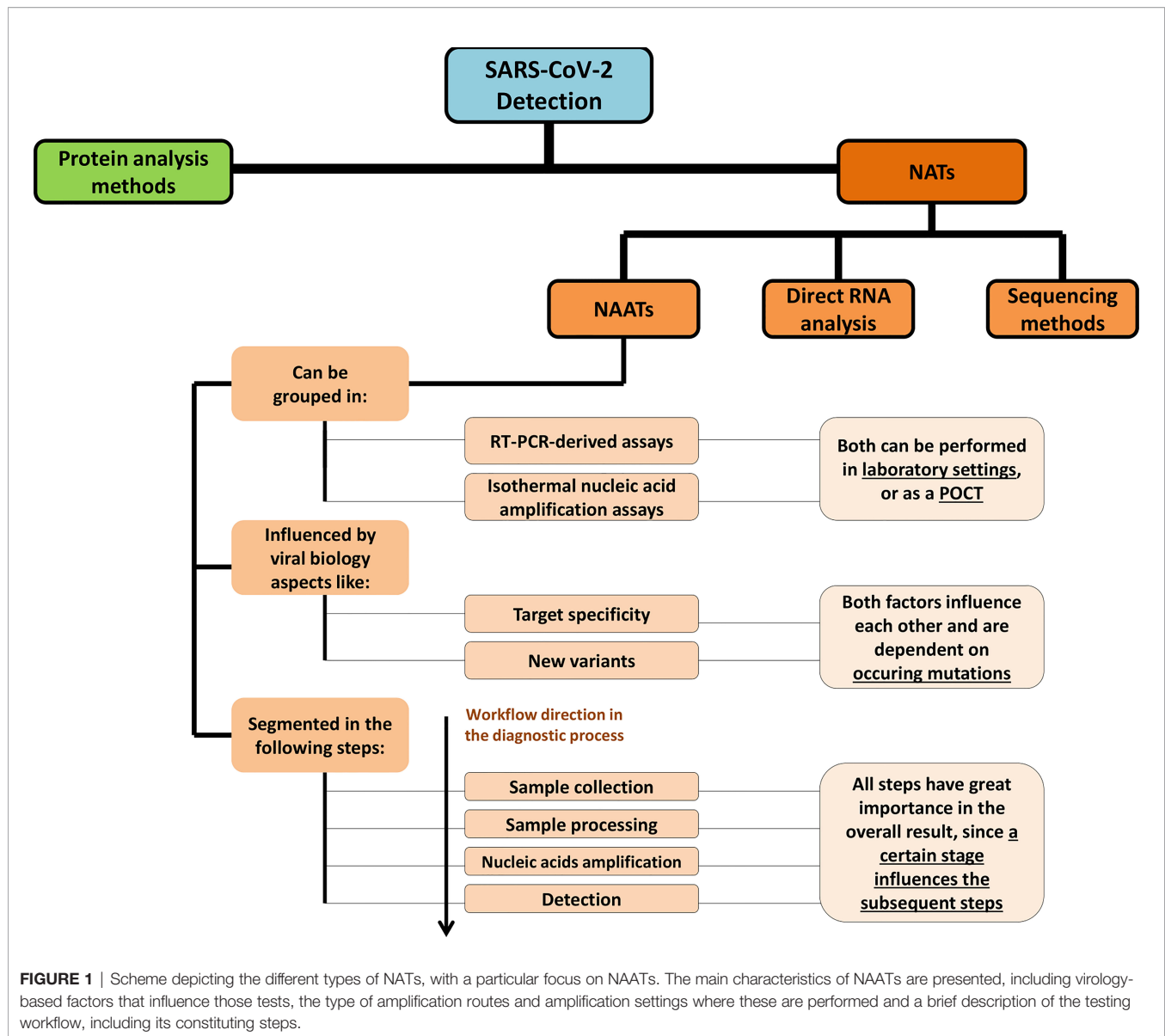
downstream impact in testing systems; followed by sample collection, including the type of clinical specimens, collection routes, adaptation of high-throughput testing and storage techniques; processing of collected samples, including virus inactivation and RNA extraction procedures; nucleic acid amplification strategies, comprising a detailed description of both PCR-derived and isothermal amplification-based methods, as well as frequently used controls; molecular detection chemistry, which will be separated in those reactions commonly associated with PCR-derived amplification techniques and those linked with isothermal amplification; or platforms for detection, ranging from those traditionally linked with the type of diagnostics used in clinical settings to those integrated in POCTs. The sequence of main topics finally culminates in a benchmark of up-to-date commercial assays. This late stage of the article requests all the knowledge displayed in the former sections for tracing a scenario of the current NAAT-based testing options offered by companies producing IVDs that aim at SARS-CoV-2 detection. The article concludes with a critical commentary on the prospects of NAATs in the near future, focusing on its frailties, necessary improvements and the influence of emerging technologies and new research fields in enhancing the ability of these types of tests to tackle this and coming pandemics.

RELEVANT VIROLOGY ASPECTS

Genome and Virions

The genome of SARS-CoV-2 constitutes a long, single and positive sense RNA molecule comprising approximately 30Kb that contains six functional open-reading frames (ORFs) (Kim et al., 2020) (Figure 2A). The extremities of the genome are covered with a 5' cap and a 3' poly (A) tail (Miao et al., 2021). ORFs vary considerably in size, with ORF1ab spanning around two thirds of the 5' region of the genome. The path that leads to the production of non-structural proteins (NSPs), from ORF1ab, begins with the entrance of the positive sense RNA molecule in the cell (Kim et al., 2020). There, positive sense RNA undergoes replication that begins with the formation of a negative sense RNA and proceeds with the amplification of positive sense genomic RNA departing from this template (Alexandersen et al., 2020). This newly formed positive sense RNA can be used in the translation of new NSPs, or packed in new virions. ORF1ab is translated in the two large polypeptides 1a and 1ab respectively, which are then cleaved by viral proteases NSP3 (Kim et al., 2020) and NSP5 (Kim et al., 2020) and originate 16 NSPs in total, with ORF1a originating 11 and ORF1ab leading to 16 (Davidson et al., 2020; Clark et al., 2021). NSP7 and NSP8 in conjunction with NSP12 form the RNA-dependent RNA polymerase (RdRp), a complex of proteins also designated as replicase (Hillen et al., 2020).

The structural proteins of SARS-CoV-2 assemble in virions (Figure 2B), in which spike proteins (S) form the characteristic signature in form of halo that is responsible for the prefix "corona" of *Coronaviridae* family (Wertheim et al., 2013).



These proteins stem directly from the lipid membrane, exhibiting two subunits and having the adequate configuration for binding with the cell-surface receptor angiotensin-converting enzyme 2 (ACE2), mediating virus entry (Walls et al., 2020; Troyano-Hernández et al., 2021). Occupying a transmembrane position, the dimeric membrane proteins (M) are the most abundant in the virion and contribute for the maintenance of its shape and support, having a major role in the assembling mechanism, contributing as well for the budding process (Mahtarin et al., 2020; Troyano-Hernández et al., 2021). Embodied in the membrane, the envelope proteins (E) are the less abundant in the virion and also execute functions related with virion assembling and budding process, in addition to envelope formation (Hassan et al., 2020; Troyano-Hernández et al., 2021). E protein can form monomeric or pentameric arrangements (De Maio et al., 2020). While forming the pentameric construct it creates an ion channel, classified as a

viporin (Cao et al., 2020; De Maio et al., 2020). At last and deeper into the virion, N protein binds to viral RNA genome and organizes it in a helical nucleocapsid structure, the ribonucleoprotein (RNP) complex, interacting also with the M protein in the process of viral assembling and exerting important roles in virus replication and transcription (Troyano-Hernández et al., 2021; Yang et al., 2021). When it comes to accessory proteins, these molecules execute tasks mainly related to the process of infection, in interaction or within host cells (Mariano et al., 2020; Ren et al., 2020; Flower et al., 2021).

Viral Variants

The evolution of viruses is driven by the occurrence of changes in the sequence of viral nucleic acids, designated as mutations (Mascola et al., 2021; Nguyen et al., 2021). When a mutation, or group of mutations occurs, it can result in the rise of a variant,

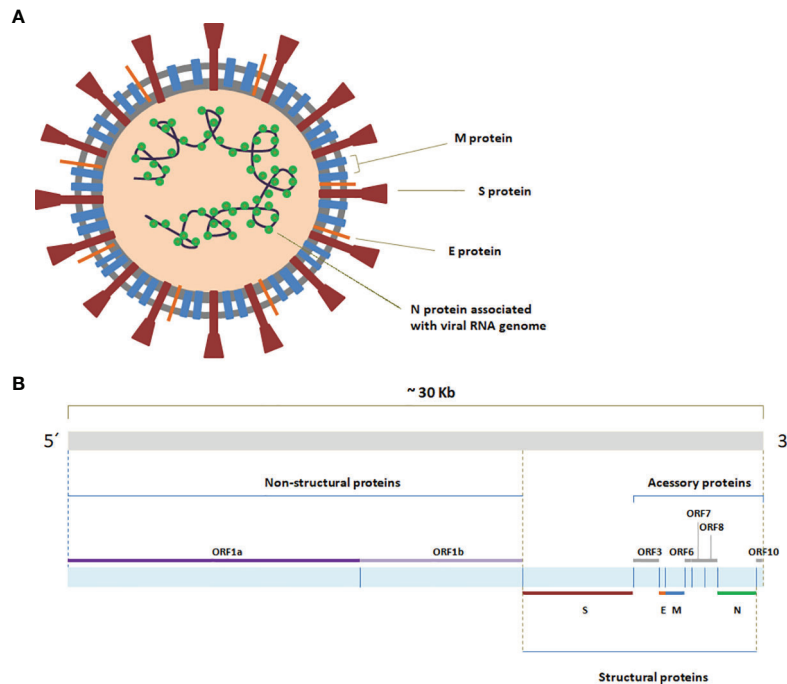


FIGURE 2 | (A) Genome organization of SARS-CoV-2. The ORFs constituting the SARS-CoV-2 RNA genome (from 5' to 3') encode the non-structural proteins (NSPs), which originate from ORF1a and ORF1b (ORF1ab), the spike (S), envelope (E), membrane (M) and nucleocapsid (N), as well as more than a handful of other dispersed and not fully characterized accessory proteins (Kim et al., 2020; Hu et al., 2021). The ORFs that lead to accessory proteins include mainly ORF3, ORF6, ORF7, ORF8 and ORF10 (Michel et al., 2020; Giri et al., 2021). **(B)** Schematic representation of SARS-CoV-2 virion.

following a selection process, over several cycles of replication (Mascola et al., 2021). Generally, those mutations that confer advantage for virus survival lead to its incorporation in the population, through the spread of the new variant in the circulating viruses (Lauring and Hodcroft, 2021). The majority of the SARS-CoV-2 ORFs belonging to circulating strains registered some mutation (Nguyen et al., 2021). ORF1ab (Velazquez-Salinas et al., 2020), and S (Borges et al., 2020; Korber et al., 2020), E, M, N, ORF3a (Issa et al., 2020; Velazquez-Salinas et al., 2020), ORF6, ORF7a, ORF7b, ORF8 (Velazquez-Salinas et al., 2020) and ORF10 sequences have all been linked with mutation occurrences (Nguyen et al., 2021). As of February 2022 there is a myriad of important mutations and derived variants that demand strict surveillance (WHO, 2021). In order to categorize relevant variants, different institutions use distinct terms, according with the level of potential danger. WHO defines Variants of Interest (VOIs), like Lambda and Mu, or Variants of Concern (VOCs), which demand more attention, like Alpha, Beta, Gamma, Delta and Omicron (WHO, 2021). ECDC adds an additional category to the ones adopted by WHO, namely Variants Under Monitoring (VUMs) (ECDC, 2022), which are designated alternatively as Variants Being Monitored (VBM) by the CDC (Centers for Disease Control and Prevention, 2021).

There is an ongoing debate on the possibility of NAATs being significantly affected by these and other upcoming variants, posing challenges to the already existing diagnostics (Artesi

et al., 2020; Jain et al., 2021; Khan and Cheung, 2021; Ramírez et al., 2021; Singh et al., 2021). Early studies on the primers and probes used for RT-qPCR, revealed the existence of mutations that hampered the sensitivity of the reverse primer for RdRp described in Charité protocol and the forward primer targeting N gene in Chinese CDC test (Vogels et al., 2020). B.1.1.7 was already associated with impairment in the use of S gene as target in a commercial multi-target RT-qPCR kit by Thermo Fisher Scientific (Ramírez et al., 2021). Another study highlighted the recurrent identification of mutated genomes in the E gene region, when a RT-qPCR commercial kit produced by Roche and targeting this site was used (Artesi et al., 2020). In order to avoid these situations, conserved regions should be used whenever possible. The efficient tracking of such mutations can facilitate the elaboration of new tests, as well as its redesign and adaptation, by altering the sets of primers and probes (Jain et al., 2021), or even the necessary biosafety practices, in case the new variant becomes more dangerous to manipulate. In addition, efforts must be taken in developing more flexible platforms, recurring to multiple targets (Artesi et al., 2020; Ramírez et al., 2021). Contrarily to an entire negative effect, the failure in detecting a specific target may indicate the presence of a well-characterized variant, thus having an odd, but accessible tool for tracking its spread (ECDC, 2020b; Ramírez et al., 2021). Overall, the negative impact of already circulating variants has been contained with success, in NAATs. However, the fact that SARS-CoV-2 has a medium-to-high mutation rate demands

continued surveillance, in particular for tests targeting less conserved regions (Artesi et al., 2020; Wang R. et al., 2020; Jain et al., 2021).

SAMPLE COLLECTION

Clinical Specimens and Collection Routes

The range of specimens already used for performing the diagnosis of the virus by NAAT is diverse. It includes samples collected from upper respiratory tract (URT) (Zou et al., 2020), such as nasal (NS) (Calame et al., 2020; Zou et al., 2020), mid-turbinate (M-T) (Barat et al., 2021), nasopharyngeal (NP) (Wyllie et al., 2020), throat (TH) (Zou et al., 2020) and oropharyngeal (OP) (Peng L. et al., 2020) regions, as well as saliva (Wyllie et al., 2020); while others are retrieved from lower respiratory tract (LRT) (Yang et al., 2020), such as sputum (Yang et al., 2020), endotracheal fluid (EDF) (Bergrath et al., 2020), or the liquid resulting from a bronchoalveolar lavage (BAL) (Yang et al., 2020). The major international health institutions, like CDC and ECDC recommend the use of URT specimens like NP or OP swabs as a first choice, particularly in asymptomatic, mild or moderate disease cases (CDC, 2020a; ECDC, 2020a). LRT are often recommended in severe cases (ECDC, 2020a), or when negative results are verified in URT samples, despite high suspicion of infection (Yang et al., 2020). Saliva has been a favorite research topic, being the main target of countless works, yielding generally good results (Kapoor et al., 2021; Moreira et al., 2021) as well as wide acceptance for the use in commercial assays (Vogels et al., 2021).

In the months following the declaration of a pandemic, technical and human resource requirements to collect infection-related specimens soon became scarce (Yee et al., 2021). This showed that the traditional sample collection made by medical staff wasn't ideal in the current pandemic (Tu et al., 2020). Furthermore, there is an increased risk of infection by those performing the collection (Tu et al., 2020; Karthik et al., 2020) and a great part of the samples collected demand well trained personnel, which is unavailable during a pandemic, thus resulting in asymmetric, ill performed sample collections that negatively affect the results obtained (Kinloch et al., 2020). The non-invasive, self-collection of specimens has been accessed (Hall et al., 2020) and perfected (Fernández-González et al., 2021), being increasingly adopted to further avoid the aforementioned downsides of traditional sampling (Wehrhahn et al., 2020). Its benefits suit both POCTs (Hanson et al., 2020) and clinically-based tests (Williams et al., 2020; Yee et al., 2021). Saliva is the easiest specimen to be collected this way and despite becoming viscous and difficult to process, it contains acceptable viral loads (Matic et al., 2021). The NS (Hanson et al., 2020; Valentine-Graves et al., 2020) and TH (Therchilsen et al., 2020) samples have also been explored for the same purpose, but the reports on the overall performance of such samples are controversial, in particular when compared with saliva (Hanson et al., 2020).

The more common way of collecting a sample for analysis is by swabbing it with a sterile instrument (Marty et al., 2020), both

in traditional RT-qPCR and in a great part of POCT (Zasada et al., 2020). Aspiration is an alternative to swabs and a common way of collecting some LRT samples like endotracheal secretions (Malczynski et al., 2020). Nevertheless, it is prone to technical problems provoked by sample viscosity (Malczynski et al., 2020). Bronchoscopy techniques are also executed, for instance in the extraction of BAL, but demand highly skilled personnel and pose an increased threat of infection for those doing the collection (Ng et al., 2021). The non-invasive collection must be actively performed by the subject under test, while expelling the substance to be analyzed (sputum, saliva, urine, stool, etc.), sometimes after gargling with a saline solution (Goldfarb et al., 2021), to a sterile recipient. The transport and storage of samples bridge the collection and processing moments. In the exact moment after retrieving the sample, it is placed in an appropriate medium designated as viral transport medium (VTM) (Garnett et al., 2020; Rodino et al., 2020). The formulation of this medium can change according with the commercial supplier, but generally comprises a salt-based solution, with a buffer, a carbon source, serum and antibiotics/antifungals. CDC recommends the following recipe: Anderson's modified Hanks Balanced Salt Solution (8.0 g/L NaCl, 0.4 g/L KCl, 0.05 g/L Na₂HPO₄, 0.06 g/L KH₂PO₄, 1.0 g/L Glucose, 0.7 g/L NaHCO₃, 0.2 g/L MgSO₄·7H₂O, 0.14 g/L CaCl₂·2H₂O) with 2% v/v heat-inactivated fetal bovine serum, 100 µg/mL gentamicin and 0.5 µg/mL amphotericin B (McAuley et al., 2021). The high demand for this medium led to shortages during the first year of pandemic that rapidly took the laboratories to look for other media options (Garnett et al., 2020; Radbel et al., 2020) and the FDA to recommend these options (Rogers et al., 2020). The suitable alternatives screened included distinct buffers containing different salts and denaturing agents (Radel et al., 2020; Rodino et al., 2020). The samples should be refrigerated, being placed at 2°C to 8°C up to 72h, or stored at -70°C, for longer periods (Dzung et al., 2021). The sample can remain stored in VTM during long periods (Dzung et al., 2021), although ideally it should be tested as soon as possible without recurring to storage in the freezer (Dzung et al., 2021). Freezing and thawing of samples can be critical in preserving it and repeated freeze-thawing has significant impact on viral RNA levels (Dzung et al., 2021). Nonetheless SARS-CoV-2 RNA samples have been reported to be able to maintain sufficient integrity for RT-qPCR detection, regardless of the temperature (from -30°C (Rogers et al., 2020) to 35°C (Dzung et al., 2021)), during several weeks (Dzung et al., 2021), for distinct specimens (Rogers et al., 2020).

High-Throughput Testing

The adoption of high-throughput measures that facilitate the scale-up of RT-qPCR-based diagnostic systems, enabling to expand the number of tests performed, but simultaneously limiting the consumption of reagents has been of paramount interest (Eis-Hübinger et al., 2020; Salimnia et al., 2021). Classical sample pooling consists in mixing different individual samples, generating a pool. The pool is tested and in case of a positive result, the individual samples are retested, in order to

find what sample yielded the positive result (Gupta et al., 2020; Yelin et al., 2020). Nonetheless, groundbreaking works have tried to simplify the two-stage process (test and re-test) by developing a method able to detect the individual sample yielding the positive result in a certain pool (Shental et al., 2020; Täufer, 2020). The main downside of pooling is a significant decrease in the analytical sensitivity of RT-qPCR tests (Lüsebrink et al., 2020); the pooling protocols lead to dilution of samples, putting in risk the detection of viral RNA in those containing low viral loads (Lüsebrink et al., 2020). Furthermore, well performed specimen collections are also critical for the success of the strategy, since errors can contribute for further dilution of the sample. Saliva has been a favorite target of pooling approaches (Barat et al., 2021; Pasomsub et al., 2021) due to being easily obtained through self-sampling. NP (Torres et al., 2020) have also been extensively accessed. The approach has even been tested with other non-PCR NAATs, with relative success (Ludwig et al., 2021).

SAMPLE PROCESSING

Virus Inactivation

SARS-CoV-2 has been categorized as a hazard group 3 pathogen (Patterson et al., 2020; Welch et al., 2020; Burton et al., 2021), demanding the same biosafety precautions already adopted for handling SARS-CoV (Burton et al., 2021) and MERS-CoV (Burton et al., 2021). The infectious form of the virus is as a general rule manipulated in biosafety level 3 (BSL-3) facilities. An exception to these biosafety norms is the processing of specimens collected for the purpose of diagnosing the virus, either in laboratories, or sites devoted to POC testing (Welch et al., 2020; van Bockel et al., 2020; Burton et al., 2021). Since BSL-3 facilities are scarce, the preparation of samples for testing has sometimes been carried in biosafety level 2 (BSL-2) installations (Welch et al., 2020; Genoud et al., 2021), or even in lower biosafety conditions in the case of POC testing (van Bockel et al., 2020; Welch et al., 2020). The inactivation of coronaviruses can be executed with efficiency by different routes, including physical and chemical approaches (Case et al., 2020; Auerswald et al., 2021) (**Table 1**). Nevertheless,

the virucidal agents may negatively impact the biomolecules constituting the virions, hampering the performance of certain diagnostic methods (Loveday et al., 2021). In order to avoid the degradation of viral RNA, preservative solutions must be found.

The use of heat has been seek due to being an easy (Smyrlaki et al., 2020), minimally harmful (Loveday et al., 2021), environmentally safe (Thompson et al., 2021) and low price (Calvez et al., 2020; Smyrlaki et al., 2020; Thompson et al., 2021) solution for sample inactivation. A vast range of temperatures were tested in distinct works, in the interval between 56°C (Auerswald et al., 2021) and 100°C (Jureka et al., 2020), including several intermediate values (Burton et al., 2021; Loveday et al., 2021; Pryce et al., 2021; Thompson et al., 2021), during periods ranging from 1 (Burton et al., 2021) to 90 (Burton et al., 2021) minutes. Heat treatment consistently proved to be efficient in the neutralization of 100% of the virions, when the temperatures used were equal or above 80°C (Burton et al., 2021; Batéjat et al., 2021); while 80°C of temperature required one hour (Patterson et al., 2020) or more (Burton et al., 2021) for inactivating 100% of the infectious particles, 95°C were able to inactivate the sample in only one minute (Burton et al., 2021). The temperatures between 56°C and 80°C also demonstrated some degree of virucidal activity (Burton et al., 2021), since the reduction in the number of infectious particles was sufficient to meet the requirements for considering a factor as a virucidal agent (Pastorino et al., 2020b) [$\geq 4 \text{ Log}_{10} \text{ TCID}_{50}$, according with the European norm NF EN 14476-A2 (Pastorino et al., 2020b)]. The unit used in the context of RT-qPCR for expressing the load of viral RNA is defined as quantification cycle (Cq). Nonetheless, these Cq value is strongly affected by the inactivation conditions of time and temperatures applied to the clinical specimens. Incubation above 90°C during 5 (Burton et al., 2021) or 15 (Pastorino et al., 2020b) minutes was less impacting in the increase of Cq ($\Delta \text{Cq} > 5$) than 60 minutes or more at 80°C ($\Delta \text{Cq} > 9$) (Burton et al., 2021), considering the same viral load. The aforementioned observations suggest that short periods at higher temperatures are preferable.

Chemical methods constitute the other major alternative for inactivating viral samples before NAATs (Richard-Greenblatt et al., 2021). Non-ionic detergents, (Welch et al., 2020; Auerswald et al., 2021), in addition to chaotropic guanidine

TABLE 1 | Comparison of methods used for complete inactivation of SARS-CoV-2 in clinical samples.

| | Type | Stage | | Protocol/Reagent used | Reference |
|--------------------|-------------------------|---------------------------------------------------------------------------------------|----------------------------------------|---------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| Virus inactivation | Heat treatment | Before direct analysis, or before RNA extraction | Lower temperature, longer duration | Temperature above 80°C, during at least 1 hour; | (Burton et al., 2021) |
| | | | Higher temperature, brief duration | Temperatures above 90°C can inactivate samples in a few minutes; | (Burton et al., 2021) |
| | Chemical methods | Transport | VTM | Primestore MTM, 4M GITC/Tx TM, COPAN eNAT; | (Welch et al., 2020; Richard-Greenblatt et al., 2021) |
| | | Before analysis without full RNA extraction, or in the process of full RNA extraction | Non-anionic detergents Lysis buffer | Triton X-100; ATL, VXL, AVL, Phanter fusion specimen lysis tubes, MagNA Pure External LB, RLT, E&O Lab LB; | (Welch et al., 2020) |
| | | Before direct analysis, | Extraction reagents Other | Trizol, Trizol LS; TCEP+ EDTA + Heat. | (Pastorino et al., 2020b; Welch et al., 2020) (Patterson et al., 2020) (Rabe and Cepko, 2020) |

salts like guanidine thiocyanate (Welch et al., 2020; Auerswald et al., 2021) and guanidine hydrochloride (Welch et al., 2020) are examples of reagents commonly used as chemical inactivating agents. The referred chemicals can be used for inactivating samples at different stages of the workflow, either in transport (Welch et al., 2020; Richard-Greenblatt et al., 2021), or during the RNA extraction process (Patterson et al., 2020; Auerswald et al., 2021), since these can be incorporated in the formulation of VTM (Welch et al., 2020; Richard-Greenblatt et al., 2021), or in other reagents (Patterson et al., 2020; Welch et al., 2020) used to treat the samples. There are at least two commercial VTM, which already proved to completely neutralize the virus. Furthermore, the range of VTM that revealed to have $\geq 4 \text{ Log}_{10} \text{ TCID}_{50}$, in spite of not being completely inactivating is vast (Welch et al., 2020). In what concerns the inactivation of viral particles in the process of RNA extraction, detergents (Welch et al., 2020), lysis buffers (Welch et al., 2020) and other extraction reagents have been used (Patterson et al., 2020; Welch et al., 2020; Auerswald et al., 2021). While the systematic use of chemical methods as an inactivation strategy before NAATs can be hampered by supply chain disruption (Auerswald et al., 2021), an increasing number of works have proved the utility of such approach, either on its own (Patterson et al., 2020; Welch et al., 2020; Auerswald et al., 2021), or conjugated with high temperatures (Arizti-Sanz et al., 2020; Rabe and Cepko, 2020).

Viral RNA Extraction

The extraction of viral RNA comprised in clinical specimens is performed to obtain sufficient target RNA available for reverse transcription and cDNA amplification, aiming to improve the sensitivity of the diagnosis (Wozniak et al., 2020), besides avoiding inhibitors of amplification that can be present in transport media (Klein et al., 2020; Graham et al., 2021). It is usually initiated by submitting the samples to detergent treatment, which promotes the disintegration and solubilization of the viral lipid envelope, in addition to the use of chaotropic agents, like guanidinium salts, or non-specific proteases like proteinase K, which provoke denaturation of RNases (Klein et al., 2020; Genoud et al., 2021; Graham et al., 2021). Following the disruption of virions, RNA is separated and purified from the reagents and products resulting on the disintegration of viral particles (Klein et al., 2020). The methods typically used to obtain this separation include liquid phase extraction using organic-aqueous emulsions (Graham et al., 2021) and solid-phase purification using columns with glass fiber, silica (Klein et al., 2020; Graham et al., 2021) and even magnetic beads (Bektaş et al., 2021; Graham et al., 2021). These approaches result in the concentration of viral RNA (Graham et al., 2021). The commercial solutions for performing full viral RNA extraction include sophisticated and automated instruments (Dimke et al., 2021; Genoud et al., 2021), or more simple, labor-intensive and ready-to-use extraction kits (Wozniak et al., 2020; Ambrosi et al., 2021; Dimke et al., 2021) based on extractions with organic solvents, as well as solid-phase purification (Klein et al., 2020; Graham et al., 2021) (Table 2). The assessment of these instruments and kits already proved in

numerous studies that a full extraction process plays a significant role in maximizing the recovery of SARS-CoV-2 RNA, increasing the sensitivity of the diagnostic process (Israeli et al., 2020; Lübke et al., 2020). The automated systems usually lead to more standardized assays and originate faster results than handmade extractions (Ulloa et al., 2020; Lázaro-Perona et al., 2021). In general, all specimens can be analyzed through the mentioned methods, without much differences in processing. Sputum and other highly viscous samples can be subject to pre-extraction treatment with sputasol (dithiothreitol) (Peng J. et al., 2020), proteinase K (Peng J. et al., 2020), or acetyl-L-cysteine (Peng J. et al., 2020).

While RNA extraction is relevant, it is not indispensable and constitutes a time-consuming step. The great urgency to optimize the duration of diagnostic tests has given rise to an increasing number of papers concerned with extraction and purification-free approaches in NAATs for SARS-CoV-2 detection (Fomsgaard and Rosenstjerne, 2020; Panpradist et al., 2021) (Table 2); a tendency closely accompanied by commercial approaches (Bordi et al., 2020; Eckel et al., 2020). The protocols without full extraction methods can directly proceed to RT and cDNA amplification stages (Bruce et al., 2020; Israeli et al., 2020; Smyrlaki et al., 2020), or according with literature can simply include the submission of the collected specimens to a lysis buffer containing non-anionic detergents (Israeli et al., 2020; Smyrlaki et al., 2020; Panpradist et al., 2021), an alkaline polyethylene glycol (APG) solution (Chomczynski et al., 2021), isopropanol (Graham et al., 2021), proteinase K digestion (Genoud et al., 2021; Graham et al., 2021), or heat treatment (Fomsgaard and Rosenstjerne, 2020; Smyrlaki et al., 2020). Detergents that were already assessed for this purpose include Triton-X 100 and Tween-20, added to specimens in percentages of 0.5% (Panpradist et al., 2021), or 5% (v/v) (Smyrlaki et al., 2020) in the first case and 10% (v/v) (Smyrlaki et al., 2020) in the second. The APG solution screened contains 65% of polyethylene (v/v) and a pH value between 12.2-12.8, being added to samples in a proportion of 1:2 (Chomczynski et al., 2021). Proteinase K was added to virus-containing samples in a range of concentrations ranging from 0.1mg/mL to 1mg/mL (Genoud et al., 2021; Graham et al., 2021). Sputasol is also used in approaches non-contemplating a full extraction protocol as a pre-processing (Lübke et al., 2020) (before heat treatment, lysis, etc.), or even pre-amplification (Wee et al., 2020) reagent. As expected, literature demonstrates that non-extracted, directly analyzed specimens can render insufficient RNA loads, in particular for asymptomatic or mildly symptomatic individuals (Eckel et al., 2020; Israeli et al., 2020). The sole use of lysis buffers containing Triton-X 100 (Smyrlaki et al., 2020; Panpradist et al., 2021), or other solutions like APG (Chomczynski et al., 2021) and isopropanol (Graham et al., 2021) without any subsequent purification stage appears to improve direct analysis of samples. In a similar way to lysis buffers, proteinase K (Chu et al., 2020; Genoud et al., 2021; Graham et al., 2021) and heat treatment above 95°C during 5 minutes are good solutions for situations in which supply chain shortages are verified and extraction kits aren't available (Fomsgaard and Rosenstjerne,

TABLE 2 | Comparison of distinct strategies for extracting viral RNA from clinical specimens.

| | Type | Operation | Stage | Method used | | Reagents and equipments | Reference |
|-----------------------|----------------|-----------|--------------------|-----------------------------------------------------------------|------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------|
| RNA Extraction | Full | Automated | Lysis | Buffer containing detergents, caotropic agents, or proteinase K | | MagNA Pure External Lysis Buffer (for use with MagNA pure system, Roche), easyMAG Lysis Buffer (for use with EMAG® and NUCLEISENS®EasyMAG® system, Biomérieux) | (Hindiye et al., 2019) |
| | | | Purification | Solid phase purification, with columns, or moving beads | | easyMAG Magnetic silica (for use with NUCLEISENS®EasyMAG® system, Biomérieux), Viral NA Small Volume kit (used with MagNA Pure 96 DNA), QIAGEN EZ1 Kits (used with EZ1 Advanced XL, Qiagen) | (Hindiye et al., 2019; Ransom et al., 2020) |
| | | Manual | Lysis | Buffer containing detergents, caotropic agents, or proteinase K | | AVL, VXL, ATL,RLT (Qiagen) | (Pastorino et al., 2020a; Welch et al., 2020) |
| | | | Purification | Liquid phase extraction, with organic-aqueous emulsions | | Trizol, Trizol LS, or TRI Reagent (Thermo fisher), EXTRAzol (Blirt) | (Wozniak et al., 2020; Ambrosi et al., 2021; Dimke et al., 2021) |
| | | | | Solid phase purification, with columns, or moving beads | Silica beads Glass fiber filter (Silica) magnetic beads | QIA amp Viral RNA Mini Kit (Qiagen) High Pure Viral RNA Kit (Roche) MagMAX Viral RNA Isolation Kit (Thermo fisher) | (Klein et al., 2020) (Wozniak et al., 2020) (Klein et al., 2020) |
| | | | | | | | |
| | Partial | Manual | Lysis | Non-anionic detergents | Triton X-100 | Common, available through a wide range of suppliers | (Smyrlaki et al., 2020) |
| | | | | | Tween 20 | Common, available through a wide range of suppliers | (Smyrlaki et al., 2020) |
| | | | | | APG solution | Common, available through a wide range of suppliers | (Chomczynski et al., 2021) |
| | | | RNase inactivation | Proteinase K | | Common, available through a wide range of suppliers | (Genoud et al., 2021) |
| | | | Purification | Isopropanol | | Common, available through a wide range of suppliers | (Graham et al., 2021) |
| | | | Other | Heat | | – | (Genoud et al., 2021) |

2020; Beltrán-Pavez et al., 2021); these two methods proved more efficient if used in parallel (Genoud et al., 2021). The specimens that constitute the main targets of non-extraction strategies are NP (Cameron et al., 2021; Chomczynski et al., 2021) and saliva (Chomczynski et al., 2021; Lalli et al., 2021), but OP (Merindol et al., 2020), NS (Panpradist et al., 2021) and TH (Fomsgaard and Rosenstjerne, 2020) samples, as well as BAL (Lübke et al., 2020) were also assessed with favorable outcomes.

NUCLEIC ACIDS AMPLIFICATION

RT-PCR and Derivates

PCR is a simple and elegant reaction based on the action of a DNA polymerase enzyme and a pair of primers, driven by thermal cycles that sequentially provoke the separation of double helix strands, annealing of primers and its extension, forming new double strands, thus yielding exponential DNA amplification (Green and Sambrook, 2019). The standardization of this process, perfected since the 80's assured its establishment as the dominant method for DNA amplification (Green and Sambrook, 2019). PCR, initially an end-point, non-quantitative analytic technique turned a real-time tracking tool, with the

addition of a fluorescent marker, producing fluorescence proportionally to the number of DNA molecules generated (Green and Sambrook, 2019). This change created a merging between amplification and detection steps, enabling to visualize the profiles of amplification associated with a certain product of amplification, or amplicon, turning the process quantitative (qPCR), faster and even more resistant to nonspecific amplification (Green and Sambrook, 2019). In the context of SARS-CoV-2, RT-qPCR, which results from the inclusion of a reverse transcriptase (RT) in the reaction, in order to convert viral RNA in cDNA has been the first option for performing the diagnosis, mainly due to its increased sensitivity and specificity (Bustin et al., 2021). These advantageous characteristics of the RT-qPCR-related approaches had already been proved in the context of the diagnosis of other RNA virus (Bustin et al., 2021). It was easily adapted due to the robustness of the underlying amplification technique and its popularity, being already widely known in the medical and life science laboratories (Bustin et al., 2021).

RT-qPCR is a technology ready for quantitative analysis, but its quantitative potential has often been misused, or neglected in the context of SARS-CoV-2 diagnostics (Bustin et al., 2021; Han et al., 2021). While a yes or no response about the existence of infection has been found enough as technical feedback resulting

from routine diagnosis (all EUA-approved assays for SARS-CoV-2 detection are described as qualitative (Cheema and Blumberg, 2021)), the interpretation of results relies on the Cq values, which are an ambiguous quantification route (Bustin et al., 2021; Han et al., 2021). The performance of a RT-qPCR for objective quantification purposes demands the construction of a calibration curve, with quantified standards, including Cq values in function of known concentrations, thus enabling the determination of the viral concentration in a certain sample (Bustin et al., 2021; Han et al., 2021). When Cq values are taken in consideration without establishing a relation with concentration values displayed by standards, diagnosis isn't straightly quantitative, since the values of viral load are not harmonized (Bustin et al., 2021). Corman, from Charité developed the first protocol in mid-January 2020 following the divulgation of the viral genome sequence (Corman et al., 2020), prompting other WHO referral laboratories to do the same (Etievant et al., 2020). CDC (CDC, 2020b), CCDC (Etievant et al., 2020), HKU (Etievant et al., 2020) and Pasteur institute (Etievant et al., 2020) also developed important protocols, which together with Charité protocol orientated the implementation of a great part of the tests conducted in multiple laboratories around the world, as well as many commercial kits (Etievant et al., 2020). While RT-qPCR was implemented worldwide for SARS-CoV-2 diagnosis using roughly the same method, there is some variation in the basic reagents and guiding protocols used in the amplification reactions by different routine laboratories, including the primers, probes, RT, reaction enhancers and controls beside specific reaction features, such as single and multiple targeting (that is called multiplex, when several sites are amplified at the same time) (Bustin et al., 2021), or the use of nested amplification (Wang J. et al., 2020) (Table 3). There were also reports of the use of other techniques that somehow are based on PCR, like digital RT-PCR (Deiana et al., 2020) and qSTAR technology (In, Diagnostic and Only, 2021) (Table 3).

Nested RT-PCR (N-RT-PCR), is one of the offshoots of traditional RT-PCR used for SARS-CoV-2 diagnostic (Wang J. et al., 2020; La Rosa et al., 2021). It differs from RT-PCR in the fact that there are two runs of PCR, using two independent sets of primers (Yip et al., 2020). The two sets of primers are arranged in such a way that the outer set of primers amplifies a first fragment and the inner set of primers amplifies a second amplicon within the product of the first reaction (Yip et al., 2020). According with different works this strategy can be used to prevent false negatives (Wang J. et al., 2020), or can be adapted for targeting the detection of distinct variants of concern, contributing for an improved tracking of the disease (La Rosa et al., 2021). Another alternative, further away from traditional PCR-based methods has been digital RT-PCR (RT-dPCR) (Poggio et al., 2021). It differs from RT-qPCR in the way of quantifying the products of amplification generated following thermal cycling (Quan et al., 2018). In this type of strategy, PCR solution is partitioned in thousands of aliquots prior to thermal cycling (Quan et al., 2018). This leads to the nonexistence of any DNA copy in some of the fractions; then the portion of aliquots where amplification occurred enable the relative quantification of the target sequence recurring to a Poisson statistic (Quan et al., 2018). Droplet digital RT-PCR (RT-ddPCR) is a particular case of RT-dPCR in which the partitioning is achieved with the production of droplets, creating isolated microreactors through the emulsification of the reactional mixture with immiscible oils (Quan et al., 2018). Chip-based digital RT-PCR (RT-Chip-based dPCR) is another variation of the technique (Poggio et al., 2021). The RT-ddPCR has been the main type of RT-dPCR explored in the context of SARS-CoV-2 detection (Deiana et al., 2020; Suo et al., 2020; de Kock et al., 2021). This variation of the standard RT-PCR aims to solve some problems associated with false negative results (Alteri et al., 2020; Suo et al., 2020), since it exhibits enhanced sensitivity (de Kock et al., 2021; Vasudevan et al., 2021). Furthermore, RT-ddPCR proved to be sensitive in

TABLE 3 | Compilation of strategies of non-isothermal amplification explored for SARS-CoV-2 diagnostic.

| | Method | | Method variation | Region targeted | Single or multiple targeting | Duration of amplification (min) | Included in assay issued with EUA | Source |
|------------------------------|-----------|------------------|-----------------------|-----------------------|------------------------------|---------------------------------|-----------------------------------|---------------------------------------------------------------------------------|
| Non-isothermal amplification | PCR-based | RT-qPCR | Single target RT-qPCR | ORF1ab, RdRp, N, E, S | Single | 50 | Yes | (Jung et al., 2020; Park et al., 2020) |
| | | | Multiplex RT-qPCR | ORF1ab, RdRp, N, E, S | Multiple | 40-50 | Yes | (Jung et al., 2020; Kudo et al., 2020; Park et al., 2020; Mancini et al., 2021) |
| | | | N-RT-qPCR | ORF1ab, RdRp, N, E, S | Both | 50-120 | Yes | (Wang J. et al., 2020; Yip et al., 2020; La Rosa et al., 2021) |
| | Non-PCR | RT-dPCR | RT-ddPCR | ORF1ab, RdRp, N, E N | Both | 70-170 | Yes | (Vasudevan et al., 2021) |
| | | | RT - Chip-based dPCR | | Single | 80 | Yes | (Poggio et al., 2021) |
| | | qSTAR Technology | | ORF1a | Single | 20 | Yes | (In, Diagnostic and Only, 2021) |

*Retrieved from the instructions for use of approved diagnostic products available in FDA website (<https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas-molecular-diagnostic-tests-sars-cov-2>).

the direct detection of viral RNA in specimens, without a RNA extraction step (Deiana et al., 2020; Vasudevan et al., 2021). There is still a distant cousin of PCR-based tests, consisting on a distinct amplification reaction, which is designated as quantitative selective temperature amplification reaction (qSTAR) (In, Diagnostic and Only, 2021). It is a significantly faster approach than other non-isothermal amplification methods, constituting a recent innovation (In, Diagnostic and Only, 2021).

Methods Based on Isothermal Amplification

The isothermal amplification of nucleic acids comprehends an array of strategies that exclusively make use of enzymes for driving the amplification of DNA, or RNA at a constant temperature (Piepenburg et al., 2006). Addition of a RT possibilities detection of RNA, following its conversion to cDNA in methods originally designed for the amplification of DNA (Dunbar and Das, 2019). These methods avoid thermal cycling and lead to obtaining results in less time than PCR, generally without the need for expensive equipment like thermocyclers (Dunbar and Das, 2019). SARS-CoV-2 pandemic accelerated the maturing of a great part of the strategies relying on isothermal amplification of nucleic acids (Table 4), with multiple new diagnostics relying on these methods.

LAMP (Loop-mediated isothermal amplification) is by chance the most accomplished strategy classified as isothermal amplification of nucleic acids. The apparatus for this reaction

includes primers and a DNA polymerase enzyme with strand displacement activity besides DNA template (Thompson and Lei, 2020). Primers (4 or 6) are carefully designed through a somewhat complex process that often requires the use of specific software (Jia et al., 2019). The combination of LAMP with an RT within the reaction of amplification, enable the occurrence of reverse transcription simultaneously with the amplification reaction. Overall, when comparing this method with other isothermal amplification forms, the main advantage is the robustness of results. In the context of SARS-CoV-2 it has proved useful for the establishment of strategies aiming fast detection of the virus (Dong et al., 2021), which now at best takes less than 15 minutes (Fowler et al., 2021), without subduing specificity and sensitivity (Dong et al., 2021). It is compatible with direct detection of RNA, without an extraction step (Fowler et al., 2021; Lalli et al., 2021). Method variations include the protocols of mismatch-tolerant RT-LAMP (Lu et al., 2020a), avoiding the occurrence of mismatches during primer hybridization; Penn-RAMP (Song et al., 2021), combining other isothermal method (mentioned below) to achieve nested, two-stage amplification, thus curbing false negatives; or barcoded RT-LAMP (Schmid-Burgk et al., 2020), a tool to turn sequencing more accessible.

RPA (Recombinase polymerase amplification) is another technique aiming at nucleic acids amplification departing from DNA. It requests a pair of primers and the activity of four types of enzymes, three of them originally found in bacteriophage T4, like recombinase, recombinase-mediator protein and single-strand DNA binding proteins (SSBs), or a DNA polymerase with strand displacement activity retrieved from bacteria

TABLE 4 | Compilation of strategies of isothermal amplification explored for SARS-CoV-2 diagnostic.

| | Method | Variations | Region targeted | Single or multiple targeting | Temperature of reaction (°C) | Duration of amplification (min) | Included in assay issued with EUA | Source |
|--------------------------|-----------------|---------------------------|-------------------------------|------------------------------|------------------------------|---------------------------------|-----------------------------------|----------------------------------------------------------------------------------|
| Isothermal amplification | LAMP | RT-LAMP | ORF1ab, S, N, M, ORF3a, ORF7a | Single, or Multiple | 60-65 | 40-60 | Yes* | (Schermer et al., 2020; Lalli et al., 2021) |
| | | Mismatch-tolerant RT-LAMP | ORF1ab, S, N | Single | 63 | 50 | No* | (Lu et al., 2020a) |
| | | Barcoded RT-LAMP | N | Single | 65 | 60 | No* | (Schmid-Burgk et al., 2020) |
| | TMA | — | ORF1ab | Multiple | — | — | Yes* | (Pham et al., 2020) |
| | NASBA | — | S and N | Single, or Multiple | 41 | 35-130 | No* | (Xing et al., 2020; Wu et al., 2021) |
| | | — | — | — | — | — | — | (Qian et al., 2020; Xia and Chen, 2020; El Wahed et al., 2021; Sun et al., 2021) |
| | RPA | RT-RPA | ORF1ab, S, N, E | Single, or Multiple | 42 | 15-30 | No* | (Kim et al., 2021) |
| | RCA | — | ORF1ab | Single | 23 | 5-15 | Yes* | (Tian et al., 2020) |
| | C2CA | HC2CA | RdRp | Single | 37 | 90 | No* | (Quidel, 2020) |
| | HDA | RT-HDA | — | — | — | — | Yes* | (Carter et al., 2021) |
| | EXPAR | RTF-EXPAR | ORF1ab | Single | 50 | <5-25 | No* | (Zhang et al., 2021b) |
| | SDA | AMC-SDA | N | Single | 55 | 30 | No* | (Li et al., 2020; Luu et al., 2021) |
| | MCDA | RT-MCDA | ORF1ab, N | Single or Multiple | 65 | 35-60 | No* | (Song et al., 2021) |
| | LAMP/RPA | Penn-RAMP | ORF1ab | Single | 38 (RPA) and 63 (LAMP) | 40 | No* | |

*Retrieved from the instructions for use of approved diagnostic products available in FDA website (<https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas-molecular-diagnostic-tests-sars-cov-2>).

(Piepenburg et al., 2006). When combined with a RT enzyme, this method enables the detection of RNA targets (Xia and Chen, 2020). The fact that it is a fast amplification process, with a simple amplification chemistry that avoids complex design of primers is a positive asset of this technique (Behrmann et al., 2020; Xia and Chen, 2020). Penn-RAMP is an assay that joins LAMP and RPA in the same strategy. In general, RPA has been widely described in literature reporting SARS-CoV-2 diagnostics, despite the inexistence of a commercial assay based on the technique (Behrmann et al., 2020; Qian et al., 2020; Xia and Chen, 2020; El Wahed et al., 2021; Lau et al., 2021). HDA (Helicase-Dependent Amplification) method is centered in the DNA strand displacement activity of helicase enzyme (Barreda-García et al., 2018). When coupled with reverse transcription, the method enables RNA detection (Barreda-García et al., 2018). Despite this method not being extensively covered in SARS-CoV-2-related literature, there is a commercial detection kit that is based on this technique (Quidel, 2020). MCDA (Multiple cross displacement amplification) is a method that amplifies DNA and makes use of ten primers, targeting ten distinct regions and a DNA polymerase with strand displacement activity (Wang et al., 2015). MCDA is related to LAMP and when compared with it, enables faster results, despite a decrease in sensitivity (Luu et al., 2021). This method has been explored for diagnostic of SARS-CoV-2 in a consistent way, being mentioned in various works where it is combined with RT enzyme, despite the inexistence of a commercial assay based on the method (Luu et al., 2021; Zhu et al., 2021).

TMA (Transcription-mediated amplification) is a technique of isothermal amplification of nucleic acids especially suited for the detection of viral RNA, since it naturally includes reverse transcription integrated in the mechanism of amplification. The main advantage of this method is its enhanced sensitivity, in some cases detecting even quantities that can't be traced with RT-qPCR (Gorzalski et al., 2020). It has been considerably explored for SARS-CoV-2 detection, with successful commercial outcomes (Gorzalski et al., 2020; Trémeaux et al., 2020; Beck et al., 2021). NASBA (Nucleic acid sequence-based amplification) is also targeted for RNA amplification and shares great similarity with TMA, since both join the performance of a RT and a RNA polymerase for generating cDNA intermediates, which are again converted in RNA transcripts by the RNA polymerase, thus prompting another amplification cycle (Wernecke and Mullen, 2014; Yan et al., 2014). It has been investigated for SARS-CoV-2 detection, being used as the basis for RNA amplification in two well reported testing strategies (Xing et al., 2020; Wu et al., 2021). RCA (Rolling circle amplification) is based in the biologic mechanism of rolling circle replication for the production of single-strand DNA (ssDNA) or individual RNA strands through the action of DNA or RNA polymerases (Dunbar and Das, 2019). In this kind of reaction, the circular template is targeted by a specific primer in the origin of replication, which is extended by a RNA, or DNA polymerase, leading to the production of ssDNA or RNA strands (Dunbar and Das, 2019). Circle-to-circle (C2CA) amplification is an independent technique derived from RCA

(Tian et al., 2020). The enhanced sensitivity is a feature of both techniques, despite the extremely long reaction times (Tian et al., 2020; Chaibun et al., 2021). There has been investigation on the potential of both methods for its incorporation in diagnostic strategies for SARS-CoV-2 (Tian et al., 2020; Chaibun et al., 2021; Kim et al., 2021). RCA was already included in a commercial test (Food and Drug Administration, 2020). EXPAR (Exponential amplification reaction) uses two types of enzymes, a DNA polymerase with strand displacement activity and a nicking endonuclease (NEase) (Reid et al., 2018). EXPAR generate around 10^8 copies of DNA in a few minutes, thus consistently possibilitating to achieve detection in a record time of less than 5 minutes (Carter et al., 2021). This method hasn't been much explored in literature, in the context of SARS-CoV-2 diagnostic, despite a single exception (Carter et al., 2021). SDA (Strand displacement amplification) is a technique used for both DNA and RNA amplification that relies on the activity of a NEase and request the use of four primers, in addition to a DNA polymerase with strand displacement activity (Dunbar and Das, 2019). While this strategy hasn't been used for the establishment of any commercial diagnostic in the context of SARS-CoV-2 detection, it is described for detection of this virus in literature, enabling detection without a reverse transcription step (Zhang et al., 2021a).

Controls Used for NAAT Diagnostics

The adoption of controls and other reference materials for quality assessment in the diagnostic process is of utmost importance to ensure the reliability and standardization of results (Mitchell et al., 2020; Yan et al., 2020). However, the subject hasn't been covered so often in the context of SARS-CoV-2, as it would be desired, despite a few meritorious exceptions (Mitchell et al., 2020; Page et al., 2020). Therefore, with a significant diversity of NAATs in addition to RT-qPCR and multiple laboratories devoted to such tasks, it is useful to clarify some notions on the existing types of controls and reference materials, as well as the right situation for using each one (Kessler and Raggam, 2012). As a way to further enter the topic, let's categorize the controls in two main branches: internal controls or external control (**Table 5**), with both being useful when performing all types of NAATs (Yan et al., 2020), regardless of we are talking about isothermal or non-isothermal methods.

External controls are designated as "external" due to being run in other well than that of the sample. These controls can give a quality measure of the entire workflow (Kessler and Raggam, 2012) or of independent stages, such as extraction, reverse transcription and amplification (Yan et al., 2020). External positive controls of the whole testing process, also designated as external run controls or batch controls are ideally inactivated viral samples (Wang et al., 2021; Yan et al., 2021) (either cell cultures or human specimens (Corman et al., 2020)), but since these may be difficult to access (SoRelle et al., 2020; Yan et al., 2021) they can be substituted by synthetic controls simulating the viral particles (Chan et al., 2021; Goncharova et al., 2021; Wang et al., 2021); Virus-Like-Particles (VLPs) (Chan et al.,

TABLE 5 | Compilation of the types of controls and corresponding examples used in the context of NAATs targeted for SARS-CoV-2 detection.

| Classification | | Stage screened | | Common examples | | Source |
|-----------------|-----------------|----------------|---------------|-----------------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------|
| Controls | External | Positive | Whole process | Entire workflow | Inactivated whole virus, armored SARS-CoV-2 RNA, VLPs | (Wang et al., 2021; Yan et al., 2021) |
| | | | | RNA extraction | Inactivated whole virus, armored SARS-CoV-2 RNA, VLPs | (Wang et al., 2021) |
| | | | | Reverse transcription cDNA amplification | SARS-CoV-2 genomic RNA, <i>In vitro</i> transcribed mRNA | (SoRelle et al., 2020) |
| | | Negative | Whole process | Contamination in the entire workflow | Plasmid DNA, SARS-CoV-2 genomic RNA, <i>In vitro</i> transcribed mRNA | (Petrillo et al., 2020; SoRelle et al., 2020) |
| | | | | Specificity in the entire workflow | Non-infected, cultured human cell lines | (Petrillo et al., 2020) |
| | | | | Stage | Human specimens infected or spiked with human infecting RNA virus (e.g. Influenza A and B, RSV) | (Lee et al., 2020) |
| | Internal | Positive | Endogenous | Contamination during RNA extraction | Nuclease-free water | (Petrillo et al., 2020) |
| | | | | Contamination associated with reverse transcription | Nuclease-free water | (Petrillo et al., 2020) |
| | | | | Contamination associated with cDNA amplification | Nuclease-free water | (Petrillo et al., 2020) |
| | | | | RNA extraction | Human cells mRNA (e.g. β actin, RNase P), 18S RNA | (Yan et al., 2020) |
| | | | Exogenous | Collection of human samples | Human cells mRNA (e.g. β actin, RNase P), 18S RNA | (Yan et al., 2020) |
| | | | | Reagent/Equipment malfunction | Human cells mRNA (e.g. β actin, RNase P), 18S RNA | (Yan et al., 2020) |
| | | | | RNA extraction | Non-pathogenic virus (e.g. AoGV, MS2 phage), armored non-SARS-CoV-2 RNA, VLPs | (Calvez et al., 2020; Hasan et al., 2020; Yan et al., 2020) |
| | | | | Reverse transcription cDNA amplification | Non-SARS-CoV-2 RNA (e.g. PVY) | (Calvez et al., 2020) |
| | | | | Inhibition of cDNA amplification | Plasmid DNA, non-SARS-CoV-2 RNA (e.g. PVY) | (Calvez et al., 2020) |
| | | | | Reagent/Equipment malfunction | Non-pathogenic virus, armored non-SARS-CoV-2 RNA, VLPs, non-SARS-CoV-2 RNA, Plasmid DNA | (Calvez et al., 2020; Hasan et al., 2020) |
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2021; Wang et al., 2021) and armored RNA (Goncharova et al., 2021; Wang et al., 2021; Yan et al., 2021) technologies enable the mimicry of viral protein structures containing packaged SARS-CoV-2 RNA (Goncharova et al., 2021; Yan et al., 2021). External positive controls can act as nucleic acid extraction controls (Wang et al., 2021), enabling to understand if the nucleic acid extraction step was well executed. Positive controls targeted for validation of reverse transcription and amplification are generally SARS-CoV-2 genomic RNA (SoRelle et al., 2020), or *in-vitro* transcribed RNA (Madala et al., 2021); plasmid DNA is also often solely used as a positive control of the stage in which cDNA is amplified (Petrillo et al., 2020). The external positive controls ideally have a known concentration and act as standards (Zhou et al., 2021). The external negative controls of the whole process are typically cultured, non-infected human cell lines (Park et al., 2020; Petrillo et al., 2020). The assay specificity may be assessed by analyzing human specimens infected, or spiked with other RNA viruses that often infect the human respiratory tract, such as Influenza A and B viruses, or respiratory syncytial virus (RSV) (Corman et al., 2020; Lee et al., 2020). However, the most common external negative control aims to validate the reverse transcription and amplification steps, substituting the extracted RNA samples by water in the reactional mixture; these are commonly called no-template controls (Petrillo et al., 2020; Buck et al., 2021) and enable to further discard contamination (or cross-contamination) and confirm the specificity of the test.

Internal controls are used to rule out eventual problems that can occur within a certain assay, being analyzed inside the same well of the sample. In the context of NAATs constructed for SARS-CoV-2 diagnosis, positive internal controls are second targets, not aimed at SARS-CoV-2 detection, which exist in the specimens under test (endogenous internal control (Wang et al., 2021)), or in whole viruses (Calvez et al., 2020), armored RNAs (Goncharova et al., 2021), RNAs (Calvez et al., 2020) and DNAs (Yan et al., 2020) spiked on purpose in the samples (exogenous internal control (Kavlick, 2018)). The range of problems being checked with these controls include ill executed RNA extraction (Goncharova et al., 2021), reverse transcription and (Petrillo et al., 2020) cDNA amplification (Petrillo et al., 2020), improper reagents (Kavlick, 2018), assay nonspecificity (Kavlick, 2018), or inhibition of amplification (Kavlick, 2018; Hasan et al., 2020) and consequent false negative results (Kavlick, 2018).

The set of controls adopted in the RT-qPCR protocol first established by the Center for Disease Control and Prevention (CDC) back in January 2020 reveals that three types of controls should be included in the usual protocol adopted for testing; namely one positive extraction control (often a positive internal control), an external positive control devoted to screen the quality of the RT and amplification steps, as well as a no-template control (CDC, 2020b). The different commercial tests also include similar controls and have strict specifications on its use, which must be followed. Nevertheless, variations exist in the

number and type of controls used in those tests. These differences can be a threat to assay comparability. WHO launched a collaborative study to designate an International Standard (IS) for SARS-CoV-2 RNA and in the aftermath of the initiative an inactivated virus standard was established as IS (Bentley et al., 2020), being currently available for purchase through the National Institute for Biological Standards and Control (NIBSC).

MOLECULAR DETECTION ROUTES

PCR-Associated Routes

TaqMan probes are fluorescence-producing oligonucleotides that specifically target amplicons generated during RT-qPCR (Navarro et al., 2015) (Table 6). These probes hybridize with single strand DNA, being labeled with both a fluorophore and a quencher (Navarro et al., 2015). The mechanism that originates

the production of fluorescence relies on the hydrolysis of the hybridized Taqman probe by 5'-3' nuclease activity displayed by Taq DNA polymerase, while extending a new complementary strand in the region where the probe first hybridized with single strand DNA (Navarro et al., 2015). These probes have been incorporated in the protocols designed, by Charité (Corman et al., 2020), or CDC (CDC, 2020b) becoming widely adopted for routine diagnostic due to its great sensitivity and specificity (Marinowic et al., 2021). Molecular beacons are another popular type of oligonucleotide probes used in this context that in contrary to TaqMan aren't hydrolysed, but constitute a stem-loop structure with a fluorophore and a quencher in the extremities, which will generate fluorescence when the probe hybridizes, as the quencher no longer is in the proximity of the fluorophore (Banada et al., 2021). DNA-binding dyes, which emit fluorescence when interacting with DNA double strand (Navarro et al., 2015), such as SYBR Green (Marinowic et al., 2021) or EvaGreen (González-González et al., 2020) have been

TABLE 6 | Depiction of distinct physicochemical routes used for performance of SARS-CoV-2 detection.

| Physicochemical detection strategy | | | | | | Real-time or End-point | Included in assay issued with EUA | Source |
|------------------------------------|--------------------------|-------------------------------|--------------|--------------------------------------------|-------------------------------|------------------------|-----------------------------------|-----------------------------------------------------------------------|
| Nucleic acids detection | PCR-associated methods | Optical | Fluorescence | Oligonucleotide Probes | Taqman probes | Real-time | Yes* | (Falzone et al., 2020) |
| | | | | | Molecular beacons | Real-time | Yes* | (Banada et al., 2021) |
| | | | | | DNA-binding dyes | Real-time | No* | (González-González et al., 2020; Toptan et al., 2020) |
| | | Electrochemical | Voltametry | CRISPR-based Intercalating redox reporters | SENA | Both | No* | (Huang et al., 2020) |
| | | | | | Methylene blue | Real-time | No* | (Nunez-Bajo et al., 2020) |
| | | | | | | | | |
| | Isothermal amplification | Optical | Fluorescence | Oligonucleotide probes | Molecular beacons | Real-time | Yes* | (Bhadra et al., 2020) |
| | | | | | SYBR Green, or SYTO-82 | Both | No* | (Ganguli et al., 2020; Garneret et al., 2021; Marinowic et al., 2021) |
| | | | | | | | | |
| | | | | CRISPR-based | SHERLOCK | Both | Yes* | (Patchsung et al., 2020) |
| | | | | | CARMEN | Both | No* | (Ackerman et al., 2020) |
| | | | | | DETECTR | Both | Yes* | (Street and Francisco, 2020) |
| | | Chemiluminescence Colorimetry | | HPA NPs + oligonucleotide probes | ENHANCE | Both | No* | (Nguyen et al., 2020) |
| | | | | | CONAN | Both | No* | (Yoshimi et al., 2020) |
| | | | | | FELUDA | Both | No* | (Azhar et al., 2021) |
| | | | | pH indicators | | Real-time | Yes* | (Inc, 2021) |
| | | | | | AuNPs | End-point | No* | (Alafeef et al., 2021) |
| | | | | | | End-point | Yes* | (González-González et al., 2021) |
| | | Scattering of light | | DNA-binding dyes | SYBR Green, or EvaGreen | End-point | No* | (García-Bernalt Diego et al., 2021; Lau et al., 2021) |
| | | | | | | | | |
| | | | | | SHERLOCK | End-point | No* | (Patchsung et al., 2020) |
| | | | | Angle-dependent light scatter analysis | | Real-time | No* | (Day et al., 2021) |
| | | | | | | | | |
| | | | | | | | | |
| | | Electrochemical detection | Amperometry | Naopore Target Sequencing | | End-Point | No* | (Ptasinska et al., 2021) |
| | | | | | Intercalating redox reporters | Real-time | No* | (Chaibun et al., 2021) |

*Retrieved from the instructions for use of approved diagnostic products available in FDA website (<https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas-molecular-diagnostic-tests-sars-cov-2>).

other common options for sensing DNA in PCR-related protocols (**Table 6**). Nevertheless, this interaction between dyes and DNA is nonspecific and enables less sensitive detection than oligonucleotide probes in RT-qPCR (Marinowic et al., 2021). The use of both SYBR Green and EvaGreen is well described for SARS-CoV-2 detection in RT-qPCR (Marinowic et al., 2021) and ddPCR protocols (Falzone et al., 2020). Electrochemical detection strategies are also noteworthy in the case of PCR-related methods, through the use of intercalating redox reporters (Nunez-Bajo et al., 2020) (**Table 6**). The intercalating redox reporters function by getting intercalated in the double strand DNA, this way yielding an eletroanalytic output that is directly associated with double strand DNA concentration in the sample under analysis. This type of reaction occurs when operating TriSilix, a lab-on-chip technology that enables to perform the RT-PCR reactions commonly used for diagnosing SARS-CoV-2, at miniature scale (Nunez-Bajo et al., 2020).

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) are DNA sequences encountered in the genome of several prokaryotes, resulting from the infection with mobile genetic elements (MGEs) like bacteriophages, plasmids, or transposons, which can be used by these same prokaryotic organisms for recognizing and destroying similar new sequences of DNA, in subsequent infections (Strich et al., 2021). In order to perform the dismantling of the residues left by bacteriophages, CRISPR are transcribed in CRISPR RNAs (crRNAs), which combine with specific endonuclease enzymes that possess great specificity and target complementary sequences (Strich et al., 2021). These endonuclease enzymes, known as CRISPR-associated proteins (Cas) are powerful gene editing tools and thus have been extensively explored for enhancing the detection of amplicons resulting from amplification reactions (Sun et al., 2021), in the scope of SARS-CoV-2 diagnostics. SENA (Specific Enhancer for detection of PCR-amplified Nucleic Acids) is a confirmatory test to be used in the aftermath of RT-qPCR (**Table 6**), when the Cq sits between 38 and 40, thus raising doubts about the final diagnosis (Huang et al., 2020). It is based in the activity of Cas12a, a fluorescent ssDNA reporter and two crRNAs that target PCR amplification products. SENA increases the sensitivity of RT-qPCR, enabling a more assertive diagnosis (Huang et al., 2020). This type of approach have been explored to overcome the loss of sensitivity associated with the occurrence of mutations in primer-binding sites, by using a version of Cas12a that is able to tolerate single mismatches when it is combined with crRNAs targeting the virus (Huang et al., 2020).

Isothermal Amplification-Associated Routes

The methods used for detection of products of amplification resulting from isothermal amplification reactions targeting SARS-CoV-2 include mainly optical (Day et al., 2021; Nawattanapaiboon et al., 2021; Reynés et al., 2021) and electrochemical strategies (Ptasinska et al., 2021) (**Table 6**). Optical detection of the virus can rely on fluorescence (Taki et al., 2021; Wu et al., 2021), colorimetry (García-Bernalt Diego

et al., 2021; Nawattanapaiboon et al., 2021; Reynés et al., 2021) and even scattering of light (Day et al., 2021). As it happens for PCR reaction, fluorescence-producing reactions are an important route for detecting these products of amplification optically, both in real-time (Ganguli et al., 2020; Alekseenko et al., 2021) and in end-point (Alekseenko et al., 2021; Sherrill-Mix et al., 2021) contexts. The mechanisms that lead to fluorescence production include intercalating dyes (Alekseenko et al., 2021), specific oligonucleotide probes (Jang et al., 2021; Ooscorbin et al., 2021), or enzymatic reactions that culminate in the production of fluorescence (Joung et al., 2020; Sun et al., 2021). Intercalating dyes, such as SYBR Green (Alekseenko et al., 2021) or EvaGreen (Alekseenko et al., 2021), are nonspecific but can be used for both real-time monitoring, or end-point measuring of amplification. The potential of nanoparticles in fluorescence-producing reactions has also been described, namely through the employment of magnetic nanoparticles in separating no-specific amplification products prior to fluorescence reading, thus enhancing established protocols (Dahiya et al., 2021).

Chemiluminescence has also been integrated in detection strategies, following isothermal amplification, in particular in TMA (Inc, 2021). Hybridization Protection Assay (HPA) is a method that allows a chemiluminescence-based readout, which relies on a specific oligonucleotide probe attached to an acridinium ester functioning as a reporter molecule by hybridizing with amplicons generated by TMA reactions (Inc, 2021). DNA-binding dyes like SYBR Green and EvaGreen, although being preferably used in approaches relying in fluorescence reading, can also be adapted for colorimetric detection of DNA following isothermal amplification, since both dyes change colors upon interacting with DNA (Bokelmann et al., 2021). Popular colorimetric reactions used for detecting the virus after isothermal amplification resort to conjugation of colored nanoparticles, like AuNPs, with oligonucleotide probes that upon interacting with specific amplicons hybridize and signal the detection of target, through color change (Alafeef et al., 2021). There are Cas-based detection approaches mentioned above that were accessed with colorimetric readouts (Joung et al., 2020; Patchsung et al., 2020), relying similarly in the interaction of oligonucleotide-labeled AuNPs and DNA amplicons. Other colorimetric alternative tested for SARS-CoV-2 detection after isothermal amplification is color change induced by pH shift, upon detection of amplified DNA, in minimal buffered media (Rabe and Cepko, 2020).

The scattering of light is a less explored method for DNA detection following isothermal amplification, which was also already used in the detection of this virus (Day et al., 2021). This route turns possible in reactions taking place in emulsions and is based on the principle that the newly generated amplicons adsorb to the water-oil interface, resulting in a diminution in the interfacial tension that traduces in a smaller diameter of the emulsion (Day et al., 2021). Then, since the light scatter intensity is directly diameter-dependent, the accumulation of amplicons resulting of amplification can be detected, by monitoring the light scatter intensity (Day et al., 2021). Electrochemical sensing

has been based on amperometry, by using nanopore target sequencing (Ptasinska et al., 2021), or voltammetry through the use of intercalating redox reporters (Chaibun et al., 2021), functioning in a similar way to what was described for the PCR-associated case (Day et al., 2021).

Enzymatic reactions engineered for fluorescence production usually rely on the use of distinct Cas endonucleases (Sun et al., 2021). The referred detection mechanisms are often based on the collateral activity of these endonucleases, which is the ability to cleave any ssDNA, or ssRNA in solution, beside the target sequences (Sun et al., 2021). SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) is a strategy developed prior to SARS-CoV-2 pandemic, which comprises a first step of isothermal amplification, following reverse transcription and a second moment where the amplified DNA is transcribed in ssRNA by RNA polymerase. Then, the ssRNA is targeted by Cas13a endonuclease coupled with a crRNA that recognizes the newly formed ssRNA (Joung et al., 2020; Patchsung et al., 2020). Following the recognition of ssRNA by Cas-crRNA complex, the collateral cleavage activity of Cas13a enables the breaking of a short ssRNA sequence labeled with a fluorophore and a quencher, producing fluorescence in the process (Joung et al., 2020; Patchsung et al., 2020). CARMEN (Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids) is a strategy also based in the action of a Cas13 that enables enhanced detection of multiple targets at the same time, thus facilitating reagent savings and testing scalability (Ackerman et al., 2020). DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter) is another detection approach created prior to pandemic that relies in a first step of isothermal amplification followed by the action of Cas12a (Sun et al., 2021). Similarly, it also comprehends a reaction driven by

a Cas endonuclease activated when crRNAs recognize target RNA, thus leading to the production of fluorescence by breaking a ssDNA reporter sequence containing a fluorophore and a quencher (Sun et al., 2021). ENHANCE (ENHanced Analysis of Nucleic acids with crRNA Extensions) involves crRNA modifications, by extending its 3' and 5' terminations with ssDNA, ssRNA and phosphorothioate ssDNA (Nguyen et al., 2020). These extensions promote self-catalysis and collateral cleavage activity of Cas12a, thus leading to enhanced specificity in target cleavage (Nguyen et al., 2020). CONAN (Cas3-Operated Nucleic Acid detectionN) requires Cas3 and is also based in the collateral activity of this endonuclease (Yoshimi et al., 2020). FELUDA (FnCas9 Editor Linked Uniform Detection Assay) relies on Cas9 for the direct detection of nucleotide sequences, without the need for the cleavage of reporter molecules, as happens in the aforementioned cases (Azhar et al., 2021; Osborn et al., 2021; Xiong et al., 2021). It aims to be an alternative to collateral cleavage activity approaches, while presenting a simpler design and higher resilience to viral mutations, by being able to detect single nucleotide variants (Azhar et al., 2021; Osborn et al., 2021; Xiong et al., 2021).

PLATFORMS FOR PERFORMING NUCLEIC ACIDS DETECTION

Fully Automated Instruments and PCR Equipment

Fully automated systems enable the analysis of large amounts of samples, in a standardized process that integrates nucleic acid

TABLE 7 | Comparison of platforms used for performing SARS-CoV-2 detection.

| | Point-of-care compatibility | Platforms | Main Physicochemical detection strategy | Other steps needed for complete diagnosis | Typical Setting | Included in assay issued with EUA | Source |
|-------------------------|-----------------------------|-------------------------------|---------------------------------------------|---------------------------------------------------|---------------------------------------------|-----------------------------------|-----------------------------------------|
| Nucleic acids detection | Mostly incompatible | Fully automated equipments | Fluorescence quantification | No | Large, well equipped clinical settings | Yes* | (Nörz et al., 2020) |
| | | PCR equipments | Fluorescence quantification | Eventually, the extraction step | Well equipped to moderate resource settings | Yes* | (Corman et al., 2020) |
| | | Plate readers | Fluorescence quantification | Yes, eventually extraction and amplification step | Moderate resource settings | Yes* | (González-González et al., 2020) |
| | Generally compatible | Portable PCR equipments | Fluorescence quantification | Eventually, the extraction step | Moderate to low resource settings | No* | (Mendoza-Gallegos et al., 2018) |
| | | Portable Fluorescence readers | Fluorescence quantification | Yes, eventually extraction and amplification step | Moderate to low resource settings | No* | (Ireta-Muñoz and Morales-Narváez, 2020) |
| | | Microfluidics | Fluorescence quantification | Eventually, the extraction step | Moderate to low resource settings | Yes* | (Garneret et al., 2021) |
| | | LFA | Colorimetry | Yes, eventually extraction and amplification step | Moderate to low resource settings | No* | (Xiong et al., 2021) |
| | | Single tube | Fluorescence quantification, or colorimetry | Eventually, the extraction step | Moderate to low resource settings | Yes* | (Aiziti-Sanz et al., 2020) |

*Retrieved from the instructions for use of approved diagnostic products available in FDA website (<https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas-molecular-diagnostic-tests-sars-cov-2>).

extraction, amplification, detection and processing of results (Mayer et al., 2020; Nörz et al., 2020) (**Table 7**). Despite being considerably expensive, these equipments enable high-throughput testing, being tailored for large laboratory settings (Mayer et al., 2020; Nörz et al., 2020). The types of extraction and amplification methods vary, according with the distinct systems. In fully automated machines, extraction approaches match those referred in **Table 2**. RT-PCR constitutes the standard route of amplification (De Luca et al., 2021), although fully automated approaches based on TMA (Trémeaux et al., 2020), or HDA (Quidel, 2020) exist.

The standard qPCR equipments are widely described as expensive (Chaibun et al., 2021; Michel et al., 2021; Panpradist et al., 2021) and impractical for use at the point of care (Panpradist et al., 2021) (**Table 7**). Conventional PCR is more affordable but always demands an end-point reading system, normally agarose gel electrophoresis (Silva Júnior et al., 2021), which adds entropy to the diagnostic process, being difficult to implement in a mass testing scenery (**Table 7**). Nonetheless, there has been an effort toward the adaptation of protocols (Panpradist et al., 2021), and construction of more democratic qPCR machines, by decreasing prices (Mendoza-Gallegos et al., 2018), turning the equipments portable (Mendoza-Gallegos et al., 2018) and miniaturizing the systems (Nunez-Bajo et al., 2020; Qin et al., 2020) (**Table 7**). These more accessible apparels can be adapted to operation at the point of care (González-González et al., 2020; Nunez-Bajo et al., 2020). There are examples of the use of qPCR equipments at the point of care with similar success to the standard systems, although the large majority of the RT-qPCR protocols for diagnostic of this virus are still being performed in standard qPCR machines, in clinical settings (Glen et al., 2021) (**Table 7**). Digital PCR is performed in specific apparels that are also associated with high costs, thus being inaccessible for every laboratory, particularly in settings that perform routine diagnosis (Tedin et al., 2021) (**Table 7**). Nonetheless, the high sensitivity of the technique has attracted increasing interest in the context of research (Tedin et al., 2021).

Plate Readers, Portable Fluorescence Readers, Luminometers and Single Tube Assays

While qPCR equipment proportionate fluorescence reading, there are various techniques used for detection of SARS-CoV-2, which demand the measuring of fluorescence and can't be performed in qPCR machines. This includes not only protocols adopting conventional fluorescence plate readers (Sun et al., 2021; Tsou et al., 2021), but also strategies suited for measuring fluorescence at the point of care. Portable, miniaturized systems for detection of fluorescence have been developed, which enable nucleic acid detection in this second case (Ganguli et al., 2020; González-González et al., 2020; Ireta-Muñoz and Morales-Narváez, 2020; Samacoits et al., 2021) (**Table 7**). In addition, there are approaches based on UV-Vis spectrophotometry (Health, 2021), which also demand the use of plate-readers, or in the detection of chemiluminescence, which require the use of a luminometer (Inc, 2021) (**Table 7**). The detection of the virus following

isothermal nucleic acid amplification can even be fully carried on in a simple tube, with only the support of a heat source able to maintain the temperature for the amplification reaction to occur and a simple visual inspection of the results after a few minutes (**Table 7**). The adaptation of smartphones as fluorescence readers, by combining its ability for data and image analysis with 3D printed components has also been a suggested option (Samacoits et al., 2021). LAMP (Pang et al., 2020) and RPA (Arizti-Sanz et al., 2020) are approaches already used in this type of NAAT. Furthermore, assays that require additional plate readers or portable fluorescence readers, instead of a smartphone may also be considered single tube assays (Behrmann et al., 2020; Lee et al., 2020).

Lateral Flow Assay

Lateral flow assay (LFA) enables the detection of analytes in a test strip, or dipstick, by submitting the sample to an unidirectional flow in a liquid medium that promotes the interaction of specific targets with capture molecules immobilized in a solid surface (Jauset-Rubio et al., 2016). The platform is designed such that immobilized molecules in a membrane recognize the signature of specific targets, thus yielding a positive or negative colorimetric result (Jauset-Rubio et al., 2016) (**Table 7**). There is one variation of the method, designated as nucleic acid lateral flow assay (NALFA) that is the main route used for end-point, point-of-care detection of DNA amplicons obtained by isothermal amplification, due to a combination of low cost and simple operation requirements (Ivanov et al., 2020). To this point, LAMP (Nguyen et al., 2020), NASBA (Wu et al., 2021), RPA (Azhar et al., 2021; Xiong et al., 2021), or MCDA (Zhu et al., 2021) are isothermal amplification methods already combined with a detection step that comprises LFA, for SARS-CoV-2 detection. The solutions that rely on simultaneous use of CRISPR-Cas systems and isothermal amplification have also been commonly combined with a LFA readout (Zhu et al., 2021; Xiong et al., 2021). A dominant approach in the NALFA systems used for SARS-CoV-2 diagnosis is based on a colorimetric detection chemistry that comprises the interaction of oligonucleotide probes labeled with AuNPs and specific amplicons (Lau et al., 2021; Xiong et al., 2021). The amplicons are often generated in the sequence of the extension of primers labeled with biotin, which are then captured on commercially available strips by the anchoring system biotin-streptavidin (Azhar et al., 2021; Lau et al., 2021), producing an intense red signal. Alternative molecular reactions reported for detection of SARS-CoV-2 with NALFA are based on in-house developed variations of the above mentioned strategy (Zhang et al., 2021a; Zhu et al., 2021), or other type of solution involving different nanoparticles, as for instance carbon nanoparticles (CNPs) (Wu et al., 2021). CRISPR-Cas-based solutions request the incubation of the amplicons obtained by isothermal amplification with a Cas-crRNA that targets its sequence, activating the collateral cleavage activity of Cas. This sequence of events provokes the cleavage of reporter oligonucleotides, which are then read in the NALFA device, this way increasing the specificity of the detection step (Nguyen et al., 2020; Xiong et al., 2021).

Microfluidic Devices

Microfluidics is based on the manipulation of fluids at a sub-millimeter scale, having multiple applications in molecular diagnostic with a growing number of solutions trusting on microfluidic-related platforms that contain nucleic acid amplification stages (Anderson et al., 2021; Fassy et al., 2021; Liu et al., 2021; Xing et al., 2021). The higher cost and complexity of these systems, which are often used at the point-of-care (**Table 7**), in comparison to LFA-based methods, are challenges that may discourage its use (Liu et al., 2021). Nonetheless, the processes of nucleic acid amplification and detection in microfluidics approaches may occur entirely in a closed system, rather than in a fragmented process always comprising closed-tube amplification and an open readout step, like it happens in methods that include LFA (de Oliveira et al., 2021; Liu et al., 2021). This fact points toward important advantages of microfluidic systems in avoiding contaminations, automatizing assays (Ramachandran et al., 2020), standardizing the different stages of reaction and enabling overall reduction in the time-to-result (de Oliveira et al., 2021; Liu et al., 2021). Microfluidic systems can be further divided in various subsets, of what rotationally-driven (Ji et al., 2020; Xiong et al., 2020; de Oliveira et al., 2021), electric field-driven (Ramachandran et al., 2020) and paper-based (Garneret et al., 2021) options were already adopted in the context of SARS-CoV-2. The normal workflow used for detecting viral RNA in other approaches, which includes extraction, amplification and detection of nucleic acids can be partially (de Oliveira et al., 2021; Fassy et al., 2021; Liu et al., 2021), or entirely (Garneret et al., 2021) conducted in microfluidic platforms, since the extraction step is often conducted out of the microfluidics apparatus. There are different types of amplification strategies already assessed in these devices that rely on PCR-related methods, generally qRT-PCR (Anderson et al., 2021; Fassy et al., 2021; Xing et al., 2021), or isothermal amplification techniques related to LAMP (Ramachandran et al., 2020; Xiong et al., 2020; de Oliveira et al., 2021; Garneret et al., 2021), NASBA (Xing et al., 2020), RPA (Liu et al., 2021) and RCA (Kim et al., 2021), including approaches based on CRISPR-Cas (Ramachandran et al., 2020). In the context of SARS-CoV-2 diagnostics, the detection of DNA following the amplification reaction is usually performed by integrated measuring of fluorescence (Ramachandran et al., 2020; Xing et al., 2020; de Oliveira et al., 2021; Fassy et al., 2021; Garneret et al., 2021), although LFAs (Liu et al., 2021), or rheometry devices (Kim et al., 2021) have already been combined with microfluidic systems for this same purpose.

BENCHMARKING OF COMMERCIAL DIAGNOSTICS

Criteria Used in the Assessment of Commercial Assays

The most frequent criteria used in the assessment and comparison of NAATs in review literature include distinct topics like the route of sample collection, the type of specimen,

the processing strategy, the type of amplification method, or the detection mechanism, including both detection chemistry and detection platforms (Khan et al., 2020). Furthermore, other important parameters to be accessed are the duration of the entire process, detection range and the possibility to perform simultaneous detection of distinct amplicons (Khan et al., 2020). The certification of the diagnostic solution is an important aspect to take into account. There are well-established and as-fast-as-possible processes for verification of diagnostic assays prior to making them available in the market, during the current pandemic (Mitchell et al., 2020). These processes vary worldwide and, while European Union (EU) countries rely on CE mark for validating new *in-vitro* diagnostics (IVDs), CE-IVD (Vermeersch and André, 2021), United States trust on EUAs (Mitchell et al., 2020). EUAs are special authorizations that enable licensing the commercialization of a certain drug, IVD, or medical device under the legislation followed by FDA, despite the non-completion of the entire validation process (Mitchell et al., 2020). This type of authorization requests less data about product performance than regular authorizations, being valid only during the pandemic and ceasing after that state is deactivated (Mitchell et al., 2020). EUAs granted by Food and Drug Administration (FDA) are widely mentioned in research papers (Basu et al., 2020; Gorzalski et al., 2020; Smith et al., 2020), being trusted as an important seal of approval.

Comparison of Different Groups of Commercial NAATs

PCR-related assays comprise standard RT-qPCR assays and other PCR-derived tests. Since it is impractical to analyze all assays granted with a EUA or other validation mark, by different regulatory bodies, a set of representative commercial assays issued with a EUA by FDA and/or CE-IVD mark by EU since December 2020 were chosen and benchmarked following the aforementioned criteria (**Table 8**). The analysis of the last mentioned table enables to verify that the most frequent route of collection among PCR-related assays relies on hetero-collection by healthcare workers, although there are options for self-collection, either with, or without supervision. URT specimens are the main source used for retrieving the RNA to be detected, being fit for all the tests assessed. NP samples are compatible with all the tests considered and BAL is the favorite LRT specimen to be collected. Processing of samples is almost always done through automated extraction, resorting to a full extraction approach. The range of amplification-based assays includes mainly RT-qPCR, although RT-ddPCR and qSTAR being also options and the targets being usually various sites, in multiplex protocols. The molecular mechanism of detection relies for the great part in real-time tracking of fluorescence, using specific oligonucleotide probes. While a significant number of tests are aimed for fully automated platforms, able to perform all testing process from sample processing to the interpretation of results, there are a greater number of tests designed for segmented analysis. Despite not being indicated in **Table 8**, the represented PCR-derived tests are all aimed to be performed at clinical settings.

TABLE 8 | Benchmarking of diagnostics for SARS-CoV-2 based on non-isothermal amplification methods issued with EUA, or CE mark.

| Tests | Producing company | Validation | Type of sample processing | Ready for Pooling | Amplification method | Detection strategy | Detection platform | Duration (min) | Detection limit (copies/μL) | Specimen under analysis | Targets, Multiplex, or Multi-species |
|-----------------------------------------------------|-------------------------------------------|------------|--------------------------------------|-------------------|----------------------|---------------------------------------------------------------|---------------------------|----------------|--------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------|
| Cobas SARS-CoV-2 | Roche Molecular Systems, Inc | EUA, IVD | CE- Automated full extraction | Yes | RT-qPCR | Fluorescence, Taqman probes | Fully automated equipment | <210 | 0.046 | Self-collected – URT swab (NS)// Collected by healthcare worker – URT swabs (NS,NP,OP) | Multi-target, ORF1ab and E and Multiplex |
| NeuMoDx SARS-CoV-2 assay | NeuMoDx Molecular, Inc | EUA, IVD | CE- Automated full extraction | No | RT-qPCR | Fluorescence, Taqman probes | Fully automated equipment | – | 0.050 (saliva) or 0.150 (NP) | Supervised self-collection – saliva collected with NeuMoDx Saliva Collection Kit//Collected by healthcare worker - URT swabs (NS, NP, OP) and BAL | Multi-target, ORF1ab (NSP2) and N and Multiplex |
| Xpert Omni SARS-CoV-2 Assay | Cepheid | EUA, IVD | CE- Automated full extraction | No | RT-qPCR | Fluorescence, Nos-specified oligonucleotide hydrolysis probes | Fully automated equipment | – | 0.4 | Collected by healthcare worker – URT swabs (NS,NP,OP, mid-turbinate) and NS wash, or aspirate | Multi-target, N and E and Multiplex |
| Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay kit | Bio-Rad Laboratories, Inc | EUA | Manual, or Automated full extraction | No | RT-qPCR | Fluorescence, No-specified oligonucleotide probes | qPCR equipment | – | 0.125-0.250 | Collected by healthcare worker – URT swabs (NS,NP, OP, mid-turbinate), nasal aspirates and nasal washes | Multi-target, two N gene regions and Multiplex |
| BioFire COVID-19 Test | BioFire Diagnostics, LLC | EUA | Automated full extraction | Yes | N-RT-qPCR | End-point Melting Curve data | qPCR equipment | 50 | 5.4 | Collected by healthcare worker – URT swabs (NS,OP, mid-turbinate), LRT samples (sputum, tracheal aspirates and BAL) | Multi-target, two ORF1ab regions and ORF8, Multiplex |
| Lyra SARS-CoV-2 Assay | Quidel, Inc | EUA, IVD | CE- Automated full extraction | No | RT-qPCR | Fluorescence, Taqman probes | qPCR equipment | 135 | 6 | Collected by healthcare worker – URT swabs (NP,OP) | Single-target, ORF1ab |
| Quest SARS-CoV-2 rRT-PCR | Quest Diagnostics Infectious Disease, Inc | EUA | Automated full extraction | Yes | RT-qPCR | Fluorescence, Taqman probes | qPCR equipment | – | 0.136 | Self-collected – URT swab (NS)// Collected by healthcare worker – URT (NP,OP) swabs and LRT samples (sputum, tracheal aspirates and BAL) | Multi-target, two N gene regions and Multiplex |
| Quest SARS-CoV-2 rRT-PCR | BioFire Diagnostics, LLC | EUA | Automated full extraction | No | N-RT-qPCR | End-point Melting Curve data | Fully automated equipment | 50 | 0.5 (SARS-CoV-2), in the range 0.01 to 3 for other pathogens | Collected by healthcare worker - URT swab (NP) | Multi-target, S and M (for SARS-CoV-2), Multiplex and Multi-species |
| Quest SARS-CoV-2 rRT-PCR | Bio-Rad Laboratories, Inc | EUA | Manual or Automated full extraction | No | RT-ddPCR | Fluorescence, Taqman probes | Digital PCR equipment | <500 | 0.4 | Collected by healthcare worker - URT swabs (NS, NP, OP, mid-turbinate), aspirates (NS,NP) and BAL | Multi-target, two N gene regions and Multiplex |
| LumiraDx SARS-CoV-2 RNA STAR Complete | Lumira Dx UK Ltd | EUA, IVD | CE- Brief lysis step | No | qSTAR | Fluorescence, Molecular beacons | qPCR equipment | <20 | 1.875 | Collected by healthcare worker - URT swabs (NS, NP, OP, mid-turbinate) and BAL | Single-target, ORF1ab |

All the information was retrieved from the instruction for use supplied in FDA website (<https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas-molecular-diagnostic-tests-sars-cov-2>) and the data sheets attached to each test validated with CE-IVD, which are listed in European Commission website (https://covid-19-diagnostics.jrc.ec.europa.eu/devices?device_id=&manufacturer=&text_name=&marking=Yes&method=&rapid_diag=&target_type=&search_method=AND&form_content). Both sites were last accessed on 02.02.2022.

TABLE 9 | Benchmarking of diagnostics for SARS-CoV-2 based on Isothermal amplification-derived methods issued with EUA, or CE mark.

| Tests | Producing company | Validation | Type of sample processing | Amplification type | Detection mechanism | Detection platform | Duration (min) | Limit of detection (copies/ μ L) | Type of specimen under analysis | Targets, Multiplex, or Multi-species | Settings |
|----------------------------------------------------|----------------------------------|------------|--------------------------------------------|--------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|----------------|--------------------------------------|--------------------------------------------------------------------------------------------|--------------------------------------------------------------------|----------|
| Lucira CHECK-IT COVID-19 Test Kit | Lucira Health, Inc | EUA | Lysis step | RT-LAMP | Colorimetry, Color change induced by pH shift upon amplification, then converted in electronic signal | Microfluidics | 30 | 0.9 | Self-collected swab (NS) | Multi-target, two N gene regions and Multiplex | Home |
| DetectTM Covid-19 | Detect, Inc | EUA | Lysis step | RT-LAMP | Colorimetry, Visual observation of specific signal upon interaction of amplicons with colored NPs and retention of the conjugates on the test point | LFA | ≈60 | 0.8 | Self-collected swab (NS) | Single target, ORF1ab | Home |
| MobileDetect Bio BCC19 Test Kit | Mobile Detect Bio Inc | EUA | Non-specified processing reagents | RT-LAMP | Colorimetry, Visual detection of color change induced by pH shift upon amplification | Single-tube | <60 | 75 | Collected by healthcare worker – URT swabs (NS,NP,OP, mid-turbinate) | Multi-target, N and E and Multiplex | Clinical |
| Color SARS-CoV-2 RT-LAMP Diagnostic Assay | Color Health, Inc | EUA | Automated full extraction | RT-LAMP | Colorimetry, Color change induced by pH shift upon amplification, then analysed spectrophotometrical | Micro-plate reader | 70 | 0.75 | Collected by healthcare worker – URT swabs (NS,NP,OP,mid-turbinate) | Multi-target, N and E, or ORF1ab,S and Multiplex | Clinical |
| SHERLOCKTM CRISPR SARS-CoV-2 Kit | Sherlock Biosciences, Inc | EUA | Manual full extraction | RT-LAMP | Fluorescence, Enzymatic-based cleavage of oligonucleotide probes containing a fluorophore and a quencher | Micro-plate reader | ≈60 | 6 | Collected by healthcare worker – URT swabs (NS,NP,OP), NS, NP aspirates and BAL | Multi-target, ORF1ab and N and Multiplex | Clinical |
| ALS SARS-CoV-2 RT-LAMP | ALS, Inc | CE -IVD | Non-specified, Non-conventional extraction | RT-LAMP | Colorimetry, Color change induced upon amplification | Single-tube | 45 | 10 | Collected by healthcare worker – URT samples | Multi-target, ORF1ab and N | Clinical |
| Procleix SARS-CoV-2 Assay | Grifols Diagnostic Solutions Inc | EUA | Automated full extraction | TMA | Chemiluminescence, using HPA | Fully automated equipment | – | 0.06 | Collected by healthcare worker – URT swabs (NS,NP,OP), NS, NP aspirates and BAL | Multi-target and Multiplex | Clinical |
| Aptima SARS-CoV-2 Assay | Hologic, Inc | EUA, IVD | Lysis step | TMA | Chemiluminescence, using HPA | Fully automated equipment | – | 0.6 | Collected by healthcare worker – URT swabs (NS,NP, mid-turbinate) | Multi-target, two ORF1ab gene regions, Multiplex | Clinical |
| Aptima SARS-CoV-2/Flu Assay | Hologic, Inc | EUA | Lysis step | TMA | Fluorescence, similar to molecular beacons | Fully automated equipment | – | 0.18 | Collected by healthcare worker – URT swabs (NS,NP, OP, mid-turbinate), NS and NP aspirates | Multi-target, two ORF1ab gene regions, Multiplex and Multi-species | Clinical |
| Solana SARS-CoV-2 Assay | Quidel, Inc | EUA, IVD | Lysis step and heat treatment | RT-HDA | Fluorescence, oligonucleotide probes hydrolysed by RNase H2 | Fully automated equipment | 30 | 54 | Collected by healthcare worker – URT swabs (NS,NP) | Multi-target, ORF1ab | Clinical |

(Continued)

TABLE 9 | Continued

| Tests | Producing company | Validation | Type of sample processing | Amplification type | Detection mechanism | Detection platform | Duration (min) | Limit of detection (copies/ μ L) | Type of specimen under analysis | Targets, Multiplex, or Multi-species | Settings |
|------------------------|-------------------------------------|-------------|---------------------------|--------------------|---------------------------------|---------------------------|----------------|--------------------------------------|---------------------------------------------------------|--------------------------------------|----------|
| ID Now COVID-19 | Abbott Diagnostics Scarborough, Inc | EUA, CE-IVD | Lysis step | Unknown | Fluorescence, molecular beacons | Fully automated equipment | ≈ 13 | 0.125 | Collected by healthcare worker – URT swabs (NS, NP, TH) | Single-target, ORF1ab | Clinical |

All the information was retrieved from the instruction for use supplied in FDA website (<https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/in-vitro-diagnostics-euas-molecular-diagnostic-tests-sars-cov-2>) and the data sheets attached to each test validated with CE-IVD, which are listed in European Commission website (https://covid-19-diagnostics.jrc.ec.europa.eu/devices?device_id=&manufacturer=&text_name=&marking=Yes&method=&rapid_diag_type=&search_method=AND#form_content). Both sites were last accessed on 02.02.2022.

Regarding isothermal nucleic acid amplification tests, these are based in distinct reactions, being logic to analyze them in a separate group from PCR-related tests. Thus, a selection of tests granted with EUAs by FDA and/or CE-IVD mark by EU since December 2020 was made according with its distinct characteristics and benchmarked (**Table 9**). The most usual path for collection of samples and the main type of specimens for analysis is similar to those used in PCR-related assays. In general, processing of samples oscillates between full RNA extraction, either automated or manual, to partial extraction through a brief lysis step. The scope of isothermal amplification techniques included in this group of diagnostics is diverse, ranging from RT-LAMP and TMA, the two most popular nucleic acid amplification methods, to RT-HAD and other undisclosed procedures. There are plenty of molecular mechanisms of detection, including either end-point colorimetry, through color change analysis following pH shift upon amplification, real-time fluorescence tracking, with fluorescence-producing oligonucleotide probes, or real-time chemiluminescence measurement, through HPA. The platforms in which detection takes place are also diverse, including fully automated equipments, microfluidic platforms, LFA, plate readers, or single tube options. Overall, a great part of the tests displayed were designed as POCT, including Lucira CHECK-IT COVID-19 Test Kit, Detect COVID-19, Mobile Detect Bio BCC19 Kit, Solana SARS-CoV-2 Assay and ID NOW COVID-19, or can easily be compatible with a wide range of laboratory settings like Color SARS-CoV-2 RT-LAMP Diagnostic Assay, SHERLOCK CRISPR SARS-CoV-2 Kit and ALS SARS-CoV-2 RT-LAMP. Nevertheless, there are still alternatives for more complex and demanding instruments, like Procleix SARS-CoV-2 Assay and Aptima SARS-CoV-2 Assay. There are various isothermal amplification tests that have a shorter duration than PCR-based assays, often without compromising sensitivity, as evidenced by comparing **Tables 8** and **9**.

CONCLUSIONS AND NEAR FUTURE PERSPECTIVES

The diversity of testing strategies for the same application mirrors the widespread response of scientific community to the ongoing pandemic. The myriad of diagnostic approaches detailed during the body of this work denounces that the detection of SARS-CoV-2 in clinical samples resorting to NAATs can vary with the type of specimens, collection practices, method of extraction, process of amplification, detection chemistry and the type of platform where the analysis is conducted. While reaction variables are abundant, the types of controls used for each diagnostic approach are also diverse, what turns it difficult to compare distinct detection methods. The semi-quantitative nature of tests that is for the greatest part supported on Cq values (for the case of RT-qPCR-derived protocols) rather than in exact viral load numbers is also a factor that contributes for impairment in the comparison of test performances (Bustin et al., 2021; Cheema and Blumberg, 2021). Therefore the standardization of NAATs, as well as reaction

controls, aimed at SARS-CoV-2 diagnosis is an issue that continues to demand attention (Bustin et al., 2021; Cheema and Blumberg, 2021).

POCTs rely on low budget solutions to proportionate wide access to essential diagnosis needs, turning these type of tests very useful in the pandemic scenery (Silva et al., 2021). However, despite countless works devoted to demonstrate the ability of NAAT-based approaches to be used as POCTs, the wide number of solutions wasn't enough to dislodge RT-qPCRs as the prime choice for performing SARS-CoV-2 detection (Bustin et al., 2021). In addition to the robustness and widespread use that is associated with RT-qPCR, the fact that most of POCTs rely on isothermal nucleic acid amplification, which are not mature technologies in the market, may explain the back position of this type of diagnostics (Mattioli et al., 2020). However, isothermal POCTs are gaining more and more traction. The continuous development of nanotechnology-based solutions that can turn isothermal amplification strategies more robust in terms of sensitivity and can promote fast adaptation to detection of rising variants will benefit these type of tests. Miniaturized PCR-based systems also haven't substituted traditional laboratory-based diagnostics, although good prospects exist of growing utilization (Gupta et al., 2021). Furthermore, the articulation of simple-functioning detection platforms with digital tools, originating faster measurements, interpretation and delivery of test results are good assets in any POCT.

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- Despite the efficient testing campaigns largely based on RT-qPCR, the potential role of POCTs as an alternative to PCR and the emergence of more robust POCT platforms would enable to better cope with a future pandemic. Also, new surging variants may challenge health systems in less favored regions, thus stressing the need for efficient POCTs. This way, more efforts need to be put in advancing POCTs for SARS-CoV-2 detection.
- ## AUTHOR CONTRIBUTIONS
- JV drafted the article, assembled and performed the analysis of data. EP, NA, and CA critically revised the article, enhancing the overall work. All authors contributed to the article and approved the submitted version.
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The COVID-19 Pandemic – A Diagnostic Industry Perspective

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The COVID-19 pandemic has brought about unprecedented changes to all facets of the healthcare system, including the diagnostic industry. The pandemic has highlighted both the challenges and strengths of the industry and has also provided valuable insights on how to be better prepared for future pandemics. In this perspective article, we describe the challenges faced by the diagnostic industry in general, particularly the difficulties encountered by Luminex Corporation, a diagnostic assay development and manufacturing company located in Austin, Texas, USA, as well as the mitigation strategies employed. In addition to discussion of the key challenges, the article provides insights on the lessons learned and steps that can be undertaken to better prepare for future outbreaks.

Keywords: COVID-19, diagnostic industry, diagnostic testing, assay development, diagnostics manufacturer

INTRODUCTION

The coronavirus-19 (COVID-19) pandemic has posed unprecedented global challenges to a multitude of sectors and industries. The pandemic not only overburdened the healthcare system but it has also significantly affected the in-vitro diagnostic industry. During the early days of the pandemic, hospitals and emergency centers were overpopulated with patients, resulting in an insufficient number of available beds and personnel, as well as an immense upsurge in need for diagnostic testing kits for identifying the SARS-CoV-2 virus, which were not yet readily available (Centers for Disease Control and Prevention, 2020). Rapid diagnosis and testing was paramount for allocating hospital resources, appropriate patient cohorting, administering effective therapeutic measures, and implementing adequate quarantining procedures (Rosenthal, 2020). Manufacturers of diagnostic assays witnessed a rapid escalation in their research and development efforts to develop tests for SARS-CoV-2. All diagnostic companies worked towards a common goal – bringing about a highly sensitive and specific test rapidly in the market and making it accessible to clinical laboratories to meet patient needs and guide the isolation practices for potentially infectious individuals. However, due to the unprecedented nature of the pandemic, the industry overall encountered tremendous challenges in terms of the available workforce, production capacity, and

ongoing supply chain issues. Additionally, the constant change in the available information and guidance from regulatory agencies created further confusion that required constant communication and monitoring of the ongoing pandemic.

Luminex[®] Corporation is a diagnostic assay development and manufacturing company located in Austin, Texas that offers a wide range of products for clinical diagnostics and biomedical research. The company offers both targeted and syndromic molecular testing panels for different disease states. Additionally, using Luminex's xMAP[®] Technology, users can perform a wide range of protein- and nucleic acid-based multiplex assays, which can simultaneously detect up to 500 targets in a single reaction. In this perspective article, we describe the unique challenges faced by the diagnostic industry during the COVID-19 pandemic, particularly focusing on the difficulties encountered by Luminex, and discuss the possible strategies that can be implemented to tackle future pandemics.

CHALLENGES IN RESEARCH AND DEVELOPMENT

During the COVID-19 pandemic, rapid and accurate diagnosis of the causative pathogen in both symptomatic and asymptomatic patients was particularly critical, as it supported appropriate patient cohorting, quarantine duration, and subsequent therapy and treatment (Rosenthal, 2020). On January 10, 2020, the viral genome sequence for SARS-CoV-2 was released for immediate public health support, and since then, hundreds of diagnostic assays have been developed commercially for the rapid detection of the novel coronavirus (Wuhan-Hu-1, GenBank accession number MN908947). From an industry perspective, developing, validating, manufacturing, and finally commercializing a diagnostic assay is an elaborate and complex process that requires interdepartmental collaboration, detailed planning, and effective time management. Several critical steps are involved in this process, including defining the need for the product, determining the clinical utility, and establishing the performance criteria of the diagnostic assay.

Similar to other diagnostic companies, Luminex offers multiple solutions for several disease states based on the needs of the clinical diagnostic laboratories. For any type of disease, the diagnostic test can be a sample-to-answer low-plex assay that may detect a few targets or a high-complexity multiplex assay that can detect many targets from a single sample. Additionally, laboratories can use extraction cassettes to design and validate their own laboratory-developed assays for clinical diagnostic purposes. Therefore, with a sudden surge in the need for SARS-CoV-2 diagnostic tests, it was essential for management and R&D to prioritize the assay platforms and chemistries for developing a SARS-CoV-2 assay based on the needs of clinical laboratories and patients. The company needed to decide whether they should prioritize the development of a molecular RT-PCR-based assay for detecting the current infection or a serological assay for determining prior exposure to the pathogen or possible immunity, or both. In addition to deciding on the type of chemistry and assay, the

R&D scientists also had to quickly develop the primer and probe designs and determine the relevant sample types and transport system/media to include in the assay claims.

The SARS-CoV-2 virus was a novel coronavirus, and therefore scientists all over the world had no access to pre-existing data regarding the pathogen. Globally, numerous studies were conducted by researchers to understand the viral structure, its pathogenicity, and transmissibility (Hsu et al., 2020). SARS-CoV-2-related available information was evolving rapidly and with the emergence of new variants and mutations, which was challenging for the R&D teams, as they had to consistently re-evaluate the developed assays to incorporate new mutations as needed. Conflicting information regarding the utility of certain assays, such as the SARS-CoV-2 serological or neutralizing assay, also caused a significant strain on research and development efforts during the early days of the pandemic. During this time, one of the primary activities of the R&D team was staying up-to-date on the constantly changing information and revising their efforts accordingly.

CHALLENGES IN MANUFACTURING AND SUPPLY CHAIN

The availability of biological materials, reagents, and other laboratory accessories is paramount for developing and commercializing any diagnostic assay. During the pandemic, laboratories and manufacturers were developing and running assays at a higher than usual pace, leading to a critical shortage of basic and essential laboratory equipment such as pipette tips, PCR reagents, tubes, and personal protective equipment (PPE), including gloves and masks. Additionally, with the World Health Organization (WHO) urging countries to ramp up testing for COVID-19, commercial assay manufacturers (including Luminex) had to surge the production of their COVID-19 assay kits which caused tremendous pressure on suppliers for sourcing assay manufacturing components such as plastics, molds, and other raw materials (World Health Organization, 2020). Most of the suppliers were not classified strictly as a healthcare business, and they were forced to shut down during the pandemic even though they were supplying critical components required for developing assay kits.

With such a massive upscale in production, another aspect to monitor and control was the quality of the assay kits being manufactured. The manufacturing team had to run a more vigorous and stringent quality control process as more kits were being produced than usual. A higher rate of manufacturing also requires adjusting the logistical chain to ensure around-the-clock production, shipping, and delivery of the testing kits. There was a tremendous demand for skilled labor and technicians, as Luminex was trying to accommodate three 8-hour manufacturing shifts in a day to meet the production demands from the customers. There were massive turnovers as demand for skilled manufacturing technicians was high, which required a constant chain of hiring and training of new personnel. The manufacturing and supply chain teams also

had to work around closed borders, canceled flights, delivery delays, and limited suppliers' stocks. Overall, the primary challenge for the team was to obtain and source the required raw material on time, produce enough kits to meet the demand, ensure the quality of the manufactured kits, and finally ship them to their destinations in a timely manner.

The high demand for testing kits also posed a significant financial burden on industries. In addition to obtaining more supplies upfront and hiring more personnel, companies also needed to invest in capital equipment to meet the production demands. At Luminex, the manufacturing team installed new automation components to speed up the processes, which required additional investment and training. Moreover, as the other instruments were working at a higher capacity than usual, it escalated the failure rates of modules and other components that demanded an increase in routine maintenance services.

CHALLENGES IN REGULATORY AFFAIRS

The Food and Drug Administration's (FDA) clearance of any medical devices and diagnostic assays is critical to obtain before product commercialization in the United States. It ensures that the device or test meets the required performance, safety, and effectiveness standards. Under normal circumstances, the Luminex regulatory affairs team works for months to obtain the necessary data required for FDA IVD-clearance by conducting rigorous clinical trials using prospective and retrospective patient samples. The approval process further requires detailed documentation of the performed clinical trial, and often takes months post-data submission to obtain the final clearance for diagnostic use. However, during emergencies, the FDA can clear diagnostic tests and devices under Emergency Use Authorization (EUA) after receiving the minimum required data or evidence regarding the safety and efficacy of the product (U.S. Food and Drug Administration, 2022).

During the COVID-19 pandemic, the FDA started issuing EUAs for SARS-CoV-2 diagnostic kits to meet the required testing needs for initiating appropriate patient isolation protocols and therapy. Although the EUA guidelines were less stringent, validation and performance evaluation using either real or contrived clinical samples is still required to obtain the FDA clearance before commercialization of the assays. Getting clinical samples from patients was challenging during the initial days of the pandemic because of their high demand from various laboratories and manufacturers, which threatened industrial assay developers with significant delays for commercialization. At Luminex, the assay development and regulatory teams had to rely on contrived samples for assay development and initial testing, and *in silico* analyses were also performed for generating the required evidence and data. During the approval process, constant communication with the FDA was essential, as reviews and suggestions for required changes were obtained almost daily, which otherwise would typically take days or months. It was further challenging to meet the FDA EUA regulatory guidelines, as in some cases

there was a need to modify requirements based on the progress on the pandemic and availability of new data.

CHALLENGES IN MARKETING AND SALES

The marketing team plays a crucial role in any product launch and development in the diagnostic industry. Under the usual business regimen, the marketing department takes part in deciding the characteristics of the diagnostic assay and determines the target market segment, price points, and the overall positioning and messaging for the product. These things needed to be handled differently with the pandemic, as there was no time for detailed and meticulous planning. The primary focus of diagnostic companies was how to provide a faster and more accurate test to all of their customers, irrespective of market segmentation, to meet patient needs and help control the pandemic. A strategic plan for commercialization that may take several months had to be implemented in a few weeks.

The seriousness of the pandemic demanded a quicker assay development timeline and a rapid product launch using limited available resources. This incurred a sudden surge of a financial burden on companies from various standpoints such as research and development, manufacturing, and shipping. Similar to other diagnostic companies, Luminex had to seek out federal government aid and apply for funding to support their research and developmental activities. The Luminex team was able to secure several millions of dollars in funding from the U.S. Department of Health and Human Services' Biomedical Advanced Research and Development Authority (BARDA) to support the development and validation of several projects, including a COVID-19 multiplex antibody test and a multiplex respiratory panel for Flu A/B, respiratory syncytial virus (RSV), and SARS-CoV-2 targets (Cision PR Newswire, 2022). Furthermore, due to the successful launch of the sample-to-answer SARS-COV-2 molecular assay, the company was able to secure additional funding for further improvements to manufacturing and alleviating supply chain issues.

The sales teams of the diagnostic companies also played a critical part during the pandemic. They had to prioritize their customers, meet their testing needs quickly, and provide adequate training and troubleshooting support. Since Luminex's sample-to-answer PCR system also supports laboratory-developed tests (LDTs), many laboratories were developing their own assays using the sequences provided by the U.S. CDC and Wuhan Institute of Virology. Therefore, in addition to providing customers with the commercially developed assay, the team also had to take care of the high complexity labs that were using the general purpose reagents and consumables with their own primers to obtain their own EUA. This challenge was global as the company had to support laboratories worldwide.

Another significant challenge faced by the company was the rapidly changing information available during the initial days of the pandemic. Laboratories implemented multiple testing platforms to accommodate the changing requirements, high patient volume, and

supply chain issues, which required a faster training time and a more demanding validation support for the assays they were using. The manufacturers had to stay on top of changing recommendations and guidelines which occurred as the pandemic evolved, and constant communication with the FDA and the CDC was vital during this time.

LESSONS LEARNED FOR FUTURE

The COVID-19 pandemic brought about a significant change in the in-vitro diagnostic industry. While the pandemic brought the laboratories and industry together to focus on a common goal, it has also highlighted numerous challenges that may be encountered during the development and commercialization of a diagnostic test under emergency and critical circumstances. The diagnostic industry must learn from this experience and implement necessary steps to ensure better preparedness for such future emergencies.

A glaring shortcoming that became evident during this pandemic was the global shortage of essential raw materials required for developing an assay. Companies faced a shortage of reagents, PPE, and other necessary laboratory equipment, such as pipette tips. For the future, companies must evaluate and implement multi-sourcing and multi-manufacturing models to ensure that critical raw materials are always available. Companies need to identify materials and products that are absolutely critical for their businesses and have a reasonable stockpile inventory of those products to ensure they are always available. It is important to understand the supplier(s), their capacity, and maintain an overall healthy working relationship. Better internal surveillance of the epidemiological data is also needed in the diagnostic industry so that they can rationally predict an outbreak and can be prepared.

Additionally, IVD industry stakeholders, including laboratories, manufacturers, and government agencies, need to communicate amongst themselves to coordinate and cooperate in developing a comprehensive national response plan for future outbreaks. Collaborative strategies should be developed to ensure that testing supplies (reagents and consumables) would be available during the different stages of a pandemic. For future preparedness, administrative bodies should also collaborate with IVD assay manufacturers and provide funding to overstock their testing supplies such as reagents, PPE, and laboratory equipment, so an extra amount of necessary test supplies is readily available (O'Connor, 2021). However, manufacturers should consider if these supplies can be used or sold before expiration.

Diagnostic companies should identify their bottlenecks and capacity trigger points from a manufacturing standpoint. Manufacturers should clearly understand their production capacity and the steps needed to escalate it on demand. It is essential that production facilities have emergency escalation plans that would aid in rapid decision-making and expansion during emergencies. Communication within different departments is also vital in establishing goals and preparing for the future.

Another critical gap was the lack of a clear research and development response strategy, which includes better

preparedness for developing an assay and collaborative efforts between different industries and stakeholders such as funding and regulatory agencies, government entities, epidemiological institutions, and researchers. Partnership efforts with different laboratories and contract research organizations for assay development can also be helpful to draw expertise from diverse areas such as biochemistry, virology, molecular biology, etc. that might assist with optimizing assay parameters. The inconsistencies observed in the diagnostic performance between different assays highlighted the need for a close partnership between assay manufacturers and component and material providers to reduce the risk of choosing suboptimal parts, specifically in sectors dealing with lateral flow based assays (Abate, 2020). Additionally, having a close relationship with clinical sample banks, government agencies, and hospitals is critical for obtaining clinical samples during emergencies for assay development and testing.

The COVID-19 pandemic has further demonstrated how regulatory timelines can be drastically improved and accelerated, and how assay developers can adapt and benefit from the accelerated pace. During an emergency, it is essential to determine the amount of oversight needed so that inadequate monitoring does not lead to inaccurate or faulty devices flooding the market or an over-extensive review creates a delay in test availability. Additionally, it is vital to establish clinical endpoints and desired performance parameters early to provide adequate transparency to the diagnostic assay developers. Regulatory agencies and policymakers should ensure that the diagnostic community can always access the required data infrastructure to evaluate different testing strategies (Wiegmann and Roca, 2021). Regulatory coordination is also of paramount importance on a global scale, and both national and international organizations should collaborate to develop comprehensive response plans and determine adequate regulatory approval processes. Evidence-based policymaking is essential during times of crisis as information changes quickly.

CONCLUSION

The COVID-19 pandemic served as a revelation for the diagnostic industry, highlighting both its strengths and weaknesses. On the one hand, there were innovations, rapid responses towards fulfilling a common goal, and immense adaptability, whereas, on the other hand, there were massive manufacturing and supply chain issues, the inability to meet the required demand, and discrepancies in the knowledge disseminated from various sources. The three critical needs identified during the pandemic were timeliness, accuracy, and availability. The adaptations and innovations that have been embraced during this pandemic will surely cause a drastic change in the overall operations and functions of the diagnostic industry. Similar to other companies, Luminex is also identifying their shortcomings and the potential bottlenecks from every departmental standpoint and are implementing better operational processes for the future. The novel circumstances

associated with the pandemic have facilitated an overall transformation and evolved the diagnostics industry towards more forward-facing patient-centric solutions that will endure even after the pandemic.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

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Large Scale SARS-CoV-2 Molecular Testing and Genomic Surveillance Reveal Prolonged Infections, Protracted RNA shedding, and Viral Reinfections

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Large-scale SARS-CoV-2 molecular testing coupled with whole genome sequencing in the diagnostic laboratories is instrumental for real-time genomic surveillance. The extensive genomic, laboratory, and clinical data provide a valuable resource for understanding cases of reinfection versus prolonged RNA shedding and protracted infections. In this study, data from a total of 22,292 clinical specimens, positive by SARS-CoV-2 molecular diagnosis at Johns Hopkins clinical virology laboratory between March 11th 2020 to September 23rd 2021, were used to identify patients with two or more positive results. A total of 3,650 samples collected from 1,529 patients who had between 2 and 20 positive results were identified in a time frame that extended up to 403 days from the first positive. Cycle threshold values (Ct) were available for 1,622 samples, the median of which was over 30 by 11 days after the first positive. Extended recovery of infectious virus on cell culture was notable for up to 70 days after the first positive in immunocompromised patients. Whole genome sequencing data generated as a part of our SARS-CoV-2 genomic surveillance was available for 1,027 samples from patients that had multiple positive tests. Positive samples collected more than 10 days after initial positive with high quality sequences (coverage >90% and mean depth >100), were more likely to be from unvaccinated, or immunosuppressed patients. Reinfections with viral variants of concern were found in 3 patients more than 130 days from prior infections with a different viral clade. In 75 patients that had 2 or more high quality sequences, the acquisition of more substitutions or deletions was associated with lack of vaccination and

longer time between the recovered viruses. Our study highlights the value of integrating genomic, laboratory, and clinical data for understanding the biology of SARS-CoV-2 as well as for setting a precedent for future epidemics and pandemics.

Keywords: SARS-CoV-2, variants, prolonged infection, prolonged shedding, reinfection

INTRODUCTION

The molecular detection of SARS-CoV-2 has been the gold standard for COVID-19 diagnosis since the beginning of the pandemic. The infrequency of diagnostic tests in March 2020 that limited testing to symptomatic patients under investigation was quickly replaced with large scale screening for both symptomatic and asymptomatic individuals (Anonymous and Centers for Disease Control and Prevention). Although a single positive result is sufficient for making a COVID-19 diagnosis, repeated testing of patients with positive test results has been a notable practice since the beginning of the pandemic. Hospitalized positive patients were re-tested to make infection control related decisions (Gniazdowski et al., 2020). In addition, re-testing is common in immunocompromised patients with symptoms, patients scheduled for certain procedures, as well as patients who develop new symptoms after resolution of COVID-19 (Dong et al., 2021).

Repeat testing revealed that detection of SARS-CoV-2 RNA can be prolonged for up to several months after the start of symptoms (Fontana et al., 2021). The significance of prolonged RNA shedding is dependent on the patient population and the clinical context. In patients with severe disease, the median duration of RNA shedding was longer than mild/asymptomatic cases (Liu et al., 2020; Xu et al., 2020; Zheng et al., 2020). Most cases of prolonged RNA shedding were associated with non-infectious viral recovery. Recovery of infectious virus from patients with extended RNA shedding was mainly reported from immunocompromised patients (Avanzato et al., 2020; Aydiello et al., 2020; Choi et al., 2020; Baang et al., 2021; Tarhini et al., 2021; Truong et al., 2021). A few cases of confirmed reinfections were reported; however, those cases are difficult to differentiate from prolonged infections or RNA shedding without whole genome sequencing. The Centers for Disease Control and Prevention (CDC) has defined the criteria of probable reinfections to either (a) a repeat positive molecular test 90 days after the initial infection regardless of symptoms; or (b) a repeat positive molecular test 45 days after the initial infection in the presence of symptoms consistent with COVID-19 (CDC. Centers for Disease Control and Prevention).

The Johns Hopkins clinical virology Laboratory started molecular testing for SARS-CoV-2 on March 11th 2020, and SARS-CoV-2 whole genome sequencing for understanding the genomic diversity started with the diagnosis of the first positives (Uhteg et al., 2020; Thielen et al., 2021). As of October 9th, 2021, the laboratory has tested a total of 555,983 specimens, identified a total of 28,904 positives, and sequenced a total of 8,027 genomes. In this study, we identified patients who had more than one positive result in our laboratory. The time between the first and subsequent tests, cycle threshold (Ct) values, clinical

data, and genomic data were examined in addition to cell culture of selected samples to differentiate cases of prolonged RNA shedding, persistent infection, and reinfections. The impact of vaccination and immune suppression on prolonged shedding was evaluated.

MATERIALS AND METHODS

Ethical Considerations and Data Availability

Ethical approval for this study was obtained from the Johns Hopkins Institutional Review Board (IRB00221396) with a waiver of consent. Whole viral genomes were made publicly available at GISAID.

Data and Sample Selection

Molecular diagnosis for SARS-CoV-2 at Johns Hopkins diagnostic laboratory is performed by different assays that include the NeuMoDx (Qiagen) (Mostafa et al., 2020a; Mostafa et al., 2020b), cobas (Roche) (Mostafa et al., 2020a), Aptima (Hologic), the Xpert Xpress SARS-CoV-2/Flu/RSV (Cepheid) (Mostafa et al., 2020c), the ePlex respiratory pathogen panel 2 (Roche) (Jarrett et al., 2021), the Accula (Hogan et al., 2020), and the RealStar SARS-CoV-2 assays (Altona diagnostics) (Uhteg et al., 2020). Testing was performed in accordance with the manufacturer instructions and the Johns Hopkins laboratory's validated protocols. Patients with more than one positive result were identified *via* the laboratory information system (SOFT). Only Ct values collected from the NeuMoDx assay were used in analysis as the majority of testing is performed using this system (target used, NSP2 gene). Samples with whole genome sequencing data were identified through our surveillance database. Whole genome sequencing and genomic data analysis were performed as we described previously (Morris et al., 2021; Thielen et al., 2021).

Post Consensus Analysis of Genomes

High quality genomes were defined as genomes with >90% coverage and a mean depth of >100. For analysis of acquired substitutions and deletions, only high-quality genomes were used and genomic areas with poor coverage were excluded. Manual reviews were performed on the acquired substitutions and deletions using the integrated genomic viewer.

Cell Culture

Aliquots of swab specimens were cultured on Vero-TMPRSS2 cells as previously described for VeroE6 cells (Gniazdowski et al., 2020). Cultures with cytopathic effect were confirmed for the presence of SARS-CoV-2 by reverse transcriptase PCR.

Clinical Data Analysis

Clinical data for the cohort were retrieved *via* bulk extraction from a data warehouse that contains all encounter-related information from hospital and outpatient visits to any Johns Hopkins Medical Institutions Facilities in addition to manual reviews of electronic medical charts. Codes associated with immunosuppression are listed in **Table S1**.

Statistical Analysis

Chi squared or Welch's t-tests were performed to show associations depending on type and number of results evaluated. Linear regression was performed with Scipy and visualized with Seaborn (Waskom, 2021).

RESULTS

Repeat Positives From March 11th 2020 to September 23rd 2021

In the time frame between March 11th 2020 and September 23rd 2021, a total of 542,948 samples were tested at the Johns Hopkins clinical virology laboratory, of which 28,521 tested positive with a total positivity rate of 5.3%. A total of 1,529 patients had more than one SARS-CoV-2 positive result for during this timeframe,

for a total of 3,650 samples (7% of the total positives, **Figure 1**). Repeat positive samples were collected between 0 and 403 days from the original positive sample (**Figure 2A**). a total of 943 of the 2,057 repeat samples were tested within the first 10 days of the first positive sample. The majority of the rest were testing that was primarily repeated between 11-20 days (N = 528) after the first positive and only 63 were tested 100 days or more after the first positive (**Figure 2A**).

As SARS-CoV-2 continued RNA positivity during the first 10 days of symptoms is expected (Sethuraman et al., 2020), and to exclude repeat testing that was performed for some patients in the same day, we limited the majority of our analysis to repeat positive samples that occurred more than 10 days from the original positive. Ct values were available for 589 of the 1,115 repeat samples > 10 days from the initial positive result (52%; **Figures 2B, C**). Although Ct values were lower prior to 11 days from the initial sample (data not shown), the majority of samples collected after 11 days from the first positive showed CT values greater than 30 (**Figures 2B, C**). The majority of samples were collected prior to 100 days, and only two Ct values were available for samples collected 300 days after the first positive (**Figure 2B**).

Of the 1,151 repeat samples more than 10 days from the initial positive, whole genome sequencing was attempted on 222 samples collected from 173 of the 795 patients from this cohort

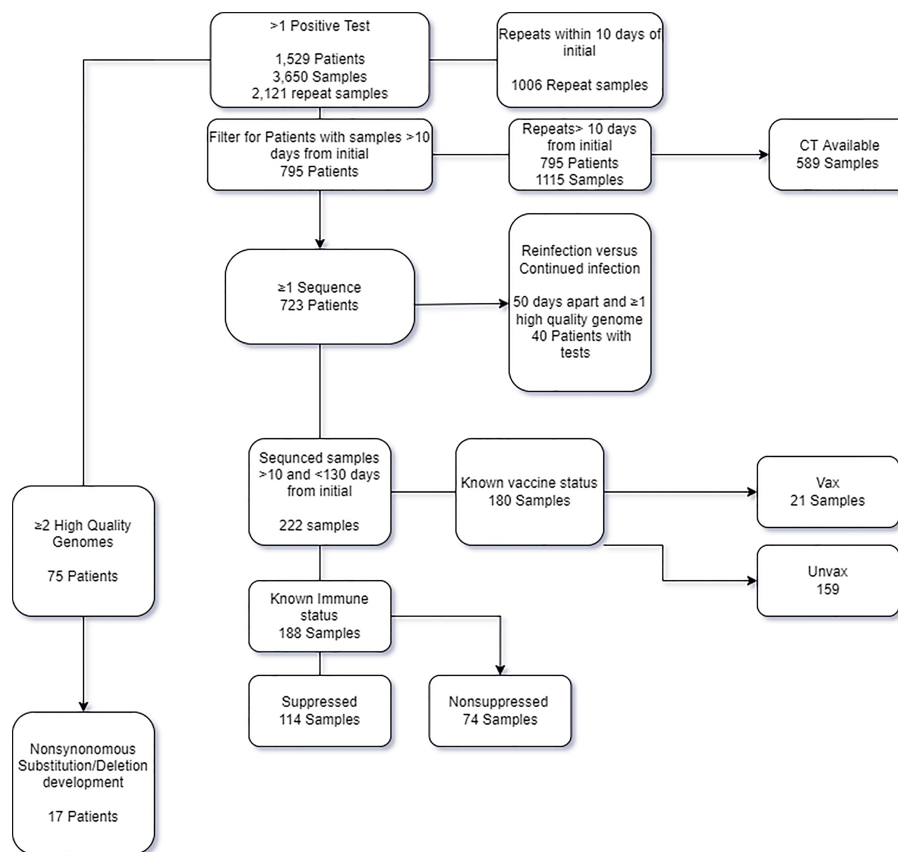


FIGURE 1 | Flow chart of the patients and samples included in the study and analysis.

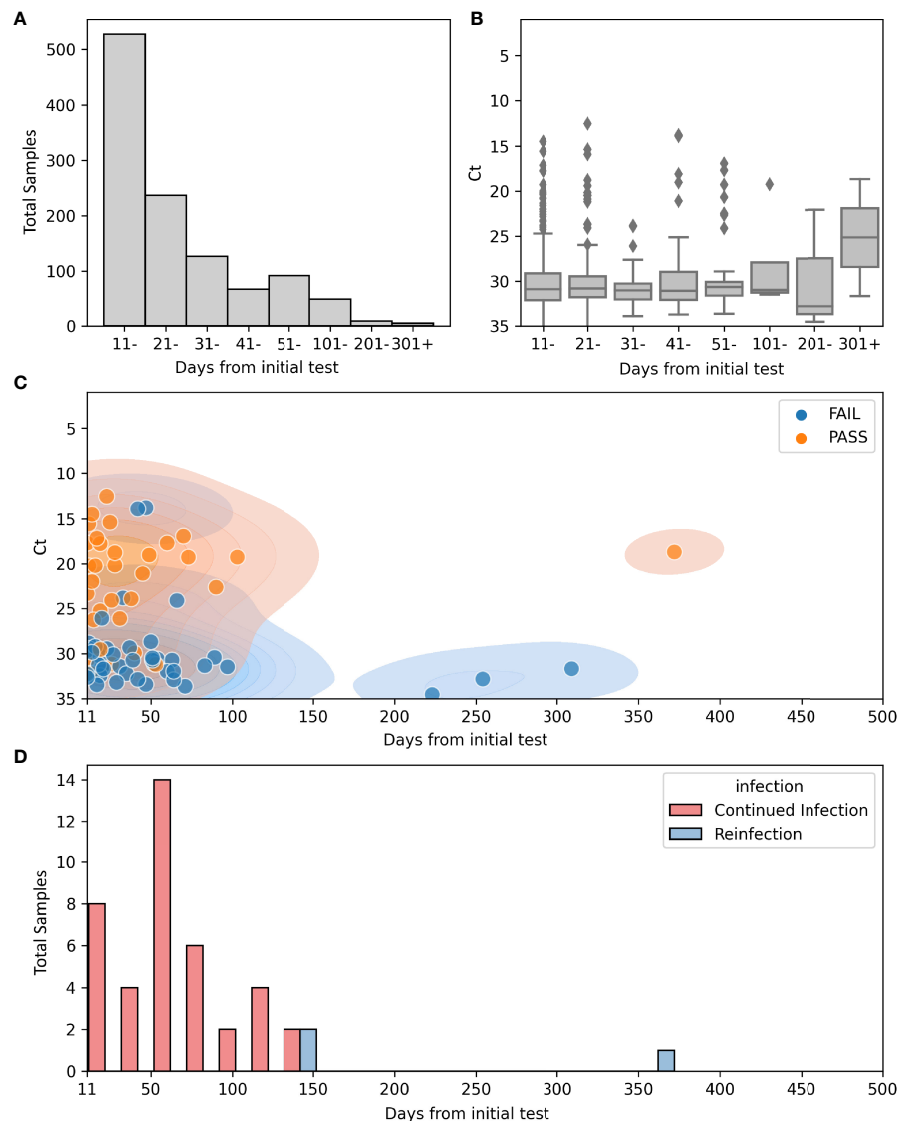


FIGURE 2 | Repeat positives from March 11th 2020 to September 23rd 2021. **(A)** Count plot of total numbers of repeat positives >10 days from the initial positive and the relation to the time after the first positive (N = 1115): 528 (11-20), 237 (21-30), 127 (31-40), 67 (41-50), 92 (51-100), 49 (101-200), 9 (201-300), 6 (301-400). **(B)** Boxplot for Ct values in repeat positive samples and the relation to the time after the first positive (N = 589): 306 (11-20), 140 (21-30), 60 (31-40), 34 (41-50), 40 (51-100), 4 (101-200), 3 (201-300), 2 (301-400). **(C)** Scatterplot of sequenced repeat samples >10 days from initial positive overlying kernel density estimate (KDE plot) of the same information. Hue indicates quality of sequences. N=222 Total, 155 failed quality metrics, 67 High quality genomes. **(D)** Histogram of days from initial positive sample in continued infection (Red, N = 40) or reinfection (Blue, N = 3).

(Figure 2C). Of the sequenced samples in this cohort, 69.8% did not meet the quality cut-off scores (155 of 222) (Table 1). The mean Ct values of samples with high quality sequences was 22.1 in contrast to a mean Ct of 30.4 for those with low quality (data detailed in Table S2 and summarized in Table 1).

Prolonged Shedding Versus Reinfections

To differentiate cases of prolonged shedding from reinfections based on viral genomics analyses, genomes from the same patients were compared. For this analysis, we focused primarily on the 40 patients in our cohort who had at least two positive

SARS-CoV-2 PCR tests >50 days apart and with at least one high quality genome (Table S3). Patients from this group tested positive between 2 and 16 times and the time between the first and last positive tests was between 51 and 372 days. In total, there were 162 positive samples of which 109 were sequenced and all available genomes were analyzed regardless of time from initial positive. Despite low quality sequences of genomes from many of the samples collected at later time points, 14 of these patients showed evidence of prolonged shedding of RNA, consistent with the initial infecting virus, and only 3 showed genomic evidence of reinfection (Table 2 and Table S3). Two were initially infected

TABLE 1 | Samples with whole genome sequencing data in our cohort of repeats collected 11 days or more after the first positive.

| Days after the first positive | Genomes with high quality | | | Genomes with low quality | | | p value |
|-------------------------------|---------------------------|------------|-------|--------------------------|------------|-------|----------|
| | Number | Average Ct | Stdev | Number | Average Ct | Stdev | |
| 11- 20 | 28 | 21.1 | 5.7 | 43 | 31.2 | 2.3 | 0.00006 |
| 21-50 | 25 | 21.1 | 5.7 | 46 | 30.4 | 6.5 | 0.02 |
| 51+ | 14 | 19.3 | 4.9 | 66 | 31.4 | 2.3 | 0.0009 |
| total | 67 | 20.24 | 5.4 | 155 | 31.24 | 4.2 | 1.00E-09 |

High quality genomes were defined as sequences with coverage >90% and a mean depth of >100. Ct, cycle threshold; stdev, standard deviation.

with one clade, [20G or 20I (Alpha)], followed by a later infection with the Delta variant. The third patient was considered a reinfection because the second infection was with the Delta variant which was not circulating when the patient first tested positive 372 days earlier (**Table 2**). The earliest positive test from a reinfection occurred 133 days following the initial infection (**Figure 2D**), and Continued RNA detection was noted as far as 139 days following the initial positive.

Of the 14 patients with genomic evidence of prolonged shedding, Cell culture was attempted for 28 samples from this cohort to assess prolonged active infection versus prolonged RNA shedding. Three immunocompromised patients showed prolonged recovery of infectious virus on cell culture (**Table 2**, patients 10, 12, and 16) for 53, 60, and 70 days after the first positive, indicating persistent infection. Notably, three additional immunocompromised patients had prolonged shedding (patients 15, 19, and 20, **Table 2**) with a notable low Ct values or recovery of complete genomes for up to 117, 61, and 31 days after the first positive. The rest of the 14 patients from this cohort had extended shedding at higher Ct values, and 6 patients had negative cell culture results suggesting protracted RNA shedding rather than persistent infection (**Table 2**).

Impact of Vaccination on Prolonged Viral Shedding

To study the impact of vaccination on prolonged viral shedding, we compared the genomic data of samples from vaccinated compared to unvaccinated patients. We limited this analysis to sequenced samples that were positive 11 to 130 days from the initial positive test (in order to remove possible repeat infections, as repeat infections started to be seen at 130 days). Patients who had received the full vaccination series at least 2 weeks prior to the first positive test were classified as vaccinated in this cohort. Only 21 samples from vaccinated patients with repeated positive tests met these criteria (**Figure 3A**) compared to 159 repeat positive samples from unvaccinated patients. Only 5 of 21(23%) genomes within this timeframe met our quality scores in vaccinated patients compared to 55 of 159(35%) in unvaccinated patients, despite older median age in the vaccinated patients (64 compared to 55, $p=0.00065$) (**Figure 3B**), and lower median days from the initial test for all samples where sequencing was attempted (17 days vaccinated, 32 days unvaccinated, $p=0.0003$, **Figure 3C**). In general, genomes of repeat positives from vaccinated patients after 20 days from the first positives were very few which caused a notable trend of a decrease in coverage with time when compared to the unvaccinated group (**Figure 3D**).

Impact of Immunosuppression on Prolonged Viral Shedding

To evaluate the impact of immunosuppression the coverage of the SARS-CoV-2 genome in 74 samples from patients without immunosuppression were compared to 114 samples from patients with immunosuppression (greater than 10 days and less than 130 days from the initial positive). High quality genomes were more represented at a higher percentage in Immunosuppressed compared to non-immunosuppressed patients (43% compared to 20%, p -value 0.0009, **Figure 4A**). Immunosuppressed patients were older than non-immunosuppressed patients (median years 60 compared to 40.5, p -value >0.0001, **Figure 4B**). However, high quality genomes were recovered for similar mean days between immunosuppressed and non-immunosuppressed patients (33.1, compared to 31.5 days, **Figure 4C**) with similar coverage over-time (**Figure 4D**). Additional analysis was performed to see how vaccination impacted Immunosuppressed patients. Of the sampled from immunosuppressed patients, 13 were from vaccinated and 94 were from unvaccinated patients. The percentages of samples that passed quality control from vaccinated and unvaccinated immunosuppressed were similar, but high-quality genomes were obtained for shorter periods of time in samples from vaccinated compared to unvaccinated patients (16.6 days compared to 35.7 days, $p=0.009$) (data not shown).

Prolonged Viral Shedding and Genomic Changes

In order to study the development of new substitutions or deletions within the genome of SARS-CoV-2 over-time, within the same patient, we identified all patients for whom we had at least 2 high quality genomes from different samples and whose genomic data was consistent with prolonged shedding or persistent infection but not reinfection. This analysis was not limited to a specific timeframe between positive samples. We identified 75 patients that met these criteria. The timeframe between the initial and subsequent high-quality genomes was between 0 and 117 days (detailed in **Table S4**). A total of 48 amino acid (AA) substitutions or deletions developed in 17 of these patients (**Table 3**). Of the 17 patients that acquired substitutions or deletions, 16 were unvaccinated and 14 were immunosuppressed. The substitutions and deletions which developed and the number of instances in our cohort can be seen in **Table S5**, along with information on how many times these specific substitutions or deletions have been seen among all the genomes sequenced in our laboratory and lineages associated

TABLE 2 | Prolonged shedding, persistent infections, versus reinfections.

| PID | Vaccinated | Immunosuppressed | Days from the first positive | HPID | Cell culture | Ct | % Coverage | Depth | Lineage | Clade |
|-----------------------------|------------|------------------|------------------------------|---------|--------------|-------|------------|--------|-----------|-----------------|
| Reinfections | | | | | | | | | | |
| 1 | Yes | No | 0 | | | | | | | |
| | | | 372 | HP08786 | | 18.7 | 98.68 | 400.00 | B.1.617.2 | 21A (Delta) |
| 13 | Yes | Yes | 0 | HP05124 | | | 99.60 | 400.00 | B.1.2 | 20G |
| | | | 11 | HP04658 | | 17.73 | 98.60 | 385.09 | B.1.2 | 20G |
| | | | 144 | HP08875 | | | 86.10 | 172.40 | AY.7.1 | 21A (Delta) |
| 28 | Yes | No | 0 | HP05001 | | | 98.87 | 333.78 | B.1.1.7 | 20I (Alpha, V1) |
| | | | 133 | HP09101 | | | 95.54 | 381.78 | B.1.617.2 | 21A (Delta) |
| Persistent infection | | | | | | | | | | |
| 10 | No | Yes | 0 | | | 24.19 | | | | |
| | | | 27 | | | 23.65 | | | | |
| | | | 33 | HP13078 | Positive | 23.83 | 0.00 | 3.00 | None | Attempted |
| | | | 38 | HP13068 | Negative | 23.93 | 97.25 | 349.00 | A.3 | 19B |
| | | | 53 | | Positive | 20.69 | | | | |
| | | | 81 | HP13137 | Negative | | 95.50 | 378.49 | A.3 | 19B |
| 12 | No | Yes | 0 | | | | | | | |
| | | | 6 | HP13142 | Positive | 16.99 | 99.60 | 400.00 | B.1 | 20C |
| | | | 42 | HP13143 | Positive | 13.93 | 2.69 | 9.15 | None | Attempted |
| | | | 46 | | | | | | | |
| | | | 60 | HP13074 | Positive | 17.71 | 98.73 | 400.00 | B.1 | 20C |
| | | | 73 | HP13075 | Negative | 19.28 | 98.75 | 400.00 | B.1 | 20C |
| | | | 84 | | | 22.47 | | | | |
| 16 | No | Yes | 0 | HP13076 | Positive | 16.5 | 99.01 | 400.00 | B.1.520 | 20C |
| | | | 39 | HP13144 | Positive | | 99.59 | 400.00 | B.1.520 | 20C |
| | | | 65 | | | | | | | |
| | | | 70 | HP13077 | Positive | 16.95 | 99.57 | 400.00 | B.1 | 20C |
| Prolonged shedding | | | | | | | | | | |
| 15 | No | Yes | 0 | HP12091 | Positive | 18.62 | 0.00 | 400.00 | B.1.1.434 | 20B |
| | | | 20 | | | | | | | |
| | | | 26 | HP12092 | Positive | 24.1 | 0.00 | 378.28 | B.1.1.434 | 20B |
| | | | 33 | HP12093 | Negative | | | 235.88 | B.1.1.434 | 20B |
| | | | 38 | HP12094 | Negative | | | 400.00 | B.1.1.434 | 20B |
| | | | 103 | HP02090 | | 19.27 | 99.57 | 400.00 | B.1.1.434 | 20B |
| | | | 117 | HP02480 | | | 98.59 | 142.00 | B.1.1.434 | 20B |
| | | | 126 | HP02621 | | | 64.60 | 63.50 | None | 20B |
| | | | 132 | HP03033 | | | 32.65 | 23.28 | None | 20B |
| | | | 139 | HP03236 | | | 9.04 | 5.85 | None | Attempted |
| 19 | Yes | Yes | 0 | HP02155 | | 13.69 | 99.60 | 400.00 | B.1.409 | 20A |
| | | | 19 | | | | | | | |
| | | | 57 | HP01654 | | | 99.59 | 400.00 | B.1.409 | 20A |
| | | | 61 | HP01599 | | | 99.59 | 400.00 | B.1.409 | 20A |
| | | | 77 | HP02479 | | | 13.62 | 9.00 | None | Attempted |
| 20 | No | Yes | 0 | | | 19.15 | | | | |
| | | | 1 | HP03788 | | 14.16 | 99.60 | 400.00 | B.1.526 | 21F (Iota) |
| | | | 7 | | | 15.31 | | | | |
| | | | 14 | HP04418 | | 14.52 | 98.60 | 400.00 | B.1.526 | 21F (Iota) |
| | | | 19 | HP04748 | | 17.78 | 99.60 | 400.00 | B.1.526 | 21F (Iota) |
| | | | 21 | HP04804 | | | 98.60 | 400.00 | B.1.526 | 21F (Iota) |
| | | | 25 | HP04922 | | 15.41 | 99.60 | 400.00 | B.1.526 | 21F (Iota) |
| | | | 28 | HP05155 | | 18.78 | 97.75 | 400.00 | B.1.526 | 21F (Iota) |
| | | | 31 | HP05251 | | 26.11 | 97.89 | 354.79 | B.1.526 | 21F (Iota) |
| | | | 35 | | | | | | | |
| | | | 39 | | | | | | | |
| | | | 64 | HP06212 | | 32.91 | 56.72 | 172.78 | None | 21F (Iota) |
| 3 | No | | 0 | HP13038 | Positive | 20.22 | 99.60 | 400.00 | B.1 | 20A |
| | | | 50 | | | 25.13 | | | | |
| | | | 50 | HP10152 | | | 87.25 | 165.00 | B.1 | 20A |
| | | | 54 | HP13039 | Negative | 30.61 | 89.76 | 102.34 | B.1 | 20A |
| 4 | No | Yes | 0 | HP13053 | Positive | 21.41 | 99.60 | 400.00 | B.1.494 | 20C |
| | | | 36 | | | 29.45 | | | | |
| | | | 37 | | | | | | | |
| | | | 64 | HP13054 | Negative | | 76.25 | 40.85 | B.1.446 | 20C |
| | | | 124 | | | 31.53 | | | | |

(Continued)

TABLE 2 | Continued

| PID | Vaccinated | Immunosuppressed | Days from the first positive | HPID | Cell culture | Ct | % Coverage | Depth | Lineage | Clade |
|-----|------------|------------------|------------------------------|---------|--------------|-------|------------|--------|---------|-----------|
| 11 | No | No | 0 | HP13040 | Negative | 25.43 | 99.60 | 400.00 | B.1.369 | 20C |
| | | | 38 | | | 31.51 | | | | |
| | | | 53 | HP13041 | Negative | 31.21 | 90.19 | 227.39 | B.1.369 | 20C |
| 27 | No | No | 0 | HP13044 | Positive | 14.47 | 99.60 | 400.00 | B.1.369 | 20C |
| | | | 25 | | | 33.14 | | | | |
| | | | 51 | HP13045 | Negative | 30.73 | 67.59 | 227.37 | None | 20C |
| 29 | Yes | Yes | 0 | HP02004 | | | 99.60 | 400.00 | B.1.2 | 20G |
| | | | 3 | HP02005 | | 15.53 | 99.60 | 400.00 | B.1.2 | 20G |
| | | | 12 | HP02006 | | | 99.60 | 387.00 | B.1.2 | 20G |
| | | | 17 | HP02007 | | | 98.60 | 400.00 | B.1.2 | 20G |
| | | | 23 | | | | | | | |
| | | | 24 | | | 20.48 | | | | |
| | | | 30 | | | 21.19 | | | | |
| | | | 37 | HP02008 | | 29.34 | 37.73 | 26.00 | None | 20C |
| | | | 44 | HP02009 | | | 81.92 | 117.00 | B.1.2 | 20G |
| | | | 51 | HP02010 | | 30.48 | 4.47 | 4.00 | None | Attempted |
| 34 | Yes | No | 0 | HP13046 | Negative | 29.6 | 99.60 | 400.00 | B.1 | 20C |
| | | | 92 | HP13047 | Negative | | 46.98 | 52.01 | None | 20C |
| 35 | Yes | No | 0 | HP13036 | Negative | 32.86 | 99.59 | 212.41 | B.1.110 | 20A |
| | | | 16 | | | 29.14 | | | | |
| | | | 57 | HP13037 | Negative | | 87.83 | 64.10 | B.1 | 20A |
| 37 | Yes | Yes | 0 | HP02713 | Positive | | 99.60 | 400.00 | B.1.2 | 20G |
| | | | 23 | HP03492 | | | 99.60 | 368.98 | B.1.2 | 20G |
| | | | 38 | | | | | | | |
| | | | 39 | | | | | | | |
| | | | 45 | HP04440 | | 21.11 | 98.60 | 387.16 | B.1.2 | 20G |
| | | | 50 | HP04626 | | 28.69 | 31.43 | 18.08 | None | 20G |
| | | | 57 | HP04882 | | 29.1 | 94.15 | 314.68 | B.1.2 | 20G |

PID, patient identifier; HPID, identification of samples with whole genome sequencing; Ct, cycle threshold.

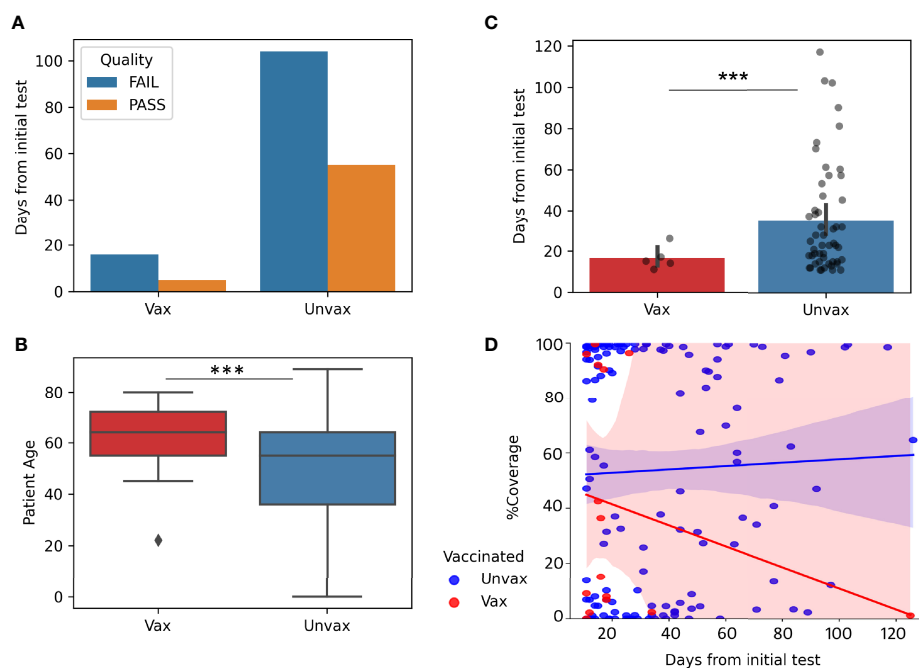


FIGURE 3 | Impact of Vaccination on prolonged viral shedding. Genomes from repeat positive samples within the first 130 days after the initial positives (N = 21 Vax “vaccinated” and 159 Unvax “unvaccinated”). **(A)** Quality of genomes in samples from vaccinated and unvaccinated patients. **(B)** Boxplot of Patient Age in samples from vaccinated (Median 64 years) and unvaccinated (median 55 years). **(C)** Barplots with overlaid strip plots of the recovery of high-quality genomes compared in relation to days after the first positive. **(D)** Implots of genome coverage compared to days from the initial positive. *** $p < 0.0001$.

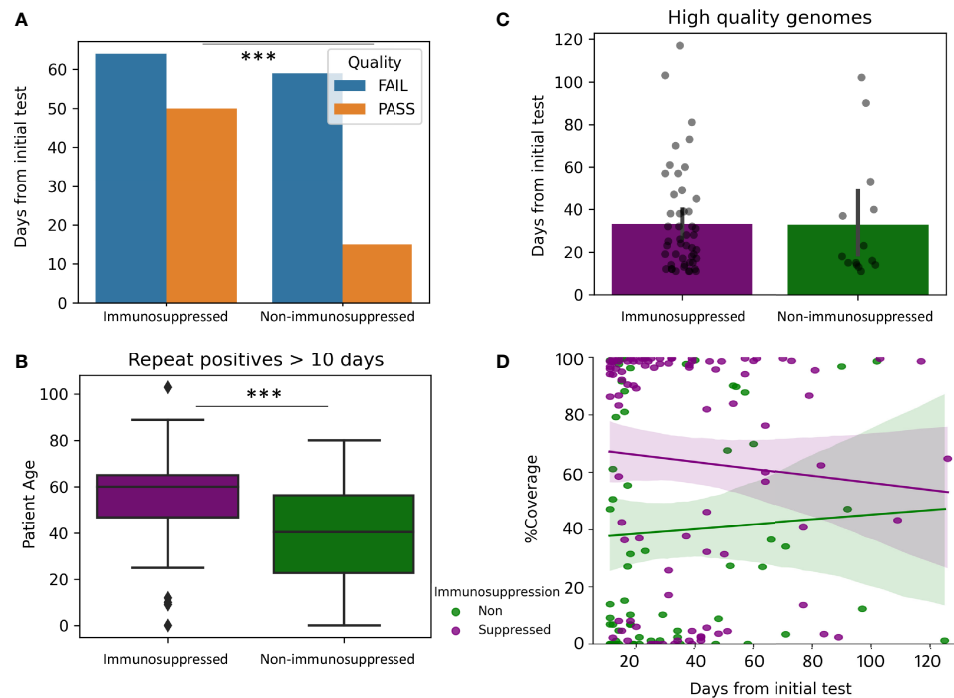


FIGURE 4 | Impact of Immunosuppression on prolonged viral shedding. Genomes from repeat positive samples within the first 130 days after the initial positives (N = 114 immunosuppressed and 74 non-immunosuppressed). **(A)** Quality of genomes in samples from immunosuppressed and non-immunosuppressed patients. **(B)** Boxplot of patient age in samples from immunosuppressed (Median 60 years) and non-immunosuppressed (median 40.5 years). **(C)** Barplots with overlaid strip plots of the recovery of high-quality genomes compared in relation to days after the first positive. **(D)** Implots of genome coverage compared to days from the initial positive in immunosuppressed and non-immunosuppressed patients. ***p < 0.00001.

with these changes in multiple instances. Of the 48 substitutions or deletions noted, three spike protein deletions (L141del, G142del, and V143del) and one spike protein substitution (E484K) occurred in two instances. Each of these substitutions or deletions that occurred more than once in this dataset was a part of disparate lineages, especially S:E484K which was seen in at least 19 lineages by our group (Table S5). The most common protein to develop substitutions or deletions was the Spike protein (Figure 5A). A correlation between AA changes and days between recoverable genomes was noted (Figure 5B, $p < 0.00001$). Recoverable genomes over 24 days from the original positive showed a minimum of two non-synonymous substitutions or deletions (Table 3).

We next examined the impact of vaccination and immune suppression on the accumulation of nonsynonymous mutations. Of the 75 patients that had multiple recoverable genomes, 17 were vaccinated (Figures 6A–C). Only one vaccinated patient developed nonsynonymous mutations (Table 3). Although vaccinated patients that had multiple recoverable genomes showed a lower mean time of recoverable genomes, and a lower mean number of developed mutations, this did not reach significance (Figures 6A–C). Immunocompromised status was associated with longer periods between the recoverable genomes (Figure 6D, $p=0.022$) and a higher number of substitutions or deletions (Figures 6E, F, $p=0.022$).

DISCUSSION

In this study, we combined large scale laboratory diagnostic data with whole genome sequencing data for surveillance to analyze prolonged SARS-CoV-2 shedding and reinfections. Clinical data analysis was performed to study the impact of vaccination and immunocompromised status on genomic changes over-time. Cell culture was performed in certain cases with extended viral shedding combined with low Ct values to characterize cases of prolonged active infection. Our data showed that the majority of patients with prolonged positive SARS-CoV-2 molecular tests have their subsequent positives collected within the first 50 days of the initial positive with Ct values that are likely higher than 30. Prolonged shedding after 50 days was largely associated with low quality genomes reflecting low viral loads except in 17 patients, 14 of whom had subsequent genomes matching the initial, indicating prolonged shedding and 3 had a second genome of a different clade, indicating reinfection. Vaccination reduced the likelihood of the recovery of good quality genomes but immunocompromised status contributed to the increased duration of viral RNA shedding and the accumulation of genomic changes. The spike gene was the region of the genome most prone to nonsynonymous changes in our cohort, but overall, the development of nonsynonymous mutations and AA changes was infrequent.

TABLE 3 | Prolonged viral shedding and genomic changes.

| PID | Positives | Sequenced | HQ Genomes | Days between first and last positives | Days between first and last HQ Genomes | First HQ genome lineage | last HQ genome lineage | Number Acquired subs/ dels | Acquired AA substitutions/deletions | Vaccinated | Immunosuppressed | Monoclonal antibody | Convalescent Plasma |
|------------|-----------|-----------|---------------|---------------------------------------------------|----------------------------------------------------|-------------------------------|------------------------------|-------------------------------------|---------------------------------------------------------------------------------------------|------------|------------------|------------------------|------------------------|
| PIDVWGDGM | 2 | 2 | 2 | 5 | 5 | B.1.2 | B.1.2 | 4 | S:G142del,S:V143del,S: Y144del,S:L141del | No | Yes | | Yes |
| PIDWUGBIZH | 5 | 4 | 3 | 14 | 7 | B.1.637 | B.1.637 | 1 | S:H146Q | No | Yes | | Yes |
| PIDCXIYIPP | 6 | 5 | 4 | 20 | 9 | B.1.243 | B.1.243 | 1 | NSP8:F6L | No | Yes | | Yes |
| PIDLUEEQYV | 5 | 4 | 2 | 13 | 13 | B.1.1.7 | B.1.1.7 | 1 | NSP14:D126Y | No | Yes | | |
| PIDEAUSZWF | 2 | 2 | 2 | 15 | 15 | B.1.2 | B.1.2 | 2 | NSP2:E490K,S:E484K | No | | | |
| PIDRSHNQNS | 12 | 8 | 5 | 51 | 17 | B.1.2 | B.1.2 | 1 | E:V70F | No | Yes | | Yes |
| PIDGBIBYYQ | 7 | 4 | 4 | 24 | 24 | Q.4 | Q.4 | 3 | NSP4:H313Y,N:F307L,S: R102G | No | Yes | | Yes |
| PIDBXQZZLV | 5 | 4 | 4 | 26 | 26 | B.1.526 | B.1.526 | 2 | S:G446V,S:E406Q | Yes | Yes | | Yes |
| PIDLCYIUUV | 2 | 2 | 2 | 37 | 37 | B.1.564 | B.1 | 2 | S:L270F,NSP2:T85I | No | | | |
| PIDFTUQFAV | 7 | 3 | 2 | 81 | 43 | A.3 | A.3 | 7 | S:R190S,S:L244del, NSP4:F317S,S:T1006I, NSP3:F1646I,NSP4: G232V,S:A243del | No | Yes | | |
| PIDSDHALQC | 5 | 4 | 4 | 49 | 49 | B.1.637 | B.1.637 | 8 | S:T95I,S:G142del, NSP13:T115K,S: V143del,S:L141del,S: E484Q,NSP8:T123I,S: W152R | No | Yes | | |
| PIDHCPHTBW | 3 | 2 | 2 | 53 | 53 | B.1.369 | B.1.369 | 2 | NSP5:Q192stop,N:T271I | No | | | |
| PIDXTTUNJN | 9 | 5 | 4 | 57 | 57 | B.1.2 | B.1.2 | 2 | S:E484K,NSP6:L37F | No | Yes | Yes | |
| PIDNXQFFZG | 6 | 4 | 3 | 77 | 61 | B.1.409 | B.1.409 | 2 | NSP8:I156L,NS6:E13K | No | Yes | Yes | Yes |
| PIDHNGFDXK | 7 | 4 | 3 | 84 | 67 | B.1 | B.1 | 4 | NSP8:T148I,S:P1079S, NSP3:T820I,N:P326L | No | Yes | | |
| PIDLYROPCD | 4 | 3 | 3 | 70 | 70 | B.1.520 | B.1 | 2 | NSP6:M86I,N:T205I | No | Yes | | |
| PIDLVBHYS | 11 | 9 | 6 | 139 | 117 | B.1.1.434 | B.1.1.434 | 4 | NSP2:K534R,S:N334K, NSP13:D56G,S:S939F | No | Yes | Yes | |

PID, patient identifier; HPID, identification of samples with whole genome sequencing; HQ, High quality genomes were defined as sequences with coverage >90% and a mean depth of >100. Subs/dels, substitutions/deletions; AA, amino acid.

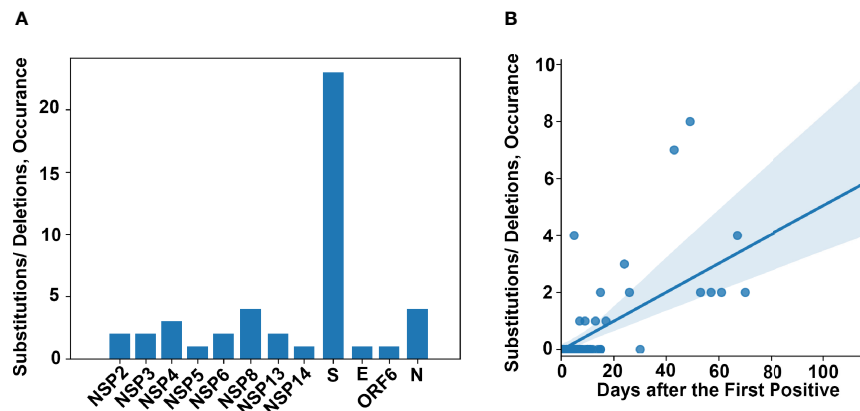


FIGURE 5 | Genomic changes in prolonged viral shedding. **(A)** Frequency of amino acid changes (substitutions and deletions) per protein in a cohort of 75 patients with two or more complete genomes from samples collected 0 - 117 days after the first positive. **(B)** Amino acid changes as a factor of time between collected positive samples.

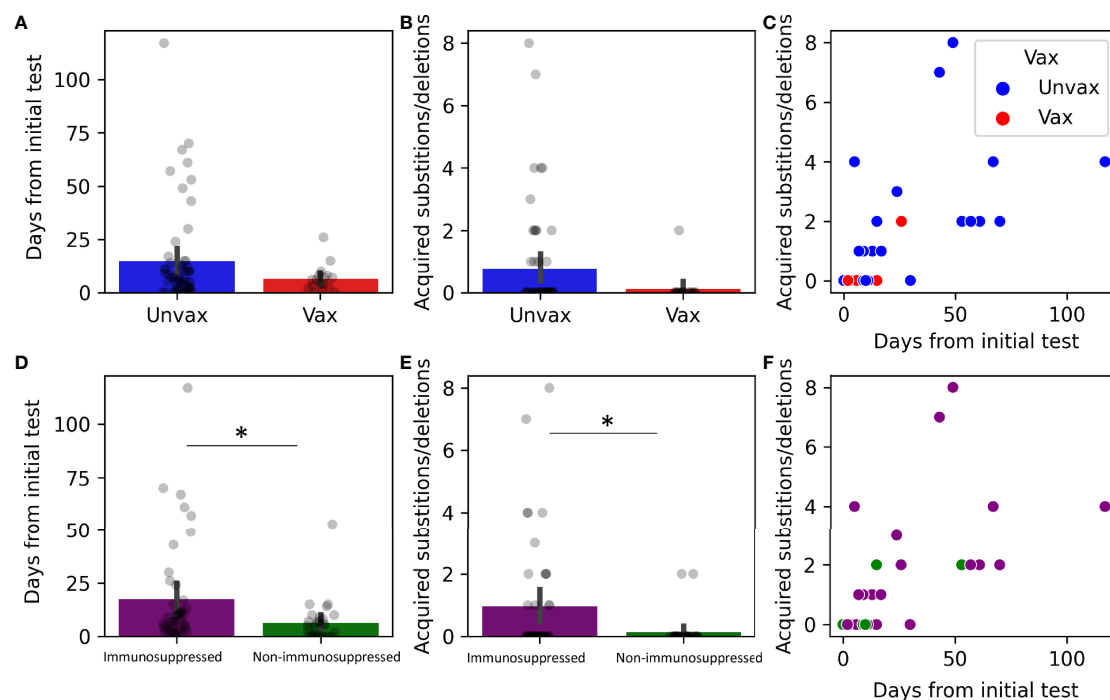


FIGURE 6 | Genomic changes in prolonged viral shedding by vaccination and immune status. Substitutions and deletions in 75 patients by status: vaccinated (N = 17, **A–C**) and immunosuppressed (N = 44, **D–F**). **(A, D)** Barplots with overlaid strip plots of mean time of prolonged shedding of complete genomes in each group, **(B, E)** Barplots with overlaid strip plots of amino acid change frequency in each group, **(C, F)** Scatterplots of the correlation of amino acid change and the days after the first positive in each group. * $p < 0.05$. Unvax, unvaccinated; Vax, vaccinated.

Prolonged SARS-CoV-2 shedding was shown to extend to multiple months after the onset of symptoms (Fontana et al., 2021). Prolonged shedding though might not correlate with prolonged infectiousness or recovery of viable virus. Previously we showed that shedding of infectious virus can extend to more than 3 weeks after the initial positive (Gniazdowski et al., 2020).

In this study, we show the recovery of infectious virus for up to 70 days after the first positive in a subset of immunocompromised patients. This data is consistent with previous reports that showed prolonged SARS-CoV-2 replication and infectiousness in immunocompromised patients (Aydillo et al., 2020; Baang et al., 2021; Tarhini et al., 2021). In general, the recovery of infectious virus

correlated with lower Ct values consistent with higher viral loads (Basile et al., 2020; Bullard et al., 2020; Gniazdowski et al., 2020). The value of differentiating prolonged shedding from prolonged infection include controlling the transmission of infection in patients with actively replicating virus as well as optimizing antiviral intervention strategies. Our data is consistent with previous reports that show that prolonged active infection is remarkably less frequent than prolonged shedding of viral RNA and are primarily associated with immunocompromised patient populations.

Reinfection with SARS-CoV-2 was previously reported and a time frame of 45 days was proposed to suggest possible reinfections when a positive test is associated with symptoms consistent with COVID-19. Genomic sequencing is the only method that can conclusively characterize reinfection cases when the initial genomes are compared to subsequent positives. When initial isolates are not available, it might be possible to characterize reinfections if the lineage identified is an emerging variant that was not circulating when the patient was previously diagnosed. Interestingly, in the time frame when the Delta variant became predominant (Morris et al., 2021), we only identified three patients who had a previous positive in our institution. Even though this data might be largely impacted by the availability of other testing sites outside of the Johns Hopkins system, within the population of patients tested in our laboratory, we identified breakthrough cases after vaccination in rates that were much higher than reinfections (Luo et al., 2021).

The evolution of SARS-CoV-2 variants associated with increased transmissibility or escape from vaccine induced or natural immune responses has been globally concerning. Specific variants showed significant reduction in neutralization by monoclonal antibodies and convalescent plasma (Hoffmann et al., 2021; Planas et al., 2021). Those variants attracted the attention to specific changes within SARS-CoV-2 genome that could be of therapeutic concern. Certain spike changes were shown to impact the action of monoclonal antibodies and convalescent sera including L452R, E484K, K417N, and K417T. In our cohort, the spike changes that we observed more than once included the E484K and the deletions 141-143. The E484K in particular was previously reported to reduce the neutralization efficacy and was identified as an escape change the could develop after treatment with certain monoclonal antibodies or convalescent plasma (Baum et al., 2020; Weisblum et al., 2020; Greaney et al., 2021). The deletions 141-144 are within the NTD and were reported to reduce binding to the monoclonal antibody 4A8 (recovered from the convalescent plasma of patients with COVID-19) as do deletions 243-244 (which occurred once in our cohort) (McCarthy et al., 2021). Other changes we detected in our cohort are within epitopes or associated with escape from convalescent sera including W152R, F157S, and G446V (Liu et al., 2021; Suryadevara et al., 2021). Although 9 of the 17 patients in our cohort whom infecting virus developed nonsynonymous mutations received monoclonal or convalescent sera treatments (Table 3), associations between those treatments and the rate of developing mutations were not performed due to the incompleteness of this data in some of the clinical charts.

In our cohort, the accumulation of genomic changes associated with the prolonged shedding time that was most remarkable in immunosuppressed individuals.

A main finding in our study was the correlation between vaccination and a lower likelihood of prolonged shedding or recovery of good quality genomes after the first 20 days of the initial positive result. Even though, vaccine breakthrough infections correlated with a bias to genomes carrying the S: E484K (Feder et al., 2021; Mostafa et al., 2021), a limited infectious virus shedding was notable when samples from patients were longitudinally analyzed (Ke et al., 2021). SARS-CoV-2 genomic diversity was also shown to decline after widespread vaccination (Niesen et al., 2021). The selected cohort for our analysis was restricted to individuals who had an initial positive when fully vaccinated. This data indicate that vaccination is interrupting the extended prolonged shedding observed since the start of the pandemic with unvaccinated individuals. Our data also show that vaccinated individuals are less likely to accumulate significant genomic changes over time.

In summary, the COVID-19 pandemic challenged the diagnostic laboratories to not only ramp up testing, but to also assist with the nationwide genomic surveillance. The diagnostic laboratories are capable of providing real-time epidemiological and clinical data that are essential for a better understanding of the biology of SARS-CoV-2. Diagnostic laboratories with limited resources can also assist by coordinating sharing real-time positive samples with public health laboratories for surveillance. The workflow of diagnosis, surveillance, and basic research we established at Johns Hopkins laboratory for characterizing SARS-CoV-2 provides a template for other evolving pathogens of concern and emphasizes the power of generating real-time data amid a quickly evolving pandemic.

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 Johns Hopkins Institutional Review Board (IRB00221396) with a waiver of consent. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CM data collection and analysis, data interpretation, figures, writing. CL, JS and ML cell culture. DG, VG, NG, AA, JN, MS, and WW. data collection. EK. Clinical data collection and analysis. AP. cell culture and scientific revision. HM study

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SUPPLEMENTARY MATERIAL

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Estimating the Neutralizing Effect and Titer Correlation of Semi-Quantitative Anti-SARS-CoV-2 Antibody Immunoassays

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For the clinical application of semi-quantitative anti-SARS-CoV-2 antibody tests, the analytical performance and titer correlation of the plaque reduction neutralization test (PRNT) need to be investigated. We evaluated the analytical performance and PRNT titer-correlation of one surrogate virus neutralization test (sVNT) kit and three chemiluminescent assays. We measured the total antibodies for the receptor-binding domain (RBD) of the spike protein, total antibodies for the nucleocapsid protein (NP), and IgG antibodies for the RBD. All three chemiluminescent assays showed high analytical performance for the detection of SARS-CoV-2 infection, with a sensitivity $\geq 98\%$ and specificity $\geq 99\%$; those of the sVNT were slightly lower. The representativeness of the neutralizing activity of PRNT ND₅₀ ≥ 20 was comparable among the four immunoassays (Cohen's kappa ≈ 0.80). Quantitative titer correlation for high PRNT titers of ND₅₀ ≥ 50 , 200, and 1,000 was investigated with new cut-off values; the anti-RBD IgG antibody kit showed the best performance. It also showed the best linear correlation with PRNT titer in both the acute and convalescent phases (Pearson's R 0.81 and 0.72, respectively). Due to the slowly waning titer of anti-NP antibodies, the correlation with PRNT titer at the convalescent phase was poor. In conclusion, semi-quantitative immunoassay kits targeting the RBD showed neutralizing activity that was correlated by titer; measurement of anti-NP antibodies would be useful for determining past infections.

Keywords: SARS-CoV-2, COVID-19, serology, immunoassay, antibody responses, neutralizing antibody, spike (S) protein, nucleocapsid (NP) protein

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing coronavirus disease-19 (COVID-19), has caused more than 5 million deaths globally as of November 2021 (WHO, 2021). While novel vaccines for SARS-CoV-2 have helped control the pandemic, there are new variants capable of escaping immunogenicity acquired by natural infection and/or vaccination (Abdool Karim and de Oliveira, 2021; Gupta, 2021; Hacisuleyman et al., 2021). Waning of acquired immunity is a concern, and whether to administer a booster vaccine is another question that requires addressing (Baraniuk, 2021). Measurement of neutralization antibody levels is useful to predict protective immunity in patients who have recovered from COVID-19 and in those who have received vaccines (Khoury et al., 2021). However, neutralizing tests are usually not applicable in clinical laboratories because they require a biosafety level (BSL) 3 facility, skilled technicians, and considerable time for testing. To overcome these limitations, elaborate immunoassay kits applying various methodologies have been developed and have suggested a correlation with neutralization activities; however, the titer correlation with neutralization tests has not been elucidated. Herein, we evaluated the performance of semi-quantitative anti-SARS-CoV-2 spike protein antibody immunoassay kits in association with the titer of the neutralization test.

MATERIALS AND METHODS

Study Population and Collected Specimens

Serum specimens were collected from three groups. First, serial serum specimens of patients with moderate-to-severe COVID-19 were collected from patients admitted to a tertiary care center (Ko et al., 2020a). Acute and convalescent specimens were collected, and sera collected after 14 days of illness were considered seroconverted-sera (Lau et al., 2021). Second, convalescent specimens from asymptomatic-to-mild COVID-19 were collected from patients staying at a residential care center at the time of discharge after two consecutive reverse transcriptase polymerase chain reaction (RT-PCR) tests (Ko et al., 2020a). Information about symptom onset and RT-PCR test results, including the cycle threshold (Ct) value, were retrospectively collected. Third, sera from healthcare workers (HCWs) collected before the spread of SARS-CoV-2 into the Korean community were used as negative control specimens. By the time of sampling, most COVID-19 cases in Korea could be epidemiologically traced, and none of the negative control patients had epidemiologic links to COVID-19 cases or the risk area. The absence of anti-SARS-CoV-2 antibodies in the negative control sera was confirmed by neutralization tests and/or multiple immunoassay kits (Ko et al., 2020b; Ko et al., 2021). While only 33 out of 126 sera from HCWs went through PRNT, the qualitative result of PRNT was imputed as negative for those without PRNT results. Written informed consent was obtained from each participant. The study was approved by the institutional review board (IRB) of each hospital (IRB No.

SMC 2020-03-113, SMC 2020-04-006, SMC 2020-04-145, and KUMC 2020-07-067).

Classification of Sera According to the Collection Time Point and Disease Severity

Specimens from the enrolled subjects with SARS-CoV-2 infection were reclassified by collection time points and disease severity. First, the sera were classified into either the acute phase specimens (collected before 21 days of illness) or convalescent phase specimens (collected since 21 days of illness), based on the time point of the peak serologic response of the present cohort. The baseline time point (day 0) was defined as follows: 1) if the patient was symptomatic before being diagnosed, the symptom onset was considered as the baseline, and 2) if the patient was asymptomatic at diagnosis, the date when the patient was diagnosed by RT-PCR was considered as the baseline. Second, for the classification of disease severity, COVID-19 patients were classified as “severe-to-critical” if the peak O₂ requirement was greater than or equal to a fraction of inspired oxygen (FiO₂) of 0.6. Otherwise, the patients were classified as “mild-to-moderate” cases.

Plaque-Reduction Neutralization Test (PRNT)

PRNT was conducted at the Korea Disease Control and Prevention Agency (KDCA). Heat-inactivated (56°C for 30 min) serum samples were serially diluted four-fold with Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Diluted serum was incubated at 37°C in a 5% CO₂ incubator for 1 h. Fifty plaque-forming units (PFU)/well of SARS-CoV-2 (βCoV/Korea/KCDC03/2020 NCCP No.43326) were mixed with serum. The mixtures were inoculated into Vero E6 cells on a 24-well plate and incubated at 37°C and 5% CO₂ for 1 h. After the inoculums were removed, the cells were overlaid with 1 ml of Minimum Essential Medium (MEM) containing 0.75% agarose and 2% FBS. The plates were incubated at 37°C in a 5% CO₂ incubator for three days, following which the cells were stained with 0.07% crystal violet, 10% formaldehyde, and 5% ethanol, and the visualized plaques were counted. The 50% neutralizing dose (ND₅₀) titer was calculated using the Kärber formula: $\log_{10} ND_{50} = m - \Delta(\Sigma p - 0.5)$ (Grist et al., 1974).

Surrogate Virus Neutralization Test (sVNT)

To detect neutralizing antibodies using an immunoassay method, the cPass sVNT kit (GenScript, Piscataway, NJ, USA) was used. sVNT measures the inhibition of interactions between the horseradish peroxidase (HRP)-conjugated SARS-CoV-2 spike protein receptor-binding domain (RBD) and the extracellular domain of the human angiotensin-converting enzyme 2 (hACE2) receptor (Taylor et al., 2021). The inhibition ratio is calculated as follows:

$$\text{Inhibition ratio} = \left(1 - \frac{\text{OD value of specimen}}{\text{OD value of control}} \right) \times 100 \%$$

The kit was approved as a qualitative test with a positive cut-off value of 30%, while the manufacturer suggested that semi-quantitative interpretation of the test would be possible (GenScript, 2020).

Anti-SARS-CoV-2 Spike Protein Total Antibody Assay

To estimate total antibody titers against the RBD of the spike protein, the Elecsys® Anti-SARS-CoV-2 S kit (Roche Diagnostics, Rotkreuz, Switzerland) was used. The kit was developed for *in vitro* qualitative and semi-quantitative measurement of anti-SARS-CoV-2 spike protein antibodies with an electro-chemiluminescence immunoassay (ECLIA) method using cobas e analyzers. A recombinant RBD of the spike protein was used with a double-antigen sandwich principle. While the antigen used in the kit was captured by IgG predominantly, IgA and IgM were detectable as well (Roche, 2020a). An anti-SARS-CoV-2 S antibody concentration ≥ 0.8 U/mL was considered positive. The linear range was 0.4–250 U/mL, and automated dilution was performed up to a 1:50 dilution in the cobas e analyzers. For results reported as <0.4 , the values were imputed as 0.4.

Anti-SARS-CoV-2 Spike Protein IgG Antibody Assay

The SARS-CoV-2 IgG II Quant kit (Abbott Laboratories, Abbott Park, IL, USA) was used for the semi-quantitative measurement of IgG antibody titers against the RBD of the spike protein. The kit was developed for *in vitro* qualitative and semi-quantitative measurement of anti-SARS-CoV-2 spike protein IgG antibodies using a chemiluminescent microparticle immunoassay (CMIA) method using the Alinity and ARCHITECT Systems (Abbott, 2020b). Test results greater than or equal to 50.0 AU/mL were considered positive. The manufacturer suggests an analytic measuring interval (AMI) from 22.0 to 25,000.0 AU/mL, with acceptable performance for linearity. We performed automated 1:2 dilutions for the specimens with $\geq 25,000.0$ AU/mL, as the manufacturer's instruction suggests extending the measuring interval (EMI) from 25,000.0 to 50,000.0 AU/mL in 1:2 dilutions (Abbott, 2020a).

Anti-SARS-CoV-2 Nucleocapsid Antibody Assay

To analyze the correlation between neutralization activity and anti-SARS-CoV-2 nucleocapsid antibody titers, Elecsys® Anti-SARS-CoV-2 kit (Roche Diagnostics) was used. A recombinant nucleocapsid protein was used to detect high-affinity antibodies against SARS-CoV-2 (Muench et al., 2020). A double-antigen sandwich principle was utilized, and the ECLIA method was applied using cobas immunoassay analyzers. The detectable isotypes included IgA and IgG, and a cut-off index (COI) ≥ 1.0 was considered positive (Roche, 2020b). The kit was approved as a qualitative test, and the manufacturer did not suggest a titer correlation between the COI value and antibody titer. Nevertheless, the measured COI values were reported to be as high as 167 in the present analysis, and we investigated the correlation between COI values and the neutralizing titer of the study specimens.

Statistical Analysis

The analytical performance for the neutralization activity of each immunoassay kit using the pre-defined cut-off value by the manufacturer was evaluated with sero-converted sera (from 14 days of illness) of confirmed COVID-19 patients as the positive group along with the negative control group. The performance was calculated for the prediction of PRNT ND₅₀ values of ≥ 20 , ≥ 40 , and ≥ 80 . Sensitivity, specificity, Cohen's kappa, and area under the receiver operating characteristic curve (ROC AUC) values were calculated. The interpretation of Cohen's kappa was as follows: values < 0.00 were considered as poor agreement, 0.00–0.20 as slight agreement, 0.21–0.40 as fair agreement, 0.41–0.60 as moderate agreement, 0.61–0.80 as substantial agreement, and 0.81–1.00 as almost perfect agreement (Landis and Koch, 1977).

Pearson's correlation coefficient (R) and P values were calculated to investigate the titer correlation between each immunoassay kit and PRNT as a continuous variable. Subgroup analyses were conducted according to the pre-defined acute/convalescent phase. For the analysis of titer correlation as a categorical variable, we calculated the optimal cut-off values with the maximal Youden's index for the prediction of PRNT ND₅₀ values of ≥ 20 , ≥ 50 , ≥ 200 , and $\geq 1,000$. The analytical performance for each PRNT titer was analyzed based on the new cut-off values of the immunoassay kits. In order to compare the titers according to the timeline obtained with each assay, Wilcoxon's test was performed.

The data were analyzed using Microsoft Excel (Microsoft, Redmond, WA, USA). Statistical analyses were performed with R 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria). Sensitivity, specificity, and 95% intervals were calculated using the epiR 2.0.38 package on R 4.0.5. The plots were depicted with the ggplot2 3.3.3 and plotROC 2.2.1 packages on R 4.0.5.

RESULTS

Characteristics of Study Population and Specimens

A total of 483 samples from 237 subjects were collected (Table 1). The median age of the subjects was 52 years (IQR 30–71 years), and the male-to-female ratio was 0.46. Among these, 357 samples from 111 patients were from COVID-19 patients with confirmed SARS-CoV-2 infection; 126 samples from 126 HCWs were used as negative controls. Specimens from the designated hospitals included 151 sera from the acute phase and 145 sera from the convalescent phase, and all specimens from the residential care center were collected at the convalescent phase. Eighty-nine patients at the residential care center and the designated hospitals experienced mild-to-moderate illness, while 19 patients at the designated hospitals progressed to severe-to-critical status.

Analytical Performance for the Discrimination of SARS-CoV-2 Infection

The analytical performance of the discrimination of SARS-CoV-2 infection was evaluated using 279 sero-converted sera from 110 confirmed-COVID-19 patients and 126 negative control sera

TABLE 1 | Characteristics of the study population and the specimens.

| Variables | Total | COVID-19 patients | | HCWs (Negative controls) |
|-------------------------------------------------|------------------|-------------------------|----------------------|--------------------------|
| | | Residential care center | Designated hospitals | |
| Number of specimens (patients) | 483 (237) | 61 (61) | 296 (50) | 126 (126)* ‡ |
| Age , years | 52.0 (30.0–71.0) | 27.0 (24.0–37.0) | 70.0 (61.0–74.0) | 33.5 (27.0–42.0) |
| Sex , male: female | 75:162 | 25:36 | 23:27 | 27:99 |
| Time point | | | | |
| Seroconverted (≥ 14 days) [†] | 279 (110) | 61 (61) | 218 (49) | NA |
| Acute (< 21 days) [†] | 151 (41) | 0 (0) | 151 (41) | NA |
| Convalescent (≥ 21 days) [†] | 206 (101) | 61 (61) | 145 (40) | NA |
| Severity of illness | | | | |
| Mild-to-moderate ($\text{FIO}_2 < 60\%$) | 206 (89) | 61 (61) | 145 (28) | NA |
| Severe-to-critical ($\text{FIO}_2 \geq 60\%$) | 151 (22) | 0 (0) | 151 (22) | NA |

Data are expressed as the number of specimens (patients) or as medians (IQR), unless indicated otherwise. *While all the sera of COVID-19 patients underwent PRNT, 33 of 126 sera of HCWs underwent PRNT. [†]For calculating the analytical performance in discriminating SARS-CoV-2 infection, sero-converted sera of COVID-19 patients were used as positive specimens and all the sera of HCWs were used as negative controls. [‡]For the investigation of titer correlation between immunoassay kits and PRNT, all specimens of COVID-19 patients were used, and subgroup analyses were conducted according to the acute/convalescent phase.

COVID-19, coronavirus disease 2019; HCW, healthcare worker; FIO_2 , fraction of inspired oxygen; PRNT, plaque reduction neutralization test.

from 126 HCWs (Table 2). Both the Roche Elecsys Anti-SARS-CoV-2 and Roche Elecsys Anti-SARS-CoV-2 S kits demonstrated 100% specificity, which implies no false positive results in these two assays. All binding assays showed higher sensitivity and specificity than the cPass sVNT kit. The AUC values were all greater than 0.98, and cPass sVNT exhibited the lowest AUC of 0.981 (Figure 1A). The performance of each assay was comparable to that claimed by the manufacturer.

Analytical Performance for Representativeness of Neutralizing Activity Using Pre-Defined Cut-Off Values

The analytical performance in terms of the representation of the neutralizing activity, with PRNT cut-off values of $\text{ND}_{50} \geq 20$, ≥ 40 , and ≥ 80 , was evaluated using 357 acute and convalescent sera of 111 confirmed-COVID-19 patients and 40 negative control sera from 40 HCWs (Table 3). For each immunoassay kit, pre-defined cut-off values suggested by the manufacturers were applied. When compared with $\text{ND}_{50} \geq 20$, which is the cut-off commonly used for designating the presence of neutralization activity, all methods exhibited results highly concordant with PRNT, with a Cohen's kappa of approximately 0.80. The Abbott

SARS-CoV-2 IgG II Quant kit demonstrated a Cohen's kappa of 0.81, showing a substantial agreement with PRNT, which was the highest value among the tested kits. The RBD-targeting semi-quantitative kits showed high sensitivity: GenScript cPass sVNT (94.68%), Roche Elecsys Anti-SARS-CoV-2 S (96.68%), and Abbott SARS-CoV-2 IgG II Quant (97.67%). While the Roche Elecsys Anti-SARS-CoV-2 assay, the only assay that targets NP in this study, showed lower sensitivity (94.35%) compared to other assays, its specificity (83.52%) was the highest among the assays compared. When pre-defined cutoffs provided by the manufacturers were applied, the ability to predict the neutralization effect (Cohen's kappa) declined for all assays as the cut-off for ND_{50} increased. While a strong agreement with $\text{ND}_{50} \geq 20$ was observed for each method, the need for a different cut-off to predict a high titer of neutralization effect was raised.

Correlation With PRNT Titers and Correspondive New Cut-Off Values

The ROC AUCs for the prediction of PRNT $\text{ND}_{50} \geq 20$ of the GenScript cPass sVNT, Roche Elecsys Anti-SARS-CoV-2, Roche Elecsys Anti-SARS-CoV-2 S, and Abbott SARS-CoV-2 IgG II Quant kits were 0.941, 0.916, 0.950, and 0.964, respectively

TABLE 2 | Analytical performance of each kit in discriminating SARS-CoV-2 infection.

| Kit, manufacturer, target protein, and Ab measured | Performance | | Kappa |
|---------------------------------------------------------|---------------------------|-----------------------------|-------|
| | Sensitivity (95% CI) | Specificity (95% CI) | |
| cPass sVNT , GenScript, RBD, total | 96.42% (93.51%–98.27%) | 95.24% (89.92%–98.23%) | 0.91 |
| Elecsys Anti-SARS-CoV-2 , Roche, NP, IgG/IgA | 98.92% (96.89%–99.78%) | 100.00% (97.11%–100.00%) | 0.98 |
| Elecsys Anti-SARS-CoV-2 S , Roche, RBD, total | 98.21% (95.87%–99.42%) | 100.00% (97.11%–100.00%) | 0.97 |
| SARS-CoV-2 IgG II Quant , Abbott, RBD, IgG | 98.92% (96.89%–99.78%) | 99.21% (95.66%–99.98%) | 0.98 |

For calculating the analytical performance in discriminating SARS-CoV-2 infection, 279 sero-converted sera of confirmed-COVID-19 patients and 126 negative control sera of HCWs were used. The pre-defined cut-off values suggested by the manufacturers were applied.

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Ab, antibody; sVNT, surrogate virus neutralization test; RBD, receptor-binding domain; NP, nucleocapsid; CI, confidence interval.

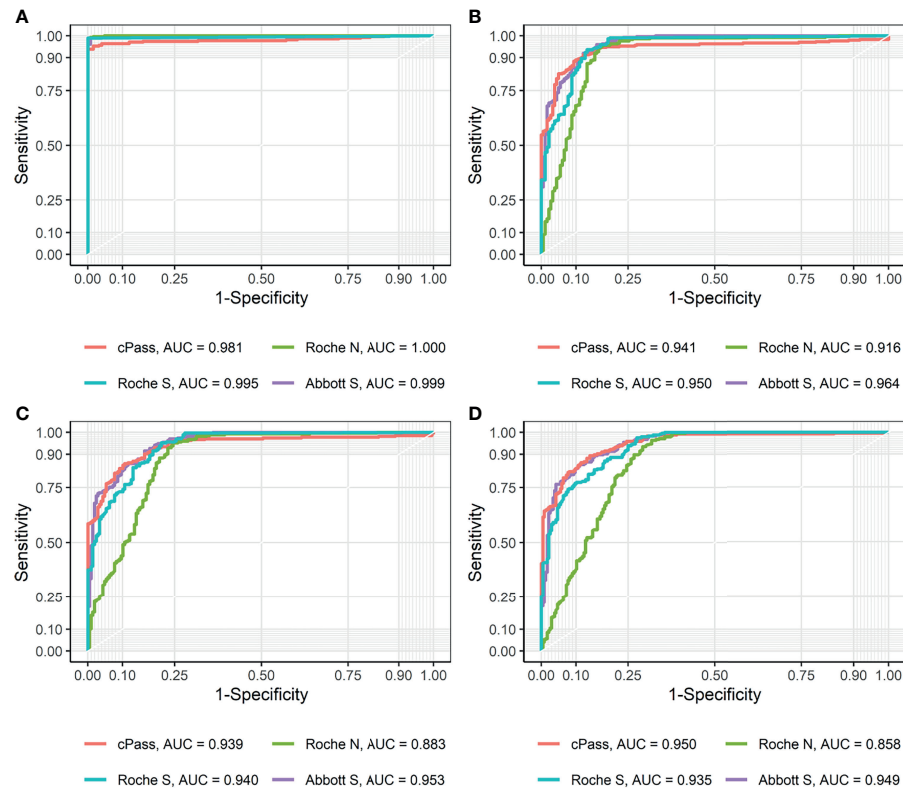


FIGURE 1 | Receiver operating characteristic (ROC) curves and area under curve (AUC) for each method (A) using positive and negative controls and (B–D) compared to PRNT ND₅₀ of (B) ≥ 20 , (C) ≥ 40 , and (D) ≥ 80 , respectively.

(Figure 1B). Based on the Youden's index found in the ROC curve, new cut-offs that represent the neutralizing activity of PRNT ND₅₀ ≥ 20 were established. The cut-offs that best predict the neutralization activity of PRNT ND₅₀ ≥ 20 were higher than the pre-defined cut-offs of the GenScript cPass sVNT (new value of 39.65% and pre-defined value of 30.0%), Roche Elecsys Anti-SARS-CoV-2 S (new value of 4.08 U/mL and pre-defined value of 0.8 U/mL), and Abbott SARS-CoV-2 IgG II Quant kits (new value of 120.1 AU/mL and pre-defined value of 50.0 AU/mL). For the Roche Elecsys Anti-SARS-CoV-2 assay, the new cut-off value was lower than the pre-defined cut-off value provided by the manufacturer (new value of 0.65 COI and pre-defined value of 1.0 COI), which showed higher sensitivity by sacrificing specificity. For other methods targeting the RBD, the new cut-offs achieved higher specificity at the cost of lower sensitivity compared to the pre-defined cut-offs. There were no significant differences in Cohen's kappa.

To investigate new cut-off values representing higher neutralizing antibody titers and analytic performances, Youden's indices in the ROC curve were utilized in the same manner to establish cut-offs for each assay that best represented ND₅₀ values ≥ 50 , ≥ 200 , and $\geq 1,000$ (Table 4 and Appendix Figure 1). While there was no significant difference between the pre-defined cut-off and the new cut-offs for the Roche Elecsys Anti-SARS-CoV-2 targeting the nucleocapsid protein, the new

cut-offs of assays targeting the RBD increased with higher PRNT cut-offs. As the target PRNT titer increased, Cohen's kappa declined, despite adopting the new cut-offs derived from Youden's indices. According to the new cut-offs, the Abbott SARS-CoV-2 IgG II Quant kit demonstrated the highest agreement in predicting high titers of neutralizing antibodies, followed by the GenScript cPass sVNT and Roche Elecsys Anti-SARS-CoV-2 S kits. Binding assays targeting the RBD demonstrated results comparable with GenScript cPass sVNT. However, the Roche Elecsys Anti-SARS-CoV-2 assay showed significantly lower Cohen's kappa compared to other assays, implying its limited use in predicting high neutralization activity.

Serial Kinetics of PRNT Titers and Semi-Quantitative Immunoassay Kits

For the categorization of the acute and convalescent phases, serial kinetics of each antibody assay were plotted using positive samples and divided by disease severity (Figure 2). According to the results of PRNT of positive samples, seroconversion was observed at 5.2 days from baseline, and peak titer was observed at 18.7 days from baseline. When this group was divided by severity, seroconversion was 6.2 days from baseline in the mild-to-moderate group (FiO₂ ≤ 0.6) and 3.9 days from baseline in the severe-to-critical group (FiO₂ > 0.6).

TABLE 3 | Analytical performance for representativeness of neutralizing activity using the pre-defined cut-off values of each immunoassay kit.

| PRNT cut-off | Kit, manufacturer, target protein, and Ab measured | Performance | | |
|-----------------------|----------------------------------------------------------------|---------------------------|---------------------------|-------|
| | | Sensitivity (95% CI) | Specificity (95% CI) | Kappa |
| ND ₅₀ ≥ 20 | cPass sVNT , <i>GenScript, RBD, total</i> | 94.68% (91.51%–96.93%) | 81.32% (74.89%–86.70%) | 0.78 |
| | Elecsys Anti-SARS-CoV-2 , <i>Roche, NP, IgG/IgA</i> | 94.35% (91.11%–96.68%) | 83.52% (77.31%–88.59%) | 0.79 |
| | Elecsys Anti-SARS-CoV-2 S , <i>Roche, RBD, total</i> | 96.68% (94.40%–98.62%) | 80.77% (74.28%–86.22%) | 0.80 |
| | SARS-CoV-2 IgG II Quant , <i>Abbott, RBD, IgG</i> | 97.67% (95.25%–99.06%) | 80.77% (74.28%–86.22%) | 0.81 |
| | cPass sVNT , <i>GenScript, RBD, total</i> | 96.00% (92.96%–97.99%) | 73.56% (67.01%–79.42%) | 0.71 |
| ND ₅₀ ≥ 40 | Elecsys Anti-SARS-CoV-2 , <i>Roche, NP, IgG/IgA</i> | 95.27% (92.05%–97.46%) | 75.00% (68.54%–80.73%) | 0.72 |
| | Elecsys Anti-SARS-CoV-2 S , <i>Roche, RBD, total</i> | 98.18% (95.81%–99.41%) | 72.60% (66.00%–78.54%) | 0.73 |
| | SARS-CoV-2 IgG II Quant , <i>Abbott, RBD, IgG</i> | 98.18% (95.79%–99.40%) | 71.63% (64.99%–77.65%) | 0.72 |
| | cPass sVNT , <i>GenScript, RBD, total</i> | 98.38% (95.91%–99.56%) | 67.80% (61.43%–73.71%) | 0.67 |
| | Elecsys Anti-SARS-CoV-2 , <i>Roche, NP, IgG/IgA</i> | 96.36% (93.20%–98.32%) | 67.80% (61.43%–73.71%) | 0.65 |
| ND ₅₀ ≥ 80 | Elecsys Anti-SARS-CoV-2 S , <i>Roche, RBD, total</i> | 99.19% (97.11%–99.90%) | 65.25% (58.80%–71.31%) | 0.65 |
| | SARS-CoV-2 IgG II Quant , <i>Abbott, RBD, IgG</i> | 99.59% (97.76%–99.99%) | 64.83% (58.37%–70.91%) | 0.65 |

For calculating the analytical performance in discriminating SARS-CoV-2 infection, 357 sero-converted sera of confirmed-COVID-19 patients and 126 negative control sera of HCWs were used. The pre-defined cut-off values suggested by the manufacturers were applied.

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sVNT, surrogate virus neutralization test; RBD, receptor-binding domain; NP, nucleocapsid; CI, confidence interval.

The peak titer was reached after 19.4 days from baseline in the mild-to-moderate group and 18.0 days in the severe-to-critical group. Compared to the kits targeting the RBD exhibiting a descending trend after reaching the peak titer at approximately 2–3 weeks, the results of the Roche Elecsys Anti-SARS-CoV-2 kit consistently increased, even after 3 weeks from the baseline. While the serial kinetics of the GenScript cPass sVNT kit was in line with other assays targeting the RBD, a decline in antibody titer after reaching the peak was not evident owing to early saturation of the method, regardless of the severity. Higher antibody titers in the severe-to-critical group were observed with the Roche Elecsys Anti-SARS-CoV-2 S and Abbott SARS-CoV-2 IgG II kits. The Abbott SARS-CoV-2 IgG II Quant kit revealed the waning of antibody titer prominently compared to the Roche Elecsys Anti-SARS-CoV-2 S assay. The antibody titers, measured with each assay, were categorized into four groups by timeline: 1) 1st week (1–6 days, before seroconversion), 2) 2nd–3rd weeks (7–13 days, acute rising), 3) 3rd–4th weeks (14–27 days, peak titers), and 4) 5th–15th weeks (28–104 days, waning titers); the results are summarized in **Table 5**. A statistically significant decrease in antibody titer after reaching the peak was observed for all kits targeting the RBD, except for the Roche Elecsys Anti-SARS-CoV-2 S kit.

Linear Correlation Between PRNT Titers and Semi-Quantitative Immunoassay Values

The assays showed significantly different correlation results when compared with PRNT. Pearson's correlation coefficients for the

GenScript cPass sVNT kit were 0.75 and 0.65 for the acute and convalescent phase, respectively. Pearson's correlation coefficients for the Roche Elecsys Anti-SARS-CoV-2 kit were 0.60 and 0.20 for the acute and convalescent phase, respectively. Pearson's correlation coefficients for the Roche Elecsys Anti-SARS-CoV-2 S kit were 0.75 and 0.67 for the acute and convalescent phase, respectively. Pearson's correlation coefficients for the Abbott AdviseDx SARS-CoV-2 IgG II kit were 0.81 and 0.72 for the acute and convalescent phases, respectively, the highest among the compared assays. All comparisons showed statistically significant *P* values (**Figure 3**).

DISCUSSION

The situation surrounding the COVID-19 pandemic has changed drastically over the last two years. Multiple serologic tests for SARS-CoV-2 have been developed and used for various indications such as: diagnosing recent or past infections, performing sero-prevalence studies assessing herd immunity, sero-epidemiologic tracing of outbreak clusters, and risk assessment of healthcare workers; preparing convalescence plasma (CP) therapy, assessing neutralizing antibodies in COVID-19 patients, and evaluating protective immunity from past infections and/or vaccinations; according to the status of the COVID-19 pandemic, the significance of each clinical implication has differed (Ko et al., 2017a; Ko et al., 2017b; Ko et al., 2017c; Ko et al., 2018; Ahn et al., 2020; Ko et al., 2020a; Ko et al., 2020b; Park et al., 2020; Yong et al., 2020; Khoury et al., 2021; Lau et al., 2021; van Kampen et al., 2021).

TABLE 4 | Titer correlation of the analytical performance of the prediction of neutralizing activity using newly calculated cut-off values determined using Youden's index.

| PRNT titers | Kit, Manufacturer, target protein, Ab measured, and pre-defined cut-off | New cut-off values | Performance | | |
|----------------------------------|----------------------------------------------------------------------------|--------------------|-----------------------------|---------------------------|-------|
| | | | Sensitivity (95% CI) | Specificity (95% CI) | Kappa |
| ND₅₀ ≥ 20 | cPass sVNT , GenScript, RBD, total, 30% | 39.65% | 91.03% (87.22%–94.01%) | 87.91% (82.27%–92.27%) | 0.79 |
| | Elecsys Anti-SARS-CoV-2 , Roche, NP, IgG/IgA, 1.0 COI | 0.65 COI | 95.68% (92.73%–97.68%) | 82.42% (76.10%–87.65%) | 0.80 |
| | Elecsys Anti-SARS-CoV-2 S , Roche, RBD, total, 1.0 U/mL | 4.08 U/mL | 93.69% (90.32%–96.16%) | 86.81% (81.02%–91.36%) | 0.81 |
| | SARS-CoV-2 IgG II Quant , Abbott, RBD, IgG, 50 AU/mL | 120.1 AU/mL | 96.00% (93.12%–97.92%) | 84.07% (77.92%–89.06%) | 0.82 |
| ND₅₀ ≥ 50 | cPass sVNT , GenScript, RBD, total, 30% | 59.7% | 85.77% (80.99%–89.73%) | 88.89% (83.92%–92.75%) | 0.74 |
| | Elecsys Anti-SARS-CoV-2 , Roche, NP, IgG/IgA, 1.0 COI | 1.1 COI | 95.51% (92.28%–97.66%) | 73.15% (66.71%–78.93%) | 0.70 |
| | Elecsys Anti-SARS-CoV-2 S , Roche, RBD, total, 1.0 U/mL | 4.1 U/mL | 94.76% (91.36%–97.10%) | 76.39% (70.15%–81.89%) | 0.72 |
| | SARS-CoV-2 IgG II Quant , Abbott, RBD, IgG, 50 AU/mL | 449.7 AU/mL | 87.22% (82.60%–90.98%) | 87.50% (82.34%–91.60%) | 0.74 |
| ND₅₀ ≥ 200 | cPass sVNT , GenScript, RBD, total, 30% | 61.7% | 93.43% (89.03%–96.46%) | 77.89% (72.62%–82.58%) | 0.69 |
| | Elecsys Anti-SARS-CoV-2 , Roche, NP, IgG/IgA, 1.0 COI | 1.1 COI | 97.47% (94.21%–99.18%) | 57.89% (51.93%–63.69%) | 0.51 |
| | Elecsys Anti-SARS-CoV-2 S , Roche, RBD, total, 1.0 U/mL | 60.6 U/mL | 83.84% (77.96%–88.68%) | 82.81% (77.92%–87.00%) | 0.66 |
| | SARS-CoV-2 IgG II Quant , Abbott, RBD, IgG, 50 AU/mL | 1665.3 AU/mL | 86.29% (80.69%–90.77%) | 90.53% (86.52%–93.66%) | 0.77 |
| ND₅₀ ≥ 1000 | cPass sVNT , GenScript, RBD, total, 30% | 86.7% | 83.84% (75.09%–90.47%) | 80.47% (76.14%–84.32%) | 0.53 |
| | Elecsys Anti-SARS-CoV-2 , Roche, NP, IgG/IgA, 1.0 COI | 1.2 COI | 100.00% (96.34%–100.00%) | 44.79% (39.74%–49.92%) | 0.25 |
| | Elecsys Anti-SARS-CoV-2 S , Roche, RBD, total, 1.0 U/mL | 127.0 U/mL | 87.88% (79.78%–93.58%) | 76.30% (71.73%–80.47%) | 0.50 |
| | SARS-CoV-2 IgG II Quant , Abbott, RBD, IgG, 50 AU/mL | 2836.2 AU/mL | 97.96% (92.82%–99.75%) | 79.69% (75.31%–83.60%) | 0.60 |

For calculating the analytical performance in discriminating SARS-CoV-2 infection, 357 sero-converted sera of confirmed-COVID-19 patients and 126 negative control sera of HCWs were used. The new cut-off value for each kit was calculated using the Youden's index.

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sVNT, surrogate virus neutralization test; RBD, receptor-binding domain; NP, nucleocapsid; CI, confidence interval.

Among these clinical implications, while the presence of binding antibodies is important for seroprevalence studies to distinguish previous infections, the detection and quantification of neutralizing antibodies are crucial for several indications, including the preparation of CP therapy, assessment of neutralizing antibodies in COVID-19 patients, and evaluation of protective immunity. The clinical utility of immunoassays would be substantiated if the magnitude of neutralization can be estimated by using it in routine clinical practice. Hence, for appropriate clinical application of serologic studies, the importance of titer correlation evaluation cannot be overemphasized.

In this study, three semi-quantitative assays targeting the RBD demonstrated a linear correlation with the neutralizing antibody titer measured using PRNT. In terms of performance in predicting the neutralization titer, the Abbott SARS-CoV-2 IgG II Quant kit, an IgG-specific binding assay, was the best, followed by other assays measuring antibody titers regardless of immunoglobulin isotypes: GenScript cPass sVNT and Roche Elecsys Anti-SARS-CoV-2 S. This could be due to IgG consisting of the majority of the antibody isotypes that target the RBD (Klein et al., 2020) and due to the different isotypes showing different epitope repertoires within the RBD (Tang et al., 2021). Moreover, the measurement

techniques utilized and the measurement range in each assay may have affected the performance. Up to a two-fold dilution was performed for the Abbott AdviseDx SARS-CoV2 IgG II Quant assay, which uses the CMIA method; up to 50-fold dilution was carried out for the Roche Elecsys Anti-SARS-CoV-2 S assay, which uses the ECLIA method due to its narrow measurement range with early saturation. For the GenScript cPass sVNT kit, dilution was not conducted because the method represents the result of the inhibition ratio calculated from the optical density measured, which does not necessarily linearly correlate with the titer of the antibodies.

Although GenScript cPass sVNT kit exhibited a lower Pearson's correlation coefficient than the Abbott AdviseDx SARS-CoV-2 IgG II, this may be due to the narrower reportable range of GenScript cPass sVNT, leading to early saturation in subjects with high antibody titers. Although it is true that GenScript cPass sVNT stands in a disadvantageous position since serial dilution was performed for results exceeding the reportable range in binding assays, while this did not occur in GenScript cPass sVNT, it is noteworthy that GenScript cPass sVNT is relatively more time-consuming and labor-intensive compared to binding assays. The GenScript cPass sVNT kit utilizes the inhibition of binding with the

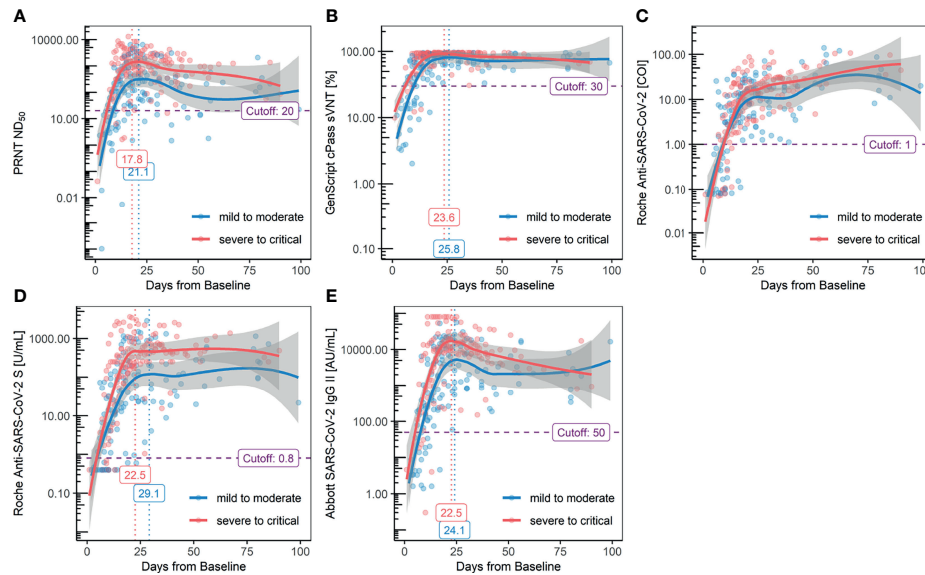


FIGURE 2 | Serial kinetics of antibody titers measured with each method: **(A)** PRNT ND₅₀, **(B.)** GenScript cPass sVNT, **(C)** Roche Elecsys Anti-SARS-CoV-2, **(D)** Roche Elecsys Anti-SARS-CoV2 S, and **(E)** Abbott AdviseDx SARS-CoV2 IgG II.

TABLE 5 | Antibody titers by timeline.

| Kit, manufacturer, target, and Ab measured | 1 st week (1–6 days) before seroconversion | 2 nd to 3 rd weeks (7–13 days) acute rising | 3 rd to 4 th weeks (14–27 days) peak titer | 5 th to 15 th weeks (28–104 days) waning titer |
|----------------------------------------------------------------|-------------------------------------------------------------|-------------------------------------------------------------------------|------------------------------------------------------------------------|----------------------------------------------------------------------------|
| PRNT ND₅₀ <i>KDCA, SARS-CoV-2, total</i> | 7.71 ± 9.68 | 938.57 ± 1889.93 | 1705.73 ± 2126.20 | 597.05 ± 870.22 |
| | └ <i>P</i> < 0.001 ┐ | | | |
| cPass sVNT , <i>GenScript, RBD, total</i> | 13.57 ± 15.64% | 46.81 ± 32.54% | 83.08 ± 17.00% | 74.53 ± 22.87% |
| | └ <i>P</i> < 0.001 ┐ | | | |
| Elecsys Anti-SARS-CoV-2 , <i>Roche, NP, IgG/IgA</i> | 2.17 ± 7.07 COI | 3.48 ± 5.07 COI | 15.62 ± 15.00 COI | 36.30 ± 33.57 COI |
| | └ <i>P</i> < 0.01 ┐ | | | |
| Elecsys Anti-SARS-CoV-2 S , <i>Roche, RBD, total</i> | 6.16 ± 19.88 U/mL | 97.18 ± 321.84 U/mL | 486.81 ± 745.27 U/mL | 373.05 ± 502.61 U/mL |
| | └ <i>P</i> < 0.001 ┐ | | | |
| SARS-CoV-2 IgG II Quant , <i>Abbott, RBD, IgG</i> | 34.65 ± 57.75 AU/mL | 5337.75 ± 15426.61 AU/mL | 16806.08 ± 21912.72 AU/mL | 5959.90 ± 8336.19 AU/mL |
| | └ <i>P</i> = 0.9394 ┐ | | | |
| | └ <i>P</i> < 0.001 ┐ | | | |

Ab, antibody; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ND₅₀, 50% neutralizing dose; KDCA, Korea Disease Control and Prevention Agency; sVNT, surrogate virus neutralization test; RBD, receptor-binding domain; NP, nucleocapsid. The bold values indicate those with statistical significance.

ACE2 receptor and this technique suggests superiority as the result itself is a surrogate of the neutralization activity. However, for a more accurate assessment of high titers with GenScript cPass sVNT, further investigation of how the inhibition ratio changes with dilutions is required as different trends in results following dilution have been reported depending on the composition of immunoglobulin isotypes (Tan et al., 2020).

There has been a study using monoclonal antibodies where sVNT was able to differentiate between neutralizing antibodies and binding antibodies, while ELISA using the identical RBD antigen

failed to distinguish neutralizing antibodies (Tan et al., 2020). Although a number of serological assays utilize the RBD as the antigenic target, which is the same as for GenScript cPass sVNT, the protein coating process can cause exposure of hidden epitopes and changes in epitopes that do not exist in the natural state, which occur due to conformational changes (Lee and Belfort, 1989; Sethuraman and Belfort, 2005; Raffaini and Ganazzoli, 2010; Guven et al., 2014; de Thier et al., 2015). This phenomenon could result in lower specificity due to antibodies binding to newly appearing epitopes (Mannik et al., 1997; Guven et al., 2014).

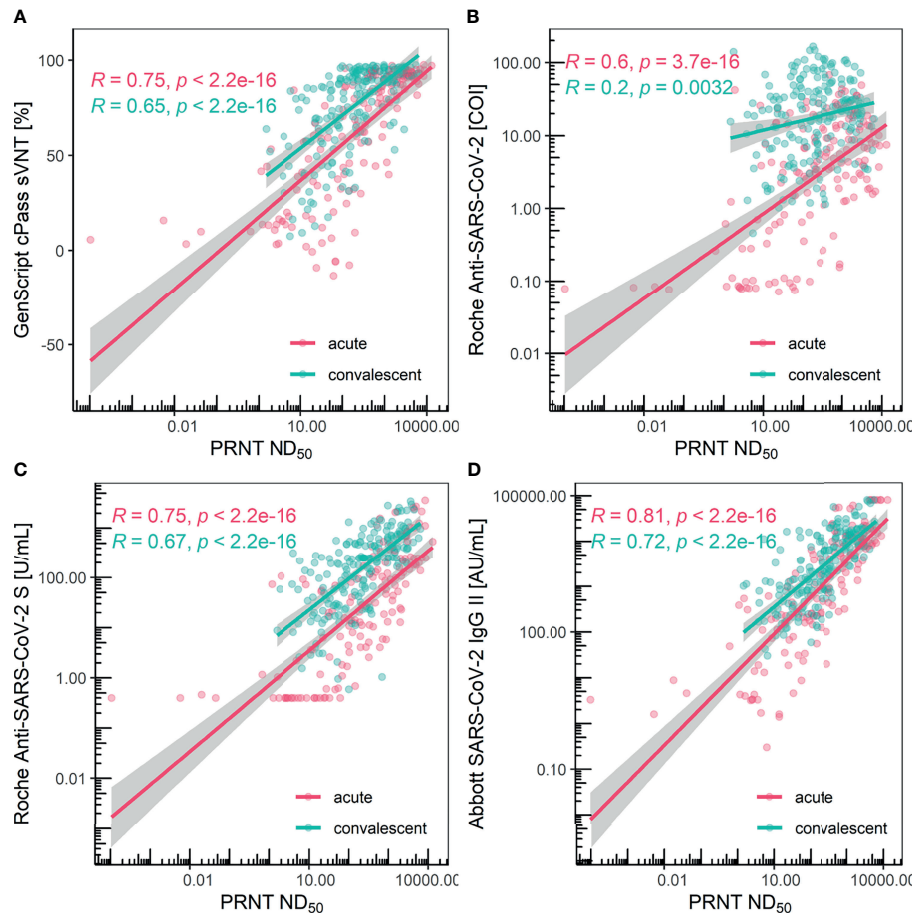


FIGURE 3 | Scatter plot and Pearson's correlation for each method grouped with acute/convalescent phase. **(A)** GenScript cPass sVNT, **(B)** Roche Elecsys Anti-SARS-CoV-2, **(C)** Roche Elecsys Anti-SARS-CoV-2 S, and **(D)** Abbott AdviseDx SARS-CoV2 IgG II were compared with PRNT, respectively. Each colored line depicts the linear regression model and the surrounding grey-colored area represents the 95% confidence interval.

However, despite this limitation of serological assays, a high correlation between anti-RBD IgG and neutralizing antibodies has been shown in previous studies (Dispinseri et al., 2021; Dogan et al., 2021), and our study shows the feasibility of using semiquantitative serologic assays targeting the RBD in predicting the neutralization titer with Abbott AdviseDx SARS-CoV2 IgG II, which measures IgG against the RBD showing the highest performance. In real-world clinical practice, there is little chance of mutually exclusive presence of binding antibodies and neutralizing antibodies. As SARS-CoV-2 infection occurs, the immune system exhibits avidity maturation over time (Luo et al., 2020); patient samples are a complex mixture of antibodies with different binding affinities. Therefore, although separated monoclonal antibodies exhibit discordant results between sVNT and binding assays (Tan et al., 2020), binding assays may be used to estimate neutralization activity in clinical practice.

It is noteworthy that there were significant differences between the acute phase and the convalescent phase in titer correlation with PRNT. During the acute phase, all assays showed a fair correlation with PRNT, since antibodies with

different isotypes targeting various epitopes exhibit a rising trend. However, in the convalescent phase, as antibody affinity maturation and titer waning occur, the difference in the correlation with PRNT of different kits becomes evident. Although the Roche Elecsys Anti-SARS-CoV-2 kit measuring nucleocapsid antibodies correlated with PRNT during the acute phase, this is primarily due to the nucleocapsid protein being the most abundant viral antigen in the early stages of infection (Satarker and Nampoothiri, 2020; Chura-Chambi et al., 2022). The poor correlation between the Roche Elecsys Anti-SARS-CoV-2 and PRNT during the convalescent phase implies that nucleocapsid antibodies are not suitable for predicting the neutralization titer. Thus, it is suggested that convalescent sera be used to evaluate the performance in predicting the neutralization titer measured with PRNT.

While the Roche Elecsys Anti-SARS-CoV-2 kit, which measures the antibodies targeting the nucleocapsid protein, performed poorly in predicting the neutralization titer, it showed the highest sensitivity and specificity in determining the diagnosis of SARS-CoV-2 infection. Furthermore, although both PRNT and the assays

targeting the RBD showed a declining trend during the convalescent phase, a persistently high value was observed with the Roche Elecsys Anti-SARS-CoV-2 kit, though the Roche Elecsys Anti-SARS-CoV-2 kit has been approved as a qualitative assay. We suggest that measuring nucleocapsid antibodies would be beneficial in seroprevalence studies in order to identify past infections and in determining breakthrough infection in vaccinated populations as well. In conclusion, the distinct characteristics of nucleocapsid antibodies compared to RBD antibodies highlight the clinical significance of measuring nucleocapsid antibodies as the vaccination rate increases.

Our study has several limitations. First, the number of acute phase samples and subjects was relatively small compared to the those in the convalescent phase because only convalescent samples were collected from patients managed at the residential care center. However, our study was able to address the analytical performance and correlation of each assay using samples with a wide range of antibody concentrations. Additionally, because whether the antibodies that last in the convalescent phase are binding antibodies or neutralizing antibodies remains unknown, the performance of each assay in the convalescent phase is a major concern. Second, although a number of samples were collected for the “severe-to-critical” group, the limited number of patients may not fully represent the disease spectrum. Patients with different clinical courses can exhibit distinguishing antibody kinetics. For instance, there could be cases where low-affinity binding antibodies persist despite the rapid waning of neutralizing antibodies, resulting in discordant results between binding assays and neutralization tests. Thus, further research should be conducted to address these limitations. In addition, the assays analyzed in this study were developed before the appearance of the new variant. Since antibodies against different variants show different affinities against the recombinant RBD used in each assay, re-validation of the assays is warranted for suitability in the current situation. Furthermore, the clinical utility of binding assays and the ability to represent neutralization activity should be assessed for vaccinees.

In summary, our study illustrates the utility of immunoassays against the RBD of SARS-CoV-2 in predicting neutralization activity. While measuring anti-NP antibodies demonstrated the best performance in determining past infections, the semi-quantitative assays targeting the RBD demonstrated linearly correlated results with PRNT, and the measurement of IgG was thought to be crucial in estimating neutralizing antibodies compared to immunoglobulins of other isotypes.

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DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study was approved by the institutional review board (IRB) of each hospital (IRB No. SMC 2020-03-113, SMC 2020-04-006, SMC 2020-04-145, and KUMC 2020-07-067). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Conceptualization, BL, J-HK, JP, E-JJ, E-SK, and KP. Investigation, BL, J-HK, JP, H-WM, JB, SJ, H-YL, K-CK, KH, SC, C-IK, DC, HH, CC, Y-JK, and E-JJ. Laboratory work, JB, SJ, H-YL, K-CK, and E-SK. Data analysis, BL and J-HK. Writing—review and editing, BL, J-HK, E-JJ, E-SK, and KP. All authors have read and agreed to the manuscript. All authors contributed to the article and approved the submitted version.

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A Tale of Two Countries: DiaSorin Molecular's Rapid Response to the COVID-19 Pandemic

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In the summer of 2019, DiaSorin Molecular started designing a multiplex respiratory panel with pan-coronavirus detection as one of the planned targets. The R&D team in Gerenzano, Italy was already searching databases, performing alignments and assessing preliminary target regions for common coronavirus RT-PCR, including SARS and MERS-CoV. In December 2019, we were vigilant and following a cluster of pneumonia cases with undetermined etiology in Wuhan, China. As we now know, the cause of the respiratory infections was the new SARS-CoV-2 virus. DiaSorin Molecular swiftly responded in line with our heritage and company history in detecting emerging infectious diseases. Early in the pandemic and in record time, using research and development teams in both Italy and the U.S. together with the U.S. manufacturing team, we were able to develop and commercialize a new diagnostic test, Simplexa™ COVID-19 Direct, to detect SARS-CoV-2. Our unique platform allowed development of a rapid diagnostic test without the need for extraction reagents. Challenges with control materials, quarantines, clinical samples, raw materials and production were overcome and the entire company worked side by side for accelerated delivery of this assay to clinical labs in Europe, the U.S. and Canada.

Keywords: COVID-19, SARS-CoV-2, Simplexa, pandemic, DiaSorin Molecular, LIAISON MDX, diagnostics manufacturer

EARLY DEVELOPMENT JANUARY – FEBRUARY 2020

The first viral genomic sequences of SARS-CoV-2 were available in public databases in mid-January 2020. At the time, it was unclear if this new virus would be a limited outbreak, an epidemic, or something more. In the “business of clinical testing,” there must be a clinical need together with a market and business case to justify commercializing a new assay, and it was challenging to dedicate resources to design an assay exclusively dedicated to a novel virus. To be prepared, the Italian R&D team pivoted from a pan-coronavirus design for a multiplex panel to a stand-alone SARS-CoV-2 assay. The design chosen employed a unique approach using a two-target algorithm that would be more robust and resistant to potential mutations in the new virus. The assay targeted the viral S and ORF1ab genes and utilized fluorescently labeled probes together with corresponding forward and reverse primers to simultaneously amplify both targets. The S gene encodes the spike glycoprotein of the virus and was targeted to specifically detect the presence of SARS-CoV-2 while the ORF1ab region encodes well-conserved non-structural proteins and therefore is less susceptible to

recombination. The design had taken advantage of the existing Simplexa™ Direct chemistry formulated for detection of other RNA viruses like influenza and enterovirus, together with LIAISON® MDX instrument parameters. The SARS-CoV-2 primers and probes were combined with the core Simplexa™ Direct chemistry which enables direct detection of pathogens without the need for a separate nucleic acid extraction step or extraction reagents. The chemistry utilizes enzymes and a special buffer system that are resistant to inhibitors commonly found in clinical specimens. The innovative and now patented design was a completely different approach than those developed by the Centers for Disease Control and Prevention (CDC) or World Health Organization (WHO). On January 22, 2020, orders were placed for the first candidate primers and probes for the new assay. However, there was still a lot of uncertainty surrounding the spread of the virus. Then, we all waited like the rest of the world, and watched as the case count started to climb.

On January 30, 2020, the WHO declared the COVID-19 outbreak a Public Health Emergency of International Concern and on the same day, DiaSorin Molecular became aware that the FDA was making an Emergency Use Authorization (EUA) template for test developers to follow. The following day, the U.S. Department of Health and Human Services Secretary declared a Public Health Emergency for the U.S. Subsequently, on February 4, 2020, the Department of Health and Human Services issued a Declaration under the Public Readiness and Emergency Preparedness Act for Medical Countermeasures Against COVID-19, which allowed the FDA to issue EUAs beginning with the CDC's 2019 Novel Coronavirus Real-Time RT-PCR Diagnostic Panel.

With the EUA path opened, DiaSorin Molecular accelerated assay development. On Saturday, February 7, 2020, at risk of impacting other development activities in favor of a virus with an uncertain future, the first pilot production lot was built.

PRIMER, PROBE, AND POSITIVE CONTROL CHALLENGES

Before the pandemic, DiaSorin Molecular had two qualified oligonucleotide vendors that supplied primers and probes. When it was apparent that the best means for detecting SARS-CoV-2 was using molecular diagnostics, commercial manufacturers, hospitals, and reference labs began ordering from the main oligonucleotide suppliers, which were quickly overwhelmed with demand. In addition, our primary oligonucleotide supplier was awarded a government contract to produce CDC SARS-CoV-2 assay components, and shifted their manufacturing and attention accordingly. We switched to several smaller suppliers to circumvent this challenge and validated a slightly lower purity scale that could deliver equivalent performance. While this had the benefit of actual delivery of material to finish development and produce kits, many smaller oligonucleotide lots were constantly received from multiple vendors, requiring a high level of attention for supply chain management, incoming testing and inventory management.

For typical assay development projects, there are commercial sources or repositories of characterized viral strains for testing,

benchmarking assay performance, and kit positive controls. One of the first development challenges encountered with this new virus was obtaining characterized reference material for testing. Initially, there were no sources of virus other than those obtained from patient samples. Because of extreme biosafety concerns and a level of discomfort handling a novel virus, DiaSorin EH&S did not initially approve of stocks of live virus or infected patient specimens in any company facilities. This presented challenges for completing kit development, considering the need for reliable reference material and material for a commercial kit positive control. Our initial solution for a kit positive control was to use synthetic gBlocks™ containing the assay target regions. This material had the advantages of being rapidly available and simple to order and obtain in large quantities. DiaSorin Molecular had previous experience with gBlocks and had confidence that they could be reproducibly manufactured, were stable and were compatible with the Simplexa™ Direct chemistry.

On February 21, 2020, contacts at NIH made us aware that the World Reference Center for Emerging Viruses and Arboviruses, Department of Microbiology and Immunology, University of Texas Medical Branch (UTMB) was culturing the virus isolated from the first U.S. patient in the state of Washington (USA_WA1/2020) and had small amounts of purified genomic RNA available. The material was requested through UTMB and was received by DiaSorin Molecular's U.S. R&D in Cypress, California, on February 27. Simultaneously, the Italian R&D team gained access to purified viral genomic RNA from a positive patient sample on February 26. These sources of authentic positive control material that could be handled safely were desperately needed to complete development and evaluate assay performance. However, using this material presented a unique challenge for the Simplexa™ chemistry because naked viral RNA is not the typical assay specimen type for our direct assay format. The solution was to conduct limit of detection testing using the viral RNA in Universal Transport Medium (UTM) with the addition of RNasin® RNase inhibitor to protect the viral RNA during the assay processing steps.

CLINICAL PERFORMANCE CHALLENGES

With a working prototype Simplexa™ COVID-19 Direct assay, the next step was to test clinical specimens and find an appropriate comparator assay to evaluate clinical performance. An unknown at this stage was the sensitivity required for a SARS-CoV-2 assay. The CDC's 2019 Coronavirus Diagnostic Panel was available. However, the initial lots were flawed and supplies were limited. Further, it was difficult to find labs that had the resources to test the CDC assay versus the DiaSorin Molecular Simplexa™ assay as all reagents available in hospital laboratories were being used to meet the growing testing demand. We chose to develop a panel of contrived positive samples using negative nasopharyngeal swab matrix spiked with extracted SARS-CoV-2 RNA plus RNasin® as a substitute for positive clinical specimens to demonstrate assay performance. However, we still needed access to genuine patient

samples to determine if the assay would be successful for clinical laboratory use.

CRISIS IN NORTHERN ITALY

No one could have predicted that the second global hotspot after Wuhan would be the Lombardy region of Northern Italy, where DiaSorin S.p.A. corporate headquarters is located. The first Italian cases were reported around the third week of February 2020, and cases multiplied exponentially. Our assay development needed to be accelerated even more to address the growing urgent situation in Italy. We identified a few partner laboratories in Rome and Pavia, Italy, who were willing to assist and had validated the WHO Berlin protocol by Corman et al., published January 17, 2020. The DiaSorin Molecular Italian R&D team risked their health and exposure to COVID-19 patients that were starting to crowd the hospitals and placed LIAISON[®] MDX instruments into the hospital labs to have access to fresh, positive patient specimens that would also have corresponding results with an established WHO comparator assay for performance benchmarking. This step allowed finalization of assay parameters so that by March 8, 2020, Italian R&D was successfully running the prototype assay with patient clinical specimens. These results enabled the clinical agreement studies required for initial CE marking of the assay in Italy and the European Union as well as the FDA Emergency Use Authorization.

The emergency continued to grow in Italy, and the entire country was placed on lockdown on March 9, 2020. Despite the setback, DiaSorin Molecular kept working diligently as the pandemic was taking hold, leaving families at home and risking falling ill. R&D employees were required to carry special permission to leave their homes to drive on the highway or cross the city of Milan to get to the R&D laboratory. We literally worked around the clock and around the globe, because as the Italian R&D team that designed the assay was racing to finalize the assay parameters and obtain clinical specimens, a second R&D team in California was simultaneously performing verification testing, and the manufacturing team in California was setting up kit production. Because of the lockdown and travel ban, we had to come up with creative ways to work together to take advantage of the 9-hour time difference between our locations. The Italian R&D team passed the baton to the California R&D and production teams at the end of the Italian day. The Italian team would begin the next day with results of the day of work in California. On March 11, 2020, the World Health Organization declared COVID-19 a pandemic due to cases in over 110 countries and territories worldwide. The first 12,000 Research Use Only (RUO) labeled tests were delivered to Italy the same week. The following week, DiaSorin Molecular had completed the activities required for CE mark self-declaration on March 20, 2020.

EMERGENCY USE AUTHORIZATION & U.S. GOVERNMENT SUPPORT

Concurrently with development, DiaSorin Molecular's pre-EUA submission questions were sent to the FDA on February 27,

2020. We communicated with FDA on an almost daily basis, working through challenges to establish the assay performance to the satisfaction of the FDA. DiaSorin Molecular submitted the Simplexa[™] COVID-19 Direct kit EUA on Tuesday, March 17, 2020, at 6:08 PM Pacific time – a little less than two months from the first primer and probe orders. On Thursday, March 19, 2020, Simplexa[™] COVID-19 Direct was granted Emergency Use Authorization by FDA. The assay was the fourth commercially available *in vitro* diagnostic (IVD) EUA kit and the first IVD shipments were sent to U.S. customers on Saturday, March 21, 2020. The assay performance has compared favorably against other commercially available FDA EUA and lab developed assays including those that use silica-based extraction (Cradic et al., 2020; Fung et al., 2020; Lieberman et al., 2020; Rhoads et al., 2020; Zhen et al., 2020).

With emerging infectious disease and pandemic preparedness, there is always a business risk. To help take on some of this risk, the U.S. Department of Health and Human Services' Biomedical Advanced Research and Development Authority (BARDA) supports development of medical countermeasures such as diagnostic tests, antivirals, and vaccines. BARDA offered a fast-funding pathway at the start of the pandemic named the DRIVE EZ-BAA program. DiaSorin Molecular submitted an abstract requesting funding to complete the verification and validation of the Simplexa[™] COVID-19 Direct assay using this program late on the evening of February 28, 2020. In record time, on March 11, DiaSorin Molecular was awarded a \$697K BARDA development contract.

BUSINESS GROWTH AND ALLOCATIONS

As COVID-19 cases were growing in early March 2020, there were only 12 LIAISON[®] MDX system placements in Italy and 483 LIAISON[®] MDX placements in the U.S., primarily in hospitals and reference laboratories. Our Cypress, California Simplexa[™] kit manufacturing capacity was only 14,400 tests per day from a single manufacturing shift. In contrast, by May 2021, manufacturing had expanded to three shifts, building kits seven days per week for an output of over one million tests per month. LIAISON[®] MDX placements had grown to 128 in Italy and 897 in the U.S.

Increasing production required hiring more employees in all manufacturing departments, from formulation to Quality Control testing to shipping. While the California manufacturing team was present in the facility in full force seven days a week, other departments worked from home starting from March 19, balancing the statewide stay at home order declared by California Governor Gavin Newsom with the fact that DiaSorin Molecular was an essential business in the pandemic fight. The facility was separated into three zones with separate entrances to isolate and protect kit production from other staff, with daily required symptom and temperature checks. There was a desire to test all employees with our Simplexa[™] COVID-19 Direct test, but there was no guidance provided by the State of California's Cal/OSHA even though this was necessary to protect the health of the production staff and their ability to continue kit production for the labs using our assays and Direct Amplification Discs. This challenge was solved by partnering with a testing service provider in

San Diego, CA, to provide a blanket employee testing order from their lead physician, as well as nurses for weekly specimen collection, and by partnering with a CLIA-certified lab located close to DiaSorin Molecular to perform Simplexa™ COVID-19 Direct testing of our employees.

Careful allocation of kits to customers was managed daily starting from the EUA and CE marked launches of Simplexa™ COVID-19 Direct in March 2020. We were transparent with clients regarding weekly kit shipments and only took on new clients as kit manufacturing output increased. Direct Amplification Disc (DAD) production was ramped up in tandem with kit production. Fortunately for DiaSorin Molecular, the supply chain impacts experienced by other suppliers did not affect Simplexa™ COVID-19 Direct as no extraction reagents or plastic cartridges were required for producing the kit.

CHALLENGES AFTER LAUNCH

Typically, there are sources of inactivated viral reference materials and specimens with accompanying IVD test results to support lab validation. Since SARS-CoV-2 was a novel virus, external controls were not readily available for laboratories to validate EUA assay performance. With concerns about this novel agent and the limited supply of inactivated virus, many commercial suppliers turned to safer synthetic alternatives. DiaSorin Molecular proactively worked with Exact Diagnostics on a synthetic verification panel for laboratories to use for implementing our assay. As a result, external reference verification panel material was available immediately after launch to use for verifying assay performance.

Because of the massive increase in nasopharyngeal and nasal swab testing, transport media and collection swab shortages occurred in the U.S. and globally by April 2020. Desperate to have specimen collection media, labs were forced to resort to alternative transport media or manufacture their own based on shared recipes from the CDC (Centers for Disease Control and Prevention, 2020). Labs also validated readily available plastic cotton-tipped swabs with SARS-CoV-2 RT-PCR assays instead of plastic synthetic-tipped swabs that were considered the gold standard (Freire-Paspuel et al., 2020). Commercial assay manufacturers typically validate different transport media types with their assays because transport media contain multiple components that can be present at different concentrations that can impact downstream molecular diagnostic assay performance. A benefit of the Simplexa™ Direct assay chemistry is that it does not require a separate nucleic acid extraction step. Technically this has the advantage of no loss of specimen due to extraction efficiency impact and no extraction reagent supply expense or issues. However, caution must be taken with different transport media types because the Simplexa™ chemistry comes into direct contact with the transport medium. Differences in salt, pH or media components such as those that denature proteins can impact Simplexa™ performance. Without proper validation, some of those alternative media types did, in fact, impact the Limit of Detection leading to reduced test sensitivity. Others that contained viral deactivation components also completely inactivated the reverse transcriptase and Taq polymerase enzymes

that are components of the Simplexa™ Direct chemistry active ingredients. DiaSorin Molecular formally validated 0.9% saline as a collection media alternative to allow labs a consistent and reliable option to use when the other on label transport media were in short supply.

SUPPLY CHAIN CHALLENGES FROM A MANUFACTURING PERSPECTIVE

Within 30 days of EUA kit launch in March 2020, Simplexa™ COVID-19 Direct reagent production increased by 141%, and production ramp-up was started for the DAD consumable required to perform the test. By January 2021, during peak demand period, production increased 2999% for DAD consumables and 433% for COVID-19 reagents. All in all, during the COVID-19 pandemic, DiaSorin Molecular packaged and shipped more than ten times as many kits per month compared to the highest prior production month in previous history. Remarkably, support was also maintained for the other >100 non-COVID-19 products and there were no customer backorders during this time.

Stepping up to the pandemic production demand required expansion of the supply chain base, increased production throughput, additional equipment sourcing, expansion of production areas, and headcount increases, all of which were performed simultaneously. The Supply Chain organization was transformed during 2020 to secure raw materials at a time where the global supply chain suffered severe interruptions, depletion, and heightened demand. To ensure business continuity, DiaSorin Molecular developed relationships with multiple backup suppliers for dual sourcing of all critical supplies; this effort yielded 25 new key suppliers, which were used to alleviate constraints for commodities, including plastics, pipettes, tank liners, oligonucleotides, resin, dry ice, and more. To combat supplier constraints at the contract manufacturer for discs, production was partially insourced and a new supply chain was developed to match the contract manufacturers' maximum volume in consumable production. In five months, a second DAD production line was implemented with new automation for manufacturing and inspection of the DADs, and 12 new pieces of equipment were installed and validated. With demand increasing, this was followed by implementing a third manufacturing line with automation, expanding the cleanroom to make space for this third line, and installing and validating 18 new pieces of equipment. A new DAD mold was also validated and implemented in January 2021, with a new mold maker vendor to strengthen our supply chain. Upgrades were also made to the warehouse to increase storage capacity for raw materials and finished goods. The company invested in expanded frozen storage capacity to provide critical backup capabilities to preserve finished goods. Manufacturing Engineering and Production teams further scaled-up kit production by fine-tuning the speed and accuracy of automated dispensing equipment to minimize reagent waste and increase throughput. The Technical Operations team performed process validations to

substantially increase lot sizes. These expansions required increased staff, and Production personnel increased by 150% starting from a single shift, Monday through Friday in March 2020 to three shifts, seven days a week during peak production.

FUTURE PERSPECTIVES AND RECOMMENDATIONS

Understanding the challenges faced by DiaSorin Molecular to bring a sensitive and specific SARS-CoV-2 RT-PCR assay to market as quickly as possible can help us prepare for future pandemics. We can reflect on the supply chain constraints that were faced internally, and by our customers. Rapid assay development was enabled by having a core Simplexa™ Direct chemistry in which to drop in primer and probe designs for a novel pathogen. The public sharing and access to databases with the virus sequence also allowed for the fastest possible target design for a molecular assay. The willingness of hospital labs to closely work with a commercial partner for access to precious clinical specimens was paramount. The availability of the FDA for discussions regarding the regulatory path and the support received from BARDA was also crucial. Pressure to manufacture as many kits as possible allowed us to quickly expand and add on new manufacturing lines and creatively overcome supply challenges. One aspect that remains critical is the worldwide shortage of the resin used for plastics manufacturing. Because disposable, medical-grade plastics are used throughout the healthcare industry, this is an area that needs attention in order to be prepared for surges of SARS-CoV-2 and pandemics in the future. Another consideration for future global pandemic preparedness is the availability of reference standards and mandatory rapid performance assessments to guarantee the quality of the supplies on the market for every country in

the world (Freire-Paspuel and Garcia-Bereguain, 2021). The successful rapid assay launch, production scale-up, and continuity of supply to our customers during the COVID-19 pandemic response is a testament to the DiaSorin Molecular team's capabilities, skillset, and determination across all groups within DiaSorin that supported their efforts. It would not have been possible without the courage and cooperation of the outstanding and resilient teams in Italy and the U.S.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MT was responsible for writing a majority of the article. GM was responsible for writing about DiaSorin Italy experience. VA was responsible for writing about DiaSorin Molecular manufacturing experience. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: MT and VA are all employees of DiaSorin Molecular LLC. GM is an employee of DiaSorin S.p.A.

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Humoral, Cellular and Cytokine Immune Responses Against SARS-CoV-2 Variants in COVID-19 Convalescent and Confirmed Patients With Different Disease Severities

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Objectives: To assess humoral and cellular immune responses against SARS-CoV-2 variants in COVID-19 convalescent and confirmed patients, to explore the correlation between disease severity, humoral immunity, and cytokines/chemokines in confirmed patients, and to evaluate the ADE risk of SARS-CoV-2.

Methods: Anti-RBD IgG were quantified using an ELISA. Neutralization potency was measured using pseudovirus and real virus. Cellular immunity was measured using ELISpot. Cytokine/chemokine levels were detected using multiplex immunoassays. *In vitro* ADE assays were performed using Raji cells.

Results: One-month alpha convalescents exhibited spike-specific antibodies and T cells for alpha and delta variants. Notably, the RBD-specific IgG towards the delta variant decreased by 2.5-fold compared to the alpha variant. Besides, serum from individuals recently experienced COVID-19 showed suboptimal neutralizing activity against the delta and omicron variants. Humoral immune response, IL-6, IP-10 and MCP-1 levels were greater in patients with severe disease. Moreover, neither SARS-CoV-1 nor SARS-CoV-2 convalescent sera significantly enhanced SARS-CoV-2 pseudovirus infection.

Conclusions: Significant resistance of the delta and omicron variants to the humoral immune response generated by individuals who recently experienced COVID-19. Furthermore, there was a significant correlation among disease severity, humoral immune response, and specific cytokines/chemokine levels. No evident ADE was observed for SARS-CoV-2.

Keywords: SARS-CoV-2 variants, humoral immune response, cellular immune response, disease severity, inflammatory mediators

INTRODUCTION

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to a large number of infections and deaths (Hu et al., 2021). Several variants known as variants of concern (VOCs) such as the delta and omicron variants had shown superior infectivity and even immune evasion ability (Harvey et al., 2021). Recent studies have reported that serum from recovered patients previously infected with the Wuhan strain showed a dramatic 10-fold decrease in the neutralizing efficacy against the omicron variant compared to the original strain with D614G mutation (Ma et al., 2022), and another study also indicated that no cross-neutralization towards the omicron variant was observed in unvaccinated alpha variant convalescent patients (Rössler et al., 2022). Cellular immunity is important in combating viral infections, which may provide protective immunity and limit severe disease, and it is important to assess whether individuals who have experienced SARS-CoV-2 infection are able to produce effective cellular immune memory against emerging variants (Rydzynski Moderbacher et al., 2020; Moss, 2022). In addition, some severe COVID-19 patients could have higher antibody levels and neutralizing titers, together with excess proinflammatory cytokine and chemokine levels (Fajgenbaum and June, 2020; Ling et al., 2021; Pum et al., 2021); however, the correlation between these indicators and COVID-19 disease severity is still unclear. Furthermore, previous studies have reported that antibodies against the SARS-CoV-1 spike protein could promote ACE2-independent virus entry into macrophages, monocytes, and B cells *in vitro* (Jaume et al., 2011; Wang et al., 2014; Yip et al., 2014). Whether antisera elicited by previous SARS-CoV-1 or SARS-CoV-2 infection could induce antibody-dependent enhancement (ADE) of SARS-CoV-2 entry is still unknown. In this study, we aimed to investigate the humoral and cellular immune responses from alpha variant convalescent and confirmed patients against emerging SARS-CoV-2 variants. Next, we analyzed the differences in humoral immune responses and cytokine/chemokine profiles in patients with mild/severe disease severity. Finally, we explored whether SARS-CoV-1 and SARS-CoV-2 convalescent sera would enhance SARS-CoV-2 pseudovirus variant infection in Fcγ receptor (FcγR)-expressing Raji cells.

MATERIALS AND METHODS

Cells Lines and Viruses

BHK-21 cells, a baby hamster kidney cell line (ATCC CCL-10), were cultured in RPMI 1640 medium containing 5% fetal bovine serum (FBS; HyClone). HEK293 cells, a human embryonic kidney cell line (ATCC CRL-1573), and 293T/17 cells (ATCC CRL-11268) were grown in DMEM containing 10% FBS. Raji cells, a human B lymphocyte cell line (BCRC 60116), were grown in RPMI 1640 containing 10% FBS. Vero E6 cells (an African green monkey kidney cell line, ATCC CRL-1586) were

maintained in high-glucose DMEM supplemented with 10% FBS and an antibiotic-antimycotic (Gibco) in a humidified atmosphere of 37°C and 5% CO₂. The SARS-CoV-2 WA strain (hCoV-19/Taiwan/4/2020) and the two variants of concern (VOCs), namely, B.1.1.7 (hCoV-19/Taiwan/792/2020, alpha variant), and B.1.617.2 (hCoV-19/Taiwan/1144/2021, delta variant), were kindly provided by Taiwan Centers for Disease Control, Ministry of Health and Welfare, and propagated using Vero E6 cells supplemented with 2% FBS. Passage 2 virus was used for all the studies described here. Viral stocks were free of contamination, and viral titers were determined by plaque assay followed by storage of aliquots at -80°C until further use in experiments.

Participants

A total of 48 consenting patients from the Tri-Service General Hospital (Taiwan) with laboratory-confirmed SARS-CoV-2 infections were enrolled in this study. Among them, samples from 26 unvaccinated individuals infected with the alpha variant were collected one month after discharge from the hospital. In addition, we also analyzed the samples of 22 patients hospitalized between June and July 2021 with confirmed alpha variant infection. Eleven of these patients were categorized as severe patients with pneumonia according to the following definitions: (1) SpO₂ <94% without oxygen supply, (2) respiratory frequency >30 breaths/min, and (3) respiratory failure.

Spike Plasmid Cloning and SARS-CoV-2 Pseudovirus Production

To construct a pseudovirus carrying the spike protein of SARS-CoV-2, the stocks of pseudovirus were produced by co-transfection of luciferase-expressing pLAS3w-FLuc-Ppuro (10 μg) with 2 other plasmids, the pCMV-Δ8.91 (Gag-Pol provider, 6.6 μg) and the following spike plasmids (4.8 μg) to HEK293T cells (4 × 10⁶ cells per 10-cm dish) by Lipofectamine 3000[®] transfection reagent (ThermoFisher): pcDNA3.1_spike_del19 (Addgene #155297), pcDNA3.3_CoV2_B.1.1.7 (Addgene #170451), pcDNA3.3_CoV2_501V2 (Addgene #170449), pcDNA3.3-SARS2-B.1.617.2 (Addgene #172320) and SARS-CoV-2 Omicron Strain S gene Human codon_pcDNA3.1(+) (GenScript # MC_0101274). In brief, 64 μL Lipofectamine 3000[®] transfection reagents were mixed with 500 μL serum free DMEM and sat at the room temperature for 5 minutes then mixed with three DNA plasmids that were diluted in 500 μL serum-free DMEM for another 25 minutes. This DNA-Lipofectamine mixture was then added into each well and incubated at 37°C in a 5% CO₂ incubator. After overnight incubation in a 37°C, 5% CO₂ incubator for 18 hours, the transfected cells were replenished with fresh growth media for continuous culture. At 48 hours post-transfection, the pseudovirus containing culture medium was collected by centrifugation at 1,000 × g for 10 minutes to remove unwanted cells or large debris, followed by passing the clarified medium through a 0.45 μm filter (Millipore Corporation, Billerica, MA, USA). Virus can be stored at 4°C for immediate use or frozen at -80°C. The pseudovirus was normalized by a p24 ELISA kit (Takara Bio).

Generation of Stable BHK-21/ACE2 and HEK293/ACE2 Cells

Overexpression of *ACE2* gene was carried out by infecting cells with Lentivirus. The pLAS3w-Ppuro plasmid was purchased from Academia Sinica RNAi Core (Taipei, Taiwan), and *ACE2* gene constructed into this plasmid named pLAS3w-*ACE2*-Ppuro. The stocks of Lentiviruses were produced by co-transfection of pLAS3w-*ACE2*-Ppuro (2.5 µg) with 2 other plasmids, the pCMV-Δ8.91 (Gag-Pol provider, 2.25 µg) and the pMD2.G (VSV-G pleiotropic envelope provider, 0.25 µg), to HEK293T cells (1.5×10^6 cells per 6-cm dish) by Lipofectamine 3000[®] transfection reagent (ThermoFisher). In brief, 15 µL Lipofectamine 3000[®] transfection reagents were mixed with 250 µL serum free DMEM and sat at the room temperature for 5 minutes then mixed with three DNA plasmids that were diluted in 250 µL serum-free DMEM for another 25 minutes. This DNA- Lipofectamine mixture was then added into each well and incubated at 37°C in a 5% CO₂ incubator. After overnight incubation in a 37°C, 5% CO₂ incubator for 18 hours, the transfected cells were replenished with fresh growth media for continuous culture. At 48 hours post-transfection, the Lentivirus containing culture medium was collected by centrifugation at 1,000 x g for 10 minutes to removes unwanted cells or large debris, followed by passing the clarified medium through a 0.45 µm filter (Millipore Corporation, Billerica, MA, USA). For *ACE2* overexpression, BHK-21 or HEK293 cells were seeded at a density of 1.5×10^6 cells per well in 6 cm dishes. After overnight culture, cells were incubated with lentivirus containing 8 µg/mL polybrene in 1 mL of fresh growth medium for 1-hour viral absorption at 37°C in a 5% CO₂ incubator with gently rocking per 15 minutes. After the absorption process, 3 mL growth medium containing 8 µg/mL polybrene were supplemented for continuous cell cultivation for 24 hours. The following days, puromycin was added to the culturing medium at a concentration of 10 µg/mL for at least 48 hours to select for survived *ACE2*-overexpressing cell clones.

Western Blot

To investigate the *ACE2* expression of BHK-21/*ACE2* cells and HEK293/*ACE2* cells, the cells were dissolved with RIPA buffer. The cell lysates were analyzed by western blot analysis with the anti-*ACE2* antibody (GeneTex 101395) and anti-actin antibody (Millipore). Then, membranes were probed with the secondary antibody horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch). The signals were developed by enhanced chemiluminescence (Millipore) and photographed by using Luminescent Image Analyzer (LAS-3000; Fujifilm).

RBD-Specific IgG ELISA

To quantitatively detect IgG antibodies against the SARS-CoV-2 RBD, we used an indirect ELISA using an anti-SARS-CoV-2 IgG1 monoclonal antibody (CR3022) (Yuan et al., 2020; Demonbreun et al., 2021; Moriyama et al., 2021; Thomas et al., 2021; Yuen et al., 2021). 96 well ELISA plates (Thermo Fisher) were coated for 16 hours at 4°C with purified SARS-CoV-2 alpha

variant RBD (Genetex, Cat No. GTX136014-pro) or delta variant RBD (GeneTex, Cat. No. GTX136014-pro) diluted in carbonate-bicarbonate buffer to a concentration of 1 µg/mL. The plates were washed with 0.05% PBST, incubated with a blocking buffer consisting of 1% BSA in PBS for 1 hour at room temperature, and then washed. Serum samples were diluted 1:50 with a dilution buffer consisting of 1% BSA. A six-point standard curve was created using CR3022-IgG1 starting at 2 mg/mL by performing 1:2 serial dilutions with dilution buffer (**Supplementary Figure S2**). Samples and standards were added to corresponding wells and incubated for 1 hour at room temperature, followed by washing. Human IgG antibodies were detected with anti-human IgG-HRP (1:100,000). This detection antibody was added to the plate and incubated for 30 minutes at room temperature. After washing, TMB substrate (Invitrogen) was added to each well and incubated for 5 minutes, and the reaction was stopped with 1 M H₂SO₄. Optical density (O.D.) was measured at 450 nm with subtraction of the O.D. at 570 nm as a reference wavelength on an ELISA reader (BioTek). Anti-RBD antibody levels were calculated by interpolating onto a standard curve and correcting for sample dilution; one unit per mL (U/mL) was defined as the equivalent reactivity seen with 1 mg/mL CR3022, and the cut-off value was defined as mean OD₄₅₀ values of pre-pandemic sera + 3 SD. All experiments were performed in duplicates.

Neutralization Assay (NT₅₀) With Pseudotyped SARS-CoV-2

BHK-21/*ACE2* cells were seeded with 4×10^4 in 24-well plate at 16 hours before the infection. To test the infectivity of pseudovirus, 100 ng of each variant of pseudovirus were add to BHK-21/*ACE2* cells and incubated for 48 hours. For neutralization assay, 50 µL of heat-inactivated sera were 2-fold diluted in duplicate samples in complete medium with 2% FBS starting with a 1:16 dilution followed by incubation with 50 µL of pseudovirus (1 ng p24) for 1 h at 37°C. On the day of infection, the cells were washed twice with PBS, and 100 µL of inoculum was added to the cells and incubated for 48 hours. The cells were quenched by adding 100 µL of BrightGlow luciferase substrate (Promega) directly to each well, and the luciferase activity was measured with Synergy H4 luminometer (BioTek). Background values, monitored from uninfected cells were consistently below 400 relative luminescence units, and sera collected before 2019 were used to set as the negative control for the neutralization assay, sera started diluted at 1:16, gave results in the range of the background RLU levels. An NT₅₀ > 1:16 serum dilution was regarded as positive.

Neutralization Assay (PRNT₅₀) With Real SARS-CoV-2

Serum samples were heat-inactivated for 30 minutes at 56°C; twofold serial dilutions, starting at a concentration of 1:5, were then mixed with an equal volume of viral solution containing 200 PFU of SARS-CoV-2. The serum-virus mixture was incubated for 1 hour at 37°C in a humidified atmosphere with 5% CO₂. After incubation, the mixture at each dilution was added to Vero

E6 cells and incubated at 37°C for 1 hour. Cells were subsequently cultured with DMEM containing 2% FBS and 1.4% methylcellulose for 72 hours. After culturing, plaques were stained and counted. Neutralizing antibody titers were defined as the reciprocal of the maximum dilution of serum that reduced the virus titer by 50% compared to the negative control sera, and PRNT₅₀ below 1:5 serum dilution was considered negative.

Isolation of PBMCs

PBMCs of COVID-19 convalescent individuals were isolated from anticoagulant blood using Ficoll-Paque™ PLUS density gradient medium (Cytiva #17144003). To isolate PBMCs, blood diluted with PBS was gently layered over an equal volume of Ficoll in a Falcon tube and centrifuged for 30 minutes at 400 × g without braking. Four layers formed, each containing different cell types. The second layer contained PBMCs. These cells were gently removed using a Pasteur pipette and added to warm medium or PBS to wash off any remaining platelets. The pelleted cells were then counted, and the percentage viability was estimated using Trypan blue staining. Isolated PBMCs were stored in liquid nitrogen until use in assays.

ELISpot Assay

The number of antigen-specific IFN- γ - or IL-2-secreting SFU was determined by ELISpot assays. Cryopreserved PBMCs were rapidly thawed and allowed to rest overnight. Cells were dispensed at 1×10^5 cells per well for the IFN- γ or IL-2 ELISpot assay (Human IFN- γ or IL-2 ELISpot Kit, R&D Systems). The cells were stimulated with a pool of peptides consisting mainly of 15-mer sequences with 11 amino acid overlap, covering the S protein selectively mutated regions of the SARS-CoV-2 alpha variant (PepTivator® SARS-CoV-2 Prot_S B.1.1.7, Miltenyi Biotec) or delta variant (PepTivator® SARS-CoV-2 Prot_S B.1.617.2, Miltenyi Biotec), and incubated at 37°C for 22 hours. Cells stimulated with PHA-M (Phytohemagglutinin, M form, ThermoFisher) served as the positive control and all convalescent patients were above 115 spot forming cells (SFC)/ 10^5 PBMCs for both IFN- γ and IL-2. IFN- γ or IL-2 release was detected following the instructions in the manual, and the spots were counted using an ELISPOT reader (Cellular Technology Ltd.). The mean SFC value counted in triplicate peptide pool stimulations was calculated and normalized by subtracting the mean of the negative control replicates (control medium), and the cut-off value for background T cell responses was defined as the mean SFC value of seronegative PBMCs derived from healthy unvaccinated donors + 3 SD (9.2 SFC/ 10^6 PBMCs). The results were expressed as SFC per million PBMCs.

Multi-Plex Immunoassay

To assess cytokines and chemokines concentrations of in confirmed patients, ELISA-based Bio-Plex Pro Human Cytokine, Chemokine, and Growth Factor Assays Kit was used for evaluating the production of IL-6, IP-10 and MCP-1 in sample sera following the manufacturer's instructions. The

patient sera samples were diluted (1:3) in sample diluent and cytokines or chemokines were analyzed with the Bio-Plex200 System using the Bio-Plex Manager™ software. For each cytokine and chemokine, assay ranges and LOD were provided by the manufacturer. All reagents and equipment, including washing station and shaking incubators, were from BIO-RAD Laboratories.

Antibody-Dependent Viral Entry (*In Vitro* ADE Assay)

For ADE assays, 100 μ l of serial 8-fold dilutions of heat-inactivated serum were incubated for 1 hour at 37°C with 100 μ l of pseudovirus. One hundred microliters of Raji cells (1×10^6 cell/mL) previously washed three times with serum-free RPMI were added to the antibody-SARS-CoV-2 pseudovirus mixture in a 96-well plate, and some groups of Raji cells were preincubated with 5 μ g/ml Fc γ R inhibitor (Invitrogen # 16-0329-81) for 15 minutes at 4°C. After adsorption for 24 hours at 37°C, the medium was renewed 24 hours later; the cells were incubated for an additional 24 hours, washed in PBS, and lysed; and luciferase activity was measured with a Synergy H4 luminometer (BioTek). Duplicates were performed for each tested serum and the dotted line represents the average luciferase activity of the virus-only group.

Ethics

This study was approved by the Tri-Service General Hospital (TSGHIRB No. C202005067). Informed consent was obtained from all enrolled participants.

Statistical Analysis

Statistics were determined using GraphPad Prism 5. Anti-RBD IgG titers, NT₅₀ and PRNT₅₀ were described as medians and IQRs. A nonlinear sigmoidal 4PL model was used to determine the NT₅₀ and PRNT₅₀ for each serum. Measured statistical significance for pseudovirus or real virus neutralization assays (NT₅₀ and PRNT₅₀) were calculated among experiments by one-way ANOVA with Tukey's multiple comparison test. Simple linear regression and Pearson correlation analysis were conducted to determine the correlation coefficients between anti-RBD IgG titers and NT₅₀ or PRNT₅₀. Two-tailed Student's t test was conducted for indicators of different disease severities. ROC curves were also plotted using GraphPad Prism 5, and the AUC was calculated. Asterisks indicated statistical significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

RESULTS

Clinical Characteristics of Enrolled Convalescent and Confirmed Patients in This Study

Blood samples were obtained from 26 alpha variant convalescent patients who have been discharged from hospital for 1 month and 22 alpha variant confirmed patients hospitalized between

June and July 2021. Among the 26 convalescent patients, 14 (54%) were male and 12 (46%) were female, with a median age of 58 years (54–70 years), while among the 22 confirmed patients, 17 (77%) were male and 5 (23%) were female, with a median age of 56 years (38–68 years). The detailed clinical characteristics of convalescent and confirmed patients are summarized in the **Supplementary Tables 1, 2**.

Humoral and Cellular Immune Responses of Alpha Variant Convalescent Sera Against Different SARS-CoV-2 Pseudovirus and Real Virus Variants

We first assessed anti-RBD IgG titers in the sera of alpha variant convalescent patients. The results showed that the median (25–75 interquartile range (IQR)) antibody titers against alpha variant was 512.54 U/mL (329.22–837.70). Notably, when faced with the delta variant, which has many mutations in RBD, the median (25–75 IQR) anti-RBD IgG titers was 286.32 U/mL (157.57–435.79), a significant 2.5-fold decrease compared to the alpha variant ($p = 0.0033$) (**Figure 1A**). Next, for the neutralization assay, we established different SARS-CoV-2 pseudovirus variants and ACE2-expression stable cell lines (**Supplementary Figure S1**). Lentiviral packaging plasmid, lentiviral transfer plasmid (with luciferase gene) and SARS-CoV-2-spike plasmid with indicated mutation were co-transfection into producer cell (293 cells), after 48 hours transfection, cell supernatant was harvested, purified and the pseudovirus titer was normalized using the p24 ELISA kit (**Supplementary Figure S1A**). After validation of exogenous ACE2 expression in BHK-21 and HEK293 cells by western blot (**Supplementary Figure S1B**), the infectivity assay were performed and showed that pseudovirus variants have significantly higher luciferase activities compared to the Wuhan strain (**Supplementary Figure S1C**), suggesting other variant strains exhibited more efficient ACE2-mediated infection than the wild type Wuhan strain as previously described (Arora et al., 2021; Kuzmina et al., 2021; Hoffmann et al., 2022). Then, we used this platform to evaluate the neutralizing abilities of alpha variant convalescent sera against different SARS-CoV-2 pseudovirus variants (**Figure 1B**). The results demonstrated that alpha variant convalescent patients showed the greatest neutralization efficacy (NT_{50}) for the alpha strains (**Figure 1B**, blue). However, the NT_{50} against the beta, delta and omicron variants decreased significantly, with a 5.2-fold reduction for the delta variant and a 7.7-fold reduction for the omicron variant compared to that for the alpha variant ($p < 0.0001$). We then further divided these convalescent individuals into robust neutralizers (serum diluted 1:128 could still neutralize more than half of the pseudovirus) and non-robust neutralizers (**Figure 1C**) (Planas et al., 2021). Most of the sera from the alpha convalescent individuals (24/26) robustly neutralized the alpha variant, compared to the delta variant, for which only 50% of the convalescent sera (13/26) effectively neutralized the pseudovirus. Likewise, a similar trend was found by using real SARS-CoV-2, with a remarkable decline in $PRNT_{50}$ for the delta

variant compared to that for the alpha variant ($p < 0.0001$) (**Figure 1D**). These results suggested that the beta, delta and omicron variants are more resistant to alpha convalescent serum neutralization. Furthermore, we correlated anti-RBD IgG titers with NT_{50} and $PRNT_{50}$ measured by using pseudovirus (**Figure 1E**) and real SARS-CoV-2 assays (**Figure 1F**). The results showed a significant positive correlation between the anti-RBD IgG titers and the corresponding NT_{50} or $PRNT_{50}$ in alpha variant convalescent patients, indicating that anti-RBD IgG is crucial for neutralizing SARS-CoV-2. Subsequently, we also assessed the cellular immune memory of T cells after stimulation by alpha and delta variants spike peptide pools using IFN- γ and IL-2 ELISpot assays (**Figures 1G, H**). The results showed that for alpha and delta variants the median (25–75 IQR) number of IFN- γ -secreting T cells were 64 (38.6–95.3) and 48.2 (29.1–63) SFC/ 10^6 PBMCs, while IL-2 were 35.1 (25.6–50.3) and 28.2 (19.4–42.4) SFC/ 10^6 PBMCs, respectively, indicating that alpha variant convalescent patients could produce T cells against alpha and delta variants.

Neutralizing Potency of the Sera From Alpha Variant Infection-Confirmed Patients Against Different SARS-CoV-2 Pseudovirus and Real Virus Variants

Next, we further measured anti-RBD IgG titers in alpha variant infection-confirmed patients (**Figure 2A**), and the median (25–75 IQR) antibody concentrations in these patients was 204.36 U/mL (90.61–335.60), which is a notably lower titer than that in the one-month alpha convalescent individuals, when faced with delta variant, the anti-RBD IgG titer decreased significantly by 1.6-fold compared to the alpha variant ($p = 0.0002$) (**Figure 1A**). In addition, the median (25–75 IQR) NT_{50} of these confirmed patients was 450.63 (275.63–692.84) for the Wuhan strain and 651.65 (268.84–1022.09) for the alpha variant (**Figure 2B**). However, the median (25–75 IQR) NT_{50} for the delta variant and omicron variant dropped to 74.80 (23.63–209.03) and 38.64 (19.77–147.09), indicating that the new emerging variants is more resistant to neutralizing antibodies. Additionally, 21 of 22 confirmed patients' sera (95.5%) were effective in neutralizing the alpha variant, which was reduced to 9 of 22 subjects (40.9%) for the delta variant (**Figure 2C**). Similarly, using real SARS-CoV-2 variants to measure neutralizing activity, we found that the $PRNT_{50}$ for the delta variant decreased 34.6 times compared to that for the alpha variant ($p < 0.0001$) (**Figure 2D**). Likewise, a significant positive correlation between the anti-RBD IgG titers and the corresponding NT_{50} (**Figure 2E**) or $PRNT_{50}$ (**Figure 2F**) were shown in confirmed patients.

Discriminating Distinct Characteristics Between Mild and Severe COVID-19 Patients

We further categorized the alpha variant infection-confirmed patients into mild and severe groups. The results revealed that the anti-RBD IgG titers ($p = 0.0014$), NT_{50} ($p < 0.0001$) and $PRNT_{50}$ ($p = 0.0009$) against the alpha variant were significantly

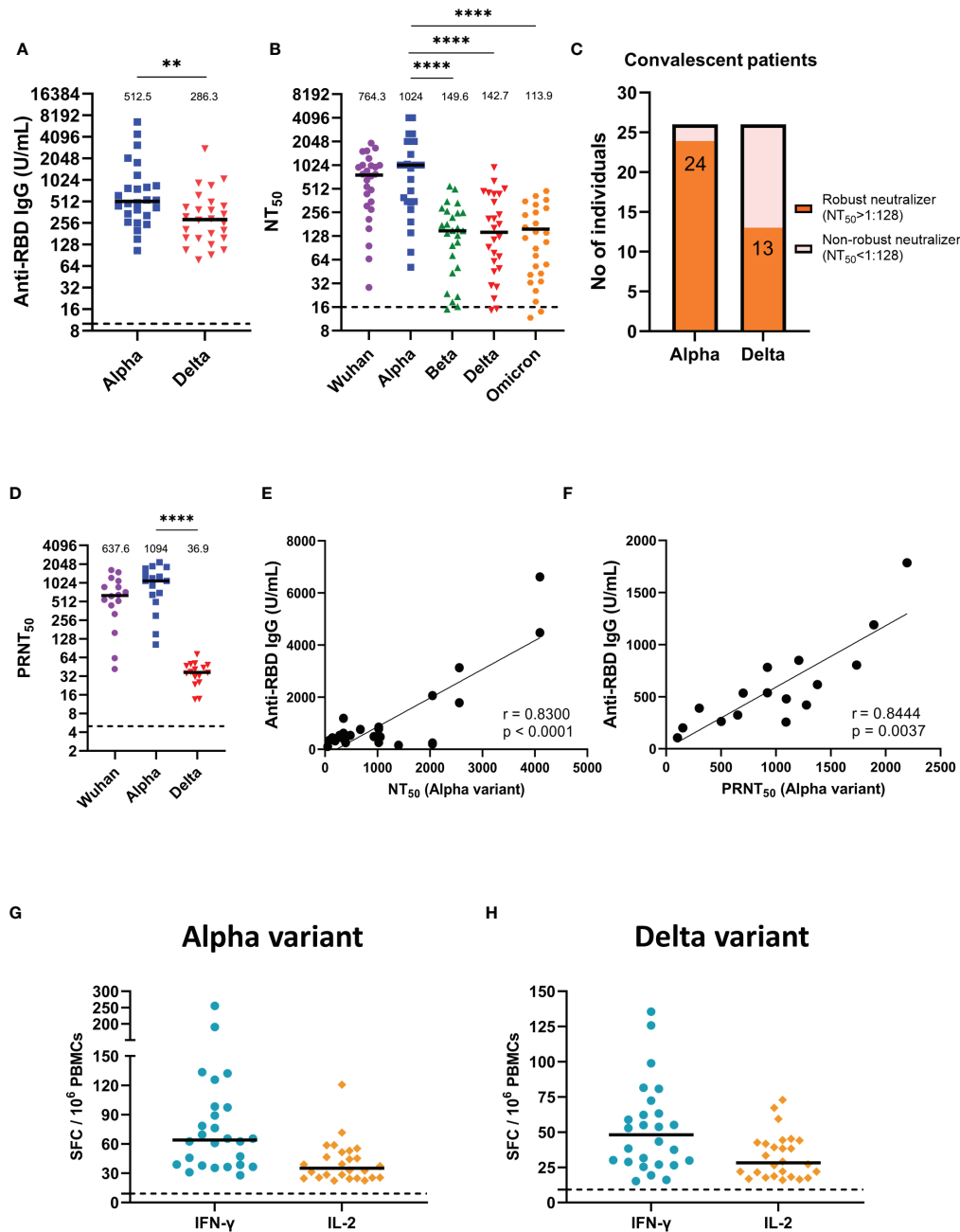


FIGURE 1 | Humoral and cellular immune responses of alpha variant convalescents. **(A)** Results of ELISA measuring serum reactivity to anti-RBD IgG ($n = 26$). **(B)** NT₅₀ of alpha variant convalescent sera ($n = 26$) measured with indicated SARS-CoV-2 pseudovirus variants. **(C)** Fraction of robust neutralizers in the convalescent cohort at 1-month post-infection. Individuals with a pseudovirus NT₅₀ above 1:128 were classified as robust neutralizers, while individuals with a pseudovirus NT₅₀ below 1:128 were classified as non-robust neutralizers. **(D)** PRNT₅₀ of alpha variant convalescent sera ($n = 16$) detected with indicated real SARS-CoV-2 variants. **(E)** Correlation between alpha variant anti-RBD IgG titers and NT₅₀. **(F)** Correlation between alpha variant anti-RBD IgG titers and PRNT₅₀. **(G, H)** IFN-γ and IL-2 ELISpot of alpha variant convalescents PBMCs stimulated with **(G)** alpha variant or **(H)** delta variant peptide pool. SFC, spot-forming cells. The dotted line represents the cut-off value for each assay. Duplicates were performed for each tested sample. Measured statistical significance was calculated among experiments by one-way ANOVA with Tukey's multiple comparison test. Simple linear regression and Pearson correlation analysis were conducted to determine the correlation coefficients. Asterisks indicate statistical significance, ** $p < 0.01$, **** $p < 0.0001$.

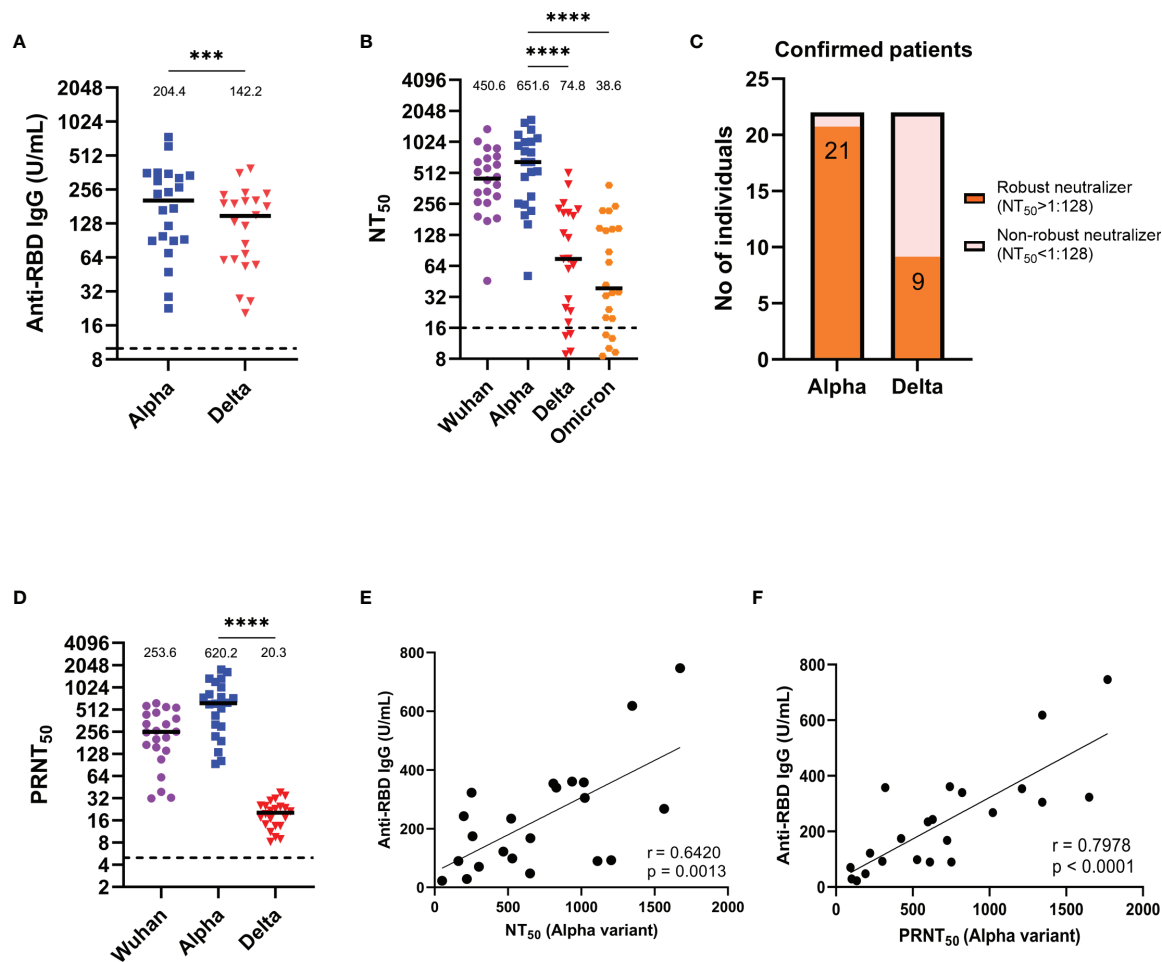


FIGURE 2 | Anti-RBD IgG titers and neutralization abilities of alpha variant infection-confirmed patient serum. **(A)** Results of an ELISA measuring serum reactivity to anti-RBD IgG ($n = 22$). **(B)** NT₅₀ of alpha variant infection-confirmed patients ($n = 22$) measured with indicated SARS-CoV-2 pseudovirus variants. **(C)** Fraction of robust neutralizers in the alpha variant infection confirmed patient cohort. Individuals with NT₅₀ above 1:128 were classified as robust neutralizers, while below 1:128 were classified as non-robust neutralizers. **(D)** PRNT₅₀ of alpha variant-confirmed patients ($n = 22$) measured with the indicated real SARS-CoV-2 variants. **(E)** Correlation between anti-RBD IgG titer and NT₅₀. **(F)** Correlation between anti-RBD IgG titer and PRNT₅₀. The dotted line represents the cut-off value for each assay. Duplicates were performed for each tested serum. Measured statistical significance was calculated among experiments by one-way ANOVA with Tukey's multiple comparison test. Simple linear regression and Pearson correlation analysis were conducted to determine the correlation coefficients. Asterisks indicate statistical significance, *** $p < 0.001$, **** $p < 0.0001$.

higher in the severe group than in the mild group (Figures 3A–C). Moreover, IL-6, IP-10, and MCP-1 levels were increased by 16.8- ($p = 0.0029$), 4.1- ($p = 0.0010$), and 5.4-fold ($p = 0.0331$) in patients with severe illness compared to those in patients with mild illness (Figures 3D–F). The area under the receiver operating characteristic (ROC) curve (AUC) for serum levels of cytokines and chemokines was used to estimate the likelihood of a patient developing severe disease (Figures 3G–I). The results showed that the AUC was 0.975 (95% CI: 0.9175–1.000) for IL-6, followed by 0.958 (95% CI: 0.8842–1.000) for IP-10 and 0.883 (95% CI: 0.8842–1.000) for MCP-1, indicating that these inflammatory indicators have excellent predictive performance for poor prognosis. Taken together, these distinct characteristics between mild and severe patients could be used as predictive markers for disease severity.

Investigate the ADE Phenomenon by Using SARS-CoV-1 and SARS-CoV-2 Convalescent Sera in FcγR-Expressing Raji Cells

ADE is usually mediated by sub- or non-neutralizing antibodies, and there are currently some variants that are resistant to the neutralizing efficacy of convalescent serum. Therefore, we established an ADE assay by using pseudoviruses and tested the infectivity of SARS-CoV-1 and SARS-CoV-2 alpha variant convalescent sera in Raji cells. As shown in Figure 4A, healthy donor sera did not increase the infection of the Wuhan strain compared to that of the virus-only group (dotted line). Although a slight increase in luciferase activity was observed when the SARS-CoV-1 convalescent serum was

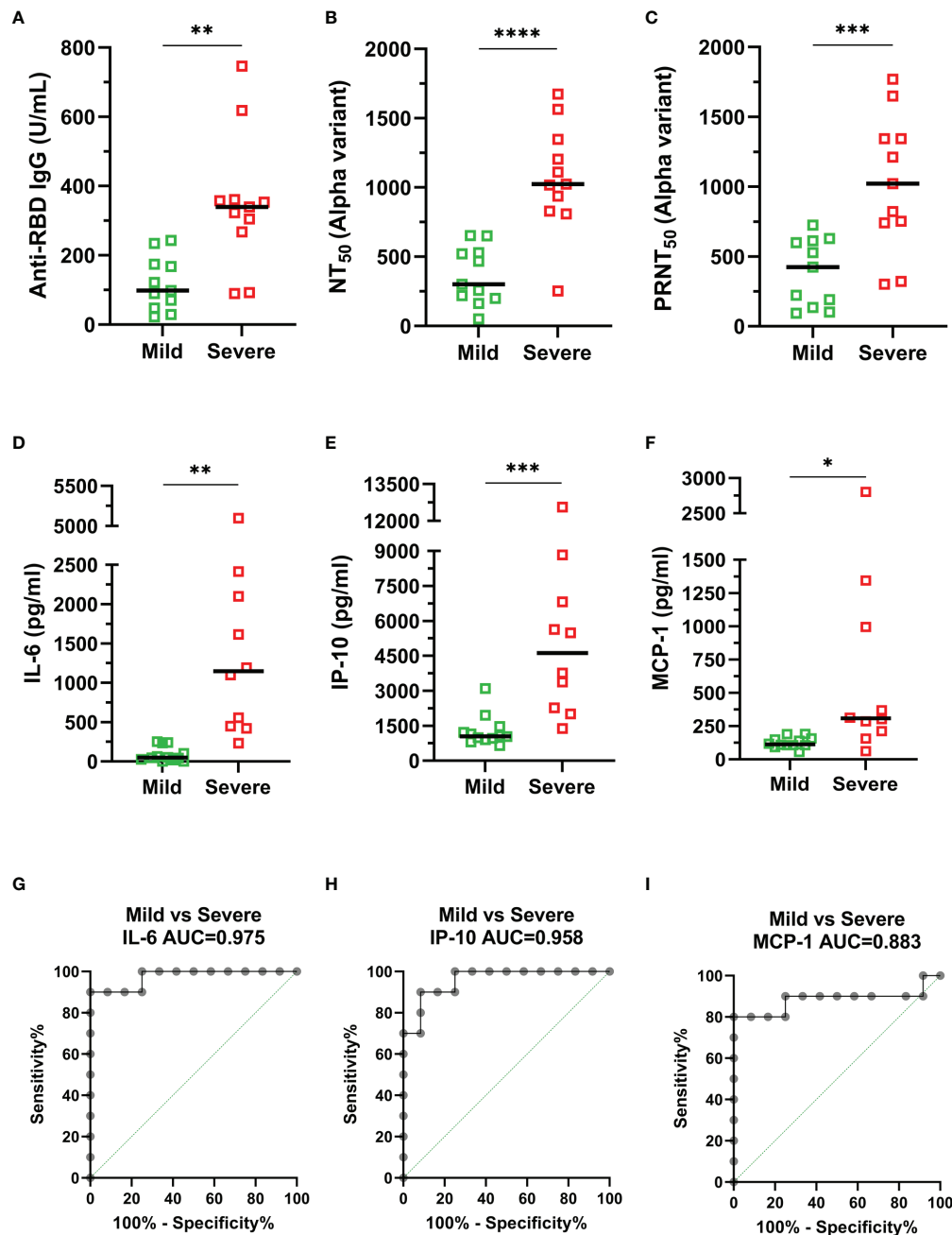


FIGURE 3 | Comparison of relevant indicators between mild (green) and severe (red) COVID-19 confirmed patients. **(A)** Anti-RBD IgG. **(B)** NT₅₀ (alpha variant). **(C)** PRNT₅₀ (alpha variant). **(D)** IL-6. **(E)** IP-10. **(F)** MCP-1. **(G–I)** The area under the receiver operating characteristic (ROC) curve (AUC) for serum cytokine levels: **(G)** IL-6, **(H)** IP-10, and **(I)** MCP-1. Duplicates were performed for each tested serum. Measured statistical significance was calculated between experiments by two-tailed Student's *t* test. Asterisks indicate statistical significance, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

serially diluted to 1:8192, and this phenomenon could be reduced by the addition of FcγR inhibitor, however, the increment was not significant compared to the virus-only group (Figure 4A). In addition, SARS-CoV-2 alpha variant convalescent sera were also examined to determine the ADE phenomenon, but no significant infection enhancement of infection by the Wuhan, alpha, and delta variants was observed (Figure 4B).

DISCUSSION

In this study, we found that individuals who recovered from the alpha variant showed a 2.5-fold decrease in antibodies against delta variant RBD and a dramatically 7.7-fold reduction in neutralizing potency when faced with the emerging omicron variant compared to the alpha variant. Furthermore, only half of

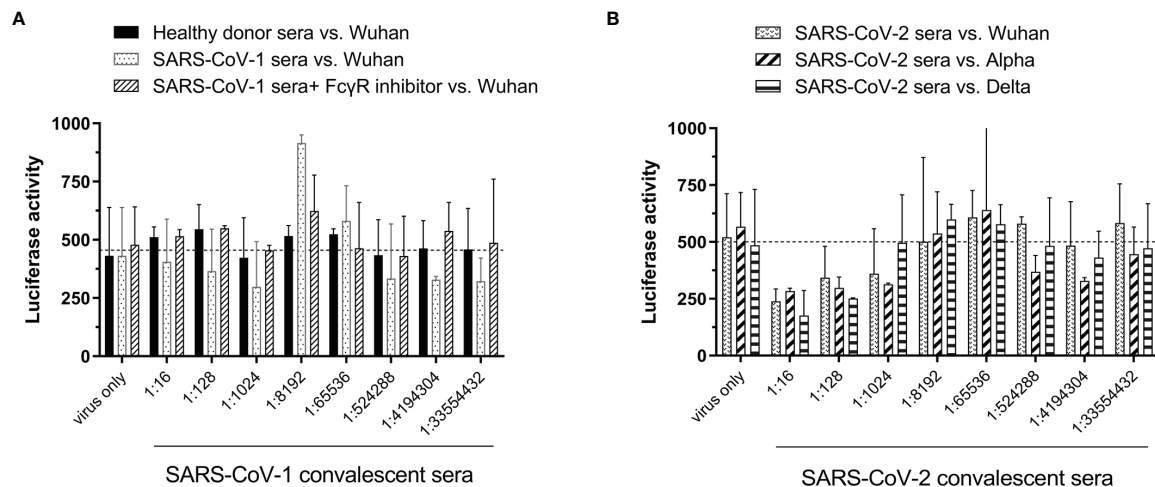


FIGURE 4 | Susceptibility of B lymphoblast Raji cells to infection by SARS-CoV-2 pseudovirus variants under different conditions. **(A)** SARS-CoV-1 convalescent serum vs. SARS-CoV-2 Wuhan strain. **(B)** SARS-CoV-2 alpha variant convalescent serum vs. SARS-CoV-2 Wuhan, alpha or delta strain.

the one-month COVID-19 convalescent patients and approximately 40% of the confirmed patients were robust neutralizers ($NT_{50} > 1:128$) against the delta variant, while all one-month convalescent PBMCs responded to alpha and delta variants spike peptide pools by producing IFN- γ and IL-2. Anti-RBD IgG and neutralizing titers against the alpha variant, as well as levels of pro-inflammatory cytokines (IL-6) and chemokines (IP-10, MCP-1) were significantly higher in the severe group than in the mild group. In addition, an *in vitro* ADE assay showed that both SARS-CoV-1 and SARS-CoV-2 convalescent serum did not significantly enhance the entry of SARS-CoV-2 pseudovirus into Fc γ R-expressing Raji cells.

Recent studies have reported numerous omicron and delta variants leading to reinfection of individuals who had previously been exposed to SARS-CoV-2 (Pulliam et al., 2021; Shastri et al., 2021) and even breakthrough infection with full vaccination (Goga et al., 2021; Kustin et al., 2021; Kuhlmann et al., 2022). Another study using the pseudotyped SARS-CoV-2 variant omicron to evaluate COVID-19 convalescent patients serum infected with the original strain also showed that compared to the D614G reference strain, the neutralization efficacy of omicron variants decreased approximately 8.4-folds (Wang et al., 2022). Our study also showed that even in newly discharged patients who recently recovered from COVID-19, the neutralizing potency against the delta variant and the omicron variant decreased nearly 5-fold and 7-fold compared to the alpha variant. (Figure 1B), suggesting a strong immune escape capability of the delta and omicron variants.

SARS-CoV-2 initiates infection by the interaction between the RBD of the spike protein and the ACE2 receptor. Previous studies have also found a high correlation between RBD-specific IgG levels and neutralizing capacity (Iyer et al., 2020; Wang et al., 2021) and confirmed that neutralizing antibody levels are highly predictive of protection (Khoury et al., 2021). Recent meta-analysis have also indicated that despite the varying degrees of

reduction in the neutralizing efficacy of the current vaccines against VOCs compared to the original Wuhan strain, there is still a robust correlation between vaccine-induced neutralizing activity and the protection capability against symptomatic SARS-CoV-2 variants infection (Cromer et al., 2022). In our study, we also found a significant positive correlation between anti-RBD IgG titers and NT_{50} for both pseudovirus (Figures 1E, 2E) and the real virus (Figures 1F, 2F). In the future, as convalescent individuals face a new epidemic of emerging SARS-CoV-2 variants, it will be possible to measure their anti-RBD IgG titers to quickly assess whether they still have sufficient protection against emerging SARS-CoV-2 variants and to serve as a basis for vaccine booster administration.

T cells can activate other immune cells during infection and kill infected cells to control disease progression (Sattler et al., 2020; Le Bert et al., 2021; Moss, 2022). As a previous study revealed that 82 COVID-19 convalescents showed a positive IFN- γ response to the spike peptide pool (Cassaniti et al., 2021), in our study, we also found that all one-month COVID-19 convalescent PBMCs responded to alpha and delta variants spike peptide pool stimulation and produced IFN- γ and IL-2 (Figures 1G, H), indicating that these individuals generated cellular immune memory against SARS-CoV-2. However, it is not entirely clear what level of antibodies and/or T cells is necessary to confer such protection against SARS-CoV-2.

Although neutralizing antibodies usually eliminate the virus and provide protection in most viral infections, previous studies have found that both anti-RBD IgG and NT_{50} titers were significantly higher in severe patients than in mild patients (Robbiani et al., 2020; Garcia-Beltran et al., 2021; Lafon et al., 2021; Legros et al., 2021). In our study, we also found that the anti-RBD IgG titer (Figure 3A) and neutralization capacity (Figures 3B, C) were significantly higher in severe patients, indicating that higher neutralizing antibodies does not appear to protect against COVID-19 progression, and that robust humoral

immunities may be a consequence of the exaggerated immune activation in severe SARS-CoV-2 infection (Legros et al., 2021).

Inflammatory mediators are thought to cause severe inflammatory responses to tissue damage, and IL-6, IP-10 and MCP-1 have been found to be associated with acute lung injury and even poor prognosis and higher risk of death in SARS-CoV-1 (Jiang et al., 2005; Chien et al., 2006) and SARS-CoV-2 infections (Chen et al., 2020; Jøntvedt Jørgensen et al., 2020; Liu et al., 2020; Hashimoto et al., 2021). Our results showed that the levels of these three inflammatory factors (IL-6, IP-10, and MCP-1) were indeed significantly higher in the severe group (Figures 3D–F). These results not only indicated that measuring the levels of these inflammatory factors could predict the disease severity but also suggested that using inhibitors of these inflammatory factors may be helpful to reduce the risk of death, such as tocilizumab, which targets IL-6R and reduces mortality in severe COVID-19 patients (Somers et al., 2021; Wei et al., 2021). In addition, these cytokines and chemokines, together with other biochemical markers (CRP, D-dimer, and ferritin), may then be used to assess the risk of disease progression in COVID-19 patients during the early stages of the disease and to advance treatment with antiviral or anticytokine/antichemokine drugs to prevent the development of severe illness.

Previous studies have found that anti-spike serum produced by mice immunized with SARS-CoV-1 spike protein can help pseudotyped or real SARS-CoV-1 enter Raji cells *via* the FcγR-dependent pathway (Yip et al., 2014). Thus, ADE is an issue of concern for SARS-CoV-2 because of worries that ADE may lead to more severe forms of the disease in recovered COVID-19 patients or vaccinated individuals (Ricke, 2021). As no infectious virus production were reported after SARS-CoV-1 or SARS-CoV-2 entered Raji cells *via* ADE (Jaume et al., 2011; Zhou et al., 2021), we also found that SARS-CoV-1 convalescent serum slightly enhanced the entry of SARS-CoV-2 pseudovirus (Figure 4A), and no significant infection enhancement was observed by using SARS-CoV-2 convalescent sera (Figure 4B). Overall, these results suggested that SARS-CoV-2 infection may not induce viral replication enhancement by ADE.

The limitations of the study include the small sample size that may not represent neutralizing potency in diverse populations, in addition to the lack of serial samples and longitudinal assessments. We will continue to follow participants and evaluate their humoral and cellular immune responses against emerging variants after subsequent vaccination. Moreover, the phenomenon observed in the ADE assay using the FcγR-expressing cell line does not necessarily mean that the same situation will be observed *in vivo*. Recent studies have found that antibodies that enhance infection *in vitro* protect mice and macaques from SARS-CoV-2 infection *in vivo* (Li et al., 2021).

In conclusion, our findings could support the evaluation of vaccination strategies for recovered COVID-19 patients in the face of newly emerging variants, facilitate the assessment of the

risk of disease progression in confirmed COVID-19 patients at an early stage of the disease, and clarify the risk of SARS-CoV-2 ADE.

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 TSGHIRB No. C202005067. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

C-HC, F-YC, L-CY, C-LL, and K-CC designed the study; C-HC, Y-HC, K-CC, and P-LT performed experiments; all authors analyzed data; K-CC, T-WC and L-CY wrote the manuscript with help from all authors. All authors contributed to the article and approved the submitted version.

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Clinical Performance of Three Commercial SARS-CoV-2 Rapid Antigen Tests for Community-Dwelling Individuals in a Tropical Setting

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During the second year of the COVID-19 pandemic, the use of Rapid Diagnosis Antigen Tests (RDAgTs) for SARS-CoV-2 detection has substantially increased as some of the brands available in the market were certified for clinical use by international regulatory agencies. RDAgTs are a fast and cheap tool for SARS-CoV-2 surveillance with great potential to improve testing capacities in middle- and low-income countries compared to the gold standard RT-qPCR. However, as the clinical performance of RDAgTs has been shown to vary greatly between the commercial brands available, evaluation studies are necessary. Moreover, the available evaluation has been done in high-income countries while SARS-CoV-2 transmission is also actively happening in developing countries, many of which are located in tropical latitudes where cross-reactivity with other infectious agents is highly prevalent, which could compromise RDAgT specificity. Moreover, unreported mutations and/or new SARS-CoV-2 variants may compromise RDAgT sensitivity as genomic surveillance is limited in these settings. Here we describe a multicenter and manufacturer-independent evaluation of the clinical performance and analytical sensitivity of three different RDAgTs brands available in South America from three companies, Rapigen (South Korea), SD-Biosensor (South Korea), and Certest (Spain), compared to the gold standard RT-qPCR. A total number of 1,646 nasopharyngeal swabs from community-dwelling individuals were included in the study, and 379 of them were SARS-CoV-2 positive by RT-qPCR. The overall sensitivity for each RDAgT was 79% (IC95%: 72 - 86.2), 64.2% (IC95%: 56.7 - 71.6), and 45.8% (IC95%: 35.8 - 55.8) for SD-Biosensor, Certest, and Rapigen, respectively. The overall specificity for each RDAgT was 100%, 97.7% (IC95%: 96.8 - 98.6), and 100% for SD-Biosensor, Certest, and Rapigen, respectively. However, the limit of detection (LoD) to achieve a sensitivity over 90% was substantially lower for Certest RDAgT (10^2 copies/uL) compared to SD-Biosensor (10^3 copies/uL) or Rapigen (10^6 copies/uL) RDAgTs, considering that the gold standard RT-

qPCR method used in this study has a high sensitivity of 97.7% and low LoD of 5 copies/uL. Additionally, the Certest RDAgT also showed an improved sensitivity up to 79.7% (IC95%: 70.2 – 89.2) for symptomatic individuals. Finally, the slight reduction in specificity for Certest RDAgTs was only associated with one of the laboratories performing this study, pointing out the need for locally assessed evaluation for RDAgTs like this one carried out in Ecuador. In conclusion, two of the three the RDAgTs tested in this study are a fast, cheap, and point of care tool for SARS-CoV-2 surveillance and reliable enough to detect SARS-CoV-2 infectious individuals.

Keywords: antigen test, RapiGEN® Ag test kit, SD-Biosensor, Certest, SARS-CoV-2, clinical performance

INTRODUCTION

After the initial COVID-19 outbreak in Wuhan, China, in December 2019, SARS-CoV-2 spread rapidly and the World Health Organization declared COVID-19 a pandemic on 11 March 2020, and this pandemic is still ongoing (Wang et al., 2020; Gorbalenya et al., 2020; Zhou et al., 2020). SARS-CoV-2 RNA detection by RT-qPCR was the gold standard for acute infection diagnosis during the first year of the COVID-19 pandemic (Corman et al., 2020). By the end of 2020 and during 2021, the use of several commercial brands of point of care or Rapid Diagnosis Antigen Tests for SARS-CoV-2 detection became endorsed by international regulatory agencies or public health authorities (Cerutti et al., 2020; Pray et al., 2021). However, RT-qPCR is still widely used to confirm SARS-CoV-2 infection though this technique has several limitations for a scenario like the current COVID-19 pandemic: it is not easy to improve as a point of care diagnosis method, it requires sophisticated laboratory infrastructure, it depends on skilled personnel with a molecular biology background, and it is also permanently dependent on reagents that have experienced supply shortages (Freire-Paspuel et al., 2020; Cubas-Atienzar et al., 2021). Moreover, both RT-qPCR effectiveness for triage and contact tracing surveillance strategies are challenged by the need for 24 to 72 hours from sample collection to diagnosis (Kretzschmar et al., 2020). Additionally, RT-qPCR is an expensive diagnostic tool in the context of middle- and low-income countries that compromise their testing capacities (Cuellar et al., 2021; Santander-Gordon et al., 2021).

By contrast, the lateral flow immunoassays for SARS-CoV-2 antigen detection, also known as Rapid Diagnosis Antigen Tests (RDAgTs), allow for the point of care identification of SARS-CoV-2 virus in nasopharyngeal, oropharyngeal, or nasal samples in a time frame of 10 to 30 minutes depending on the commercial brand (Cerutti et al., 2020; Cubas-Atienzar et al., 2021; Iglói et al., 2021; Lee et al., 2021; Pray et al., 2021). Moreover, RDAgTs can either be performed by nursing staff without any laboratory infrastructure requirements or have been validated for patient self diagnosis (Nagura-Ikeda et al., 2020; Marx et al., 2021). Additionally, the cost of RDAgTs diagnosis is substantially cheaper than RT-qPCR diagnosis, as there are currently several RDAgTs commercial brands for self diagnosis sold for less than 5 USD even at grocery stores in the USA and

some European countries. As RDAgTs are cheaper, faster, and available for point of care diagnosis, they are a powerful tool for SARS-CoV-2 surveillance, not only for triage in hospital settings for symptomatic individuals but also for the massive screening of community-dwelling individuals in middle- and low-income countries (Iglói et al., 2021; Marx et al., 2021; Pollock et al., 2021; Pray et al., 2021; Tinker et al., 2021).

Studies have addressed the clinical performance of different RDAgT brands compared to the gold standard RT-qPCR (Cubas-Atienzar et al., 2021; Lee et al., 2021). Those studies confirm that RDAgTs have reduced sensitivity and a higher limit of detection compared to RT-qPCR (Cubas-Atienzar et al., 2021; Lee et al., 2021). However, the accuracy of some RDAgTs brands has been suggested to allow the identification of the vast majority of infectious individuals, as the sensitivity is over 90% for viral loads with $> 10^6$ genomic virus copies/ml (Corman et al., 2021; Cubas-Atienzar et al., 2021; Lee et al., 2021). Additionally, under a scenario like the COVID-19 pandemic, a reduction in sensitivity is acceptable as long as it comes with an increase in testing capacities, so the final output is a higher number of SARS-CoV-2 positive individuals detected (Mina et al., 2020). RDAgTs would fulfill these requirements as they are fast, cheap, and accurate enough to allow massive and rapid detection and isolation of new cases to stop transmission chains and reduce the impact of COVID-19 (World Health Organization (WHO), 2020; Andreani et al., 2021; Corman et al., 2021; Cubas-Atienzar et al., 2021; Lee et al., 2021; Pekosz et al., 2021; Weiss and Bellmann-Weiler, 2021).

As we have described above, the SARS-CoV-2 testing capacity in developing countries has been a challenge during the COVID-19 pandemic as it has been relying on the RT-qPCR technique. Moreover, as vaccination programs have been progressing slowly in middle- and low-income countries, SARS-CoV-2 circulation is still happening very actively in those settings, threatening COVID-19 pandemic control and eradication through new SARS-CoV-2 variant appearances (Dhawan et al., 2022). RDAgTs have the necessary features to improve effective SARS-CoV-2 surveillance programs in developing countries (World Health Organization (WHO), 2020; Andreani et al., 2021; Corman et al., 2021; Lee et al., 2021; Pekosz et al., 2021; Weiss and Bellmann-Weiler, 2021). However, the clinical performance evaluation studies for RDAgTs have been done in high-income countries (Albert et al., 2021; Andreani et al., 2021; Baro et al., 2021; Cerutti et al., 2020; Corman et al., 2021; Cubas-Atienzar et al., 2021; Iglói et al., 2021; Lee et al.,

2021; Pérez-García et al., 2021; Pollock et al., 2021; Pray et al., 2021; Tinker et al., 2021; Weitzel et al., 2021). It has been already reported that low-quality COVID-19 diagnosis products are commercialized in developing countries and genomic surveillance in those settings is limited, so the tracking of new mutations or variants of SARS-CoV-2 potentially compromises the sensitivity of RDAgTs for COVID-19 diagnosis (Cota et al., 2020; Freire-Paspuel and Garcia-Bereguain, 2020; Freire-Paspuel and Garcia-Bereguain, 2021; Freire-Paspuel and Garcia-Bereguain, 2021; Freire-Paspuel et al., 2021). Moreover, as the cross reactivity with other infectious pathogens for SARS-CoV-2 serology testing has been described, this phenomenon may also happen for RDAgTs, compromising their specificity in these middle- and low-income tropical countries (Echeverría et al., 2021; Faccini-Martínez et al., 2020; Tso et al., 2021). Considering this scenario, clinical performance evaluation of RDAgTs in the context of middle- and low-income countries are mandatory.

The aim of this work was to address the clinical performance and analytical sensitivity of three RDAgT commercial brands available to community-dwelling individuals in Ecuador.

MATERIALS AND METHODS

Study Design

A total number of 1,646 community-dwelling individuals (COVID-19 asymptomatic or mildly symptomatic) were included in the study performed from 12 January to 8 May 2021 at two independent laboratories: 1,076 samples were taken at a laboratory for SARS-CoV-2 detection at “Universidad de Las Américas” in Quito, Pichincha province, Ecuador (UDLA lab); and 570 samples were taken at “OneLabt” laboratory in Ballenita, Santa Elena province, Ecuador. Overall, the study population included 1,267 individuals who tested negative and 379 who tested positive for SARS-CoV-2 detection by RT-qPCR (29.9% positivity rate).

A single nasopharyngeal swab was collected for each individual and tested for SARS-CoV-2 detection by RT-qPCR following the standard protocol in both laboratories. As the sample collection buffer volume was sufficient to perform RT-qPCR and RDAgTs, the spare sample volume was immediately processed for SARS-CoV-2 detection by RDAgT.

According to Ecuadorian regulations, all the results for SARS-CoV-2 detection made by RT-qPCR must be reported to the Ministry of Health, where a short survey is completed and information regarding COVID-19 related symptoms for individuals is stored. Based on this survey, we could classify our study groups as symptomatic or asymptomatic individuals.

SARS-CoV-2 Detection Using Rapid Diagnosis Antigen Tests

Three different commercial brands of RDAgTs were evaluated in this study: Biocredit Covid-19 Ag Detection Kit (RapiGen, South Korea), SARS-CoV-2 Ag Test (Certest Biotec, Spain), and SARS-CoV-2 Rapid Antigen Test (SD-Biosensor, South Korea).

Hereafter, we refer to the different test kits using the names “Rapigen”, “Certest”, and “SD-Biosensor”.

The three RDAgTs included in the study are based on lateral flow immunochromatography. We used the collection buffer provided for each RDAgT for sample collection and follow each manufacturer's instructions to perform the SARS-CoV-2 detection. The reading time for the RDAgT varied from 10 to 30 min depending on the commercial brand.

As only one sample was collected from each patient, there were only paired samples for each RDAgT brand and RT-qPCR: 200 samples for Rapigen; 223 samples for SD-Biosensor; 1,223 for Certest. The variability or bias of the sample size for each commercial brand was due to the total number of RDAgTs that were kindly donated by each Ecuadorian distribution company for each of those brands. For Rapigen and “SD-Biosensor”, all the samples were processed at the UDLA lab. However, for the Certest evaluation, 653 and 570 samples were processed at UDLA lab and Onelab, respectively.

SARS-CoV-2 Detection Using RT-qPCR

Both laboratories involved in the study performed SARS-CoV-2 detection by RT-qPCR with the same protocol based on an adapted version from the Centers for Disease Control and Prevention (USA) protocol by using a CFX96 BioRad instrument and a triplex PCR assays (Freire-Paspuel et al., 2020; Freire-Paspuel and Garcia-Bereguain, 2021; Freire-Paspuel et al., 2021; Freire-Paspuel et al., 2021). Briefly, the commercial kit ECUGEN SARS-CoV-2 RT-qPCR kit (UDLA-Startnewcorp, Ecuador) includes a triplex assay for N1 and N2 viral targets to detect SARS-CoV-2 and RNase P as an RNA extraction quality control (Freire-Paspuel and Garcia-Bereguain, 2021). Also, negative controls (sample collection buffer) were included as a control for carry-over contamination, one for each set of RNA extractions. For viral loads calculation, the 2019-nCoV N positive control (IDT, USA) was used and provided at 200,000 genome equivalents/mL (Freire-Paspuel et al., 2020; Freire-Paspuel et al., 2021).

This positive control is a plasmid including N1 and N2 viral gene targets sequences, and it is a SARS-CoV-2 positive control recommended by CDC guidelines (Freire-Paspuel et al., 2020; Freire-Paspuel et al., 2021). Serial dilutions of the positive control were included in each set of samples RT-qPCR running, so an internal calibration curve with known concentrations of genomic SARS-CoV-2 material was always available. A regression analysis was made for each of those calibration curves taking RT-qPCR Ct values for N1 and N2 targets and viral genomic material concentrations as variables. The equation obtained was used for viral load calculations for each set of clinical samples, finally expressed as an average of the values for N1 and N2 targets. Regression coefficients over 0.99 were obtained for the viral load calibration curves. The RT-qPCR method used in this study has a high sensitivity of 97.7% and a low LoD of 5 copies/uL (Freire-Paspuel et al., 2020; Freire-Paspuel and Garcia-Bereguain, 2021).

Statistical Analysis

We carried out a descriptive study of the characteristics of the population by sex, age, and presence or absence of symptoms. The

sensitivity, specificity, positive predictive value, and negative predictive value of the three different commercial brands of lateral flow immunochromatography based SARS-CoV-2 Rapid Diagnosis Antigen tests (RDAgT) were calculated in the general population, separating them into symptomatic and asymptomatic individuals at two different laboratories with a confidence level of 95%. Furthermore, Sensitivity and Negative Predictive Values (NPV) for different viral load detection thresholds of Limit of Detection (LoD) by RT-qPCR are presented.

All statistical analysis was carried out using SPSS Statistics 23 software.

RESULTS

A descriptive analysis was performed by age, sex, and presence or absence of symptoms in the total study population (Table 1). Most of the population was female (618/1076, 57.4%) and the highest number of participants ranged in age from 20 – 40 years (593/1076, 55.1%). It should be noted that sex and age information from one of the laboratories is not included as it was not collected. Conversely, the distribution of individuals according to the presence or absence of symptoms is provided for the whole population study, with a greater number of asymptomatic patients (1119/1646, 68%), as detailed in Table 1.

Overall Clinical Performance for the Three SARS-CoV-2 Rapid Diagnosis Antigen Tests Included in the Study

The analysis of the clinical performance for Rapigen, SD-Biosensor, and Certest RDAgTs is detailed in Table 2. The number of samples tested was 200, 223, and 1,223 for Rapigen,

SD-Biosensor, and Certest, respectively. The ratio values for the number of positive SARS-CoV-2 samples by each RDAgT compared to RT-qPCR were 44/96, 98/124, and 102/159 for Rapigen, SD-Biosensor, and Certest, respectively. So, the overall sensitivity values for the RDAgTs evaluated in the present work were 45.8% (IC95%: 35.8 - 55.8), 79% (IC95%: 72 - 86.2), and 64.2% (IC95%: 56.7 - 71.6) for Rapigen, SD-Biosensor, and Certest, respectively (Table 2).

No SARS-CoV-2 false-positive samples were found for RDAgTs from the Rapigen and SD-Biosensor brands, so the specificity in both cases was 100%. For the Certest RDAgT, a total of 1,040 SARS-CoV-2 negative samples out of 1,064 samples were correctly identified, yielding a specificity of 97.7% (Table 2).

Evaluation of the Analytical Sensitivity for the Three SARS-CoV-2 Rapid Diagnosis Antigen Tests Included in the Study

In Table 3, the analysis of the clinical performance at different limit of detection (LoD) or viral load thresholds for the three RDAgTs evaluated in this study is detailed. The values of LoD for which the sensitivity is over 90% were as follows: 100 copies/uL for Certest (90.8%, IC95%: 85.4 - 96.2), 1,000 copies/uL for SD-Biosensor (94.7%, IC95%: 90.2 - 99.2), and 1,000,000 copies/uL for Rapigen (100%). For an LoD of 1,000,000 copies/uL, the sensitivity values for Certest and SD-Biosensor were 100% and 97.4% (IC95%: 92.4 - 100), respectively.

In Figure 1, the viral load distribution for the SARS-CoV-2 positive samples by RT-qPCR included in each RDAgTs evaluation is detailed. There are statistically significant differences ($p < 0.05$) for the mean viral load between RDAgT positive and RDAgT negative samples for Certest and SD-Biosensor, but not for Rapigen.

TABLE 1 | Characteristics of the population tested with the three different commercial brands of lateral flow immunochromatography based SARS-CoV-2 Rapid Diagnosis Antigen tests (RDAgT) included in this study.

| Brand | Total samples | Age (years) | | | Sex | | Symptoms | |
|--------------|---------------|-------------|-------------|-------------|-------------|-------------|--------------|---------------|
| | | ≤ 20 | 20 and 40 | ≥40 | Female | Male | Symptomatic | Asymptomatic |
| Rapigen | 200 | 5 (6,8%) | 122 (20,6%) | 73 (17,8%) | 116 (18,8%) | 84 (18,3%) | 138 (26,2%) | 62 (5,5%) |
| Certest | 653 | 46 (63%) | 358 (60,4%) | 249 (60,7%) | 372 (60,2%) | 281 (61,4%) | 166 (31,5%)* | 1057 (94,5%)* |
| SD-Biosensor | 223 | 22 (30,1%) | 113 (19,1%) | 88 (21,5%) | 130 (21%) | 93 (20,3%) | 223 (42,3%) | 0 |
| Total | 1076 | 73 (6,8%) | 593 (55,1%) | 410 (38,1%) | 618 (57,4%) | 458 (42,6%) | 527 (32%) | 1119 (68%) |

*For CerTest, the information on gender and age does not include data from OneLabt laboratory in Ballenita, Santa Elena province, Ecuador.

TABLE 2 | Clinical performance of the three different commercial brands of lateral flow immunochromatography based SARS-CoV-2 Rapid Diagnosis Antigen tests (RDAgT) included in this study (total samples: number of samples included in the evaluation; positive samples: number of SARS-CoV-2 positive samples included in the evaluation for RDAgTs or RT-qPCR; negative samples: number of SARS-CoV-2 negative samples included in the evaluation for RDAgTs or RT-qPCR; PPV, positive predictive value; NPV, negative predictive value; parenthesis includes IC95%.

| RDAgT brand | Total samples | Positive samples (RDAgT/RT-qPCR) | Negative samples (RDAgT/RT-qPCR) | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|--------------|---------------|----------------------------------|----------------------------------|--------------------|--------------------|-------------------|--------------------|
| Rapigen | 200 | 44/96 | 104/104 | 45.8 (35.8 - 55.8) | 100 | 100 | 66.7 (59.2 - 74) |
| CerTest | 1,223 | 102/159 | 1,040/1,064 | 64.2 (56.7 - 71.6) | 97.7 (96.8 - 98.6) | 81 (74.1 - 87.85) | 94.8 (93.5 - 96.1) |
| SD-Biosensor | 223 | 98/124 | 99/99 | 79 (72 - 86.2) | 100 | 100 | 79.2 (72 - 86.3) |

TABLE 3 | Evaluation of the analytical sensitivity of the three different commercial brands of lateral flow immunochromatography-based SARS-CoV-2 Rapid Diagnosis Antigen tests (RDAgT) included in this study.

| LoD (copies/mL) | Rapigen | | | CerTest Biotec | | | SD-Biosensor | | |
|-----------------|---------|--------------------|--------------------|----------------|--------------------|--------------------|--------------|--------------------|--------------------|
| | N | Sensitivity (%) | % NPV | N | Sensitivity (%) | % NPV | N | Sensitivity (%) | %NPV |
| 10 ² | 44/76 | 57.9 (46.8 - 69) | 76.4 (69.3 - 83.6) | 99/109 | 90.8 (85.4 - 96.2) | 99 (98.5 - 99.6) | 96/108 | 88.8 (82.8 - 94.7) | 84.6 (77.9 - 91.3) |
| 10 ³ | 42/66 | 63.6 (51.2 - 75.2) | 81.2 (74.5 - 88) | 86/91 | 94.5 (89.8 - 99.1) | 99.5 (99.1 - 99.9) | 90/95 | 94.7 (90.2 - 99.2) | 95 (90.8 - 99.2) |
| 10 ⁴ | 38/50 | 76 (64.1 - 87.8) | 89.6 (84.1 - 95.2) | 57/59 | 96.6 (92 - 100) | 99.8 (99.5 - 100) | 83/86 | 96.5 (92.6 - 100) | 97 (93.7 - 100) |
| 10 ⁵ | 29/33 | 87.9 (76.8 - 99) | 96.3 (92.7 - 99.8) | 33/34 | 97.1 (91.3 - 100) | 99.9 (99.7 - 100) | 64/65 | 98.5 (95.5 - 100) | 99 (97 - 100) |
| 10 ⁶ | 11/11 | 100 | 100 | 10/10 | 100 | 100 | 38/39 | 97.4 (92.4 - 100) | 99 (97 - 100) |

Sensitivity and Negative Predictive Values (NPV) for different viral load detection thresholds of Limit of Detection (LoD) by RT-qPCR are presented next to the 95% confidence interval.

Clinical Performance for the Three SARS-CoV-2 Rapid Diagnosis Antigen Tests Included in the Study for Symptomatic and Asymptomatic Individuals

In **Table 4**, the clinical performance of the three RDAgTs in symptomatic and asymptomatic individuals is shown. For Rapigen, the sensitivity values for symptomatic and asymptomatic individuals were 48.7% and 28.6%, respectively. For Certest, the sensitivity values for symptomatic and asymptomatic individuals were 79.7% and 52.2%, respectively. For SD-Biosensor, the sensitivity values were only addressed for symptomatic individuals, as asymptomatic individuals were excluded in this evaluation, so the value and overall sensitivity (79%) are the same as reported above. In **Table 4** we included a sensitivity value of 43.6% that has been reported in another study (Weitzel et al., 2021) for SD-Biosensor with asymptomatic individuals for comparison. There was a significant reduction in sensitivity ($p < 0.05$) for

asymptomatic individuals compared to symptomatic individuals for the Certest and Rapigen RDAgTs.

Comparison of the Clinical Performance for the Rapid Diagnosis Antigen Test From Certest at Two Different Laboratories Located in Quito (Pichincha Province, Andean Region of Ecuador) and Ballenita (Santa Elena Province, Coastal Region of Ecuador)

For the clinical performance evaluation of Certest RDAgTs, there were two independent laboratories involved in the evaluation. In **Table 5**, the results of the clinical performance of the RDAgTs are presented for each of those two labs. In the UDLA lab, 653 samples were processed and the values for sensitivity and specificity were 72.2% and 95.9%, respectively. In the Onelab laboratory, 653 samples were processed and the values for sensitivity and specificity were 57.5% and 100%, respectively.

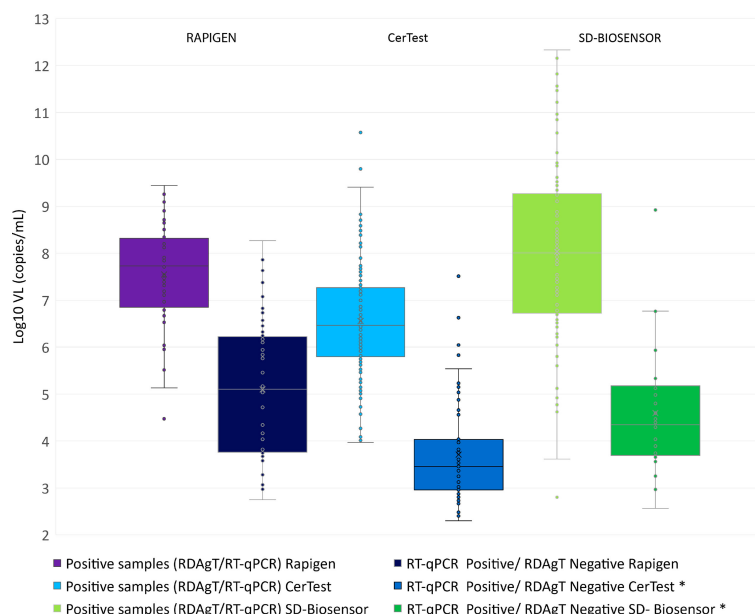


FIGURE 1 | Viral loads distribution for all the SARS-CoV-2 positive samples by RT-qPCR was included in the study. Viral loads (VL) are presented on a Log10 scale. The different sets of samples used for each Rapid Diagnosis Antigen Test (RDAgT) brand are divided into two categories: RDAgT positive and RDAgT negative. *There are statistically significant differences ($p < 0.05$) for VL between RDAgT positive and RDAgT negative only for Certest Biotec and SD-Biosensor brands.

TABLE 4 | Clinical performance of the three different commercial brands of lateral flow immunochromatography based SARS-CoV-2 Rapid Diagnosis Antigen tests (RDAGT) included in this study for symptomatic and asymptomatic individuals (total samples: number of samples included in the evaluation; positive samples: number of SARS-CoV-2 positive samples included in the evaluation for RDAGTs or RT-qPCR; negative samples: number of SARS-CoV-2 negative samples included in the evaluation for RDAGTs or RT-qPCR; PPV: positive predictive value; NPV: negative predictive value; next to the 95% confidence interval (IC95%).

| Type of individual | RDAGT Brand | Total samples | Positive samples (RDAGT/RT-qPCR) | Negative samples (RDAGT/RT-qPCR) | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|--------------------|---------------|---------------|----------------------------------|----------------------------------|--------------------|--------------------|------------------|--------------------|
| Symptomatic | Rapigen | 138 | 40/82 | 56/56 | 48.7 (37.8 – 59.5) | 100 | 100 | 57.1 (47.3 – 66.9) |
| | Certest | 166 | 55/69 | 94/97 | 79.7 (70.2 – 89.2) | 97 (93.6 – 100) | 94.8 (89.1–100) | 87 (80.6– 93.3) |
| | SD-Biosensor | 223 | 98/124 | 99/99 | 79 (71.8 – 86.2) | 100 | 100 | 79.2 (72.1– 86.3) |
| | | | | | | | | |
| Asymptomatic | Rapigen | 62 | 4/14 | 48/48 | 28.6 (5 – 52.3) | 100 | 100 | 82.8 (73.1–92.5) |
| | CerTest | 1057 | 47/90 | 946/967 | 52.2 (41.9 – 62.5) | 97.8 (96.8 – 98.7) | 69.1 (58.1–80.1) | 95.6 (94.3–96.9) |
| | SD-Biosensor* | 286 | 44/101 | 178/185 | 43.6 (33.5 – 52.9) | 96.2 (93.4 – 98.9) | 86 (76.5 – 95.5) | 75.7 (70.2– 81.2) |
| | | | | | | | | |

*For SD-Biosensor, as non-asymptomatic individuals were included in our study, we took values from the published report described in reference 26).

DISCUSSION

In this study, we describe the clinical performance of three commercial RDAGTs brands currently available in several South American countries, including Ecuador. We found differences in terms of the overall sensitivity for the three RDAGTs evaluated. While Rapigen has a substantially reduced sensitivity below 50%, Certest and SD-Biosensor have an equivalent performance of almost 80% sensitivity for symptomatic individuals. Moreover, for a viral load threshold of 100 copies/uL, only the Certest RDAGT had an overall sensitivity over 90%. Both Certest and SD-Biosensor had sensitivity values close to 95% when samples with viral loads lower than 1000 copies/uL were excluded from the analysis. However, the overall sensitivity of Rapigen only reached a value over 90% for samples with viral loads over 10^6 copies/uL. As an approximate LoD of 10^6 copies/ml has been proposed as the minimal analytical sensitivity by the WHO or the Department of Health and Social Care from the United Kingdom (Department of Health and Social Care, 2020; WHO & R&D Blue Print, 2020), only SD-Biosensor and Certest RDAGTs evaluated in this study accomplished that requirement. Moreover, as the viral load is a dynamic parameter that may grow exponentially during the incubation period, our results would support the use of either Certest or SD-Biosensor over Rapigen RDAGTs (Avanzato et al., 2020; Kawasuji et al., 2020; Kleiboeker et al., 2020; Lavezzo et al., 2020; Pekosz et al., 2021; Singanayagam et al., 2020; Walsh et al., 2020; Weiss and Bellmann-Weiler, 2021).

Additionally, we call attention to the variability of sensitivity and specificity among the two labs involved in this evaluation study. As the same protocol for sample collection and RT-qPCR

was used in both laboratories, the differences observed in sensitivity were associated at a random event such as a higher number of individuals with low viral loads in one of the locations. This difference in sensitivity occurred considering that more than 500 samples were evaluated in each lab setting, pointing out the need for extensive and multi-center studies for an accurate clinical performance evaluation of commercial RDAGTs. As reflected in **Table 6**, our results are within the range of sensitivity and specificity reported for RDAGTs, but there are substantial differences in the clinical performance between the different studies, even for the same RDAGT commercial brand (Albert et al., 2021; Baro et al., 2021; Cerutti et al., 2020; Corman et al., 2021; Cubas-Atienzar et al., 2021; Iglói et al., 2021; Lee et al., 2021; Nagura-Ikeda et al., 2020; Pérez-García et al., 2021; Pray et al., 2021; Pollock et al., 2021; Tinker et al., 2021). Moreover, the vast majority of clinical performance evaluations for RDAGTs have been carried out in high-income countries. However, SARS-CoV-2 current transmission is also happening in middle- and low-income countries where COVID-19 vaccination programs are progressing slowly. Moreover, SARS-COV-2 genomic surveillance in developing countries is limited, so new mutations or SARS-CoV-2 variants may not be well characterized. Under this scenario, locally assessed studies of the available RDAGT commercial brands are needed, as there is a concern regarding the potential reduction of sensitivity for SARS-CoV-2 variants (Frediani et al., 2021).

In terms of specificity, the three RDAGTs showed a good performance with values of 100% for Rapigen, SD-Biosensor, and also for Certest at one of the laboratories. Interestingly, there was almost a 5% reduction in specificity for the Certest RDAGT only for the UDLA lab evaluation. It is important to note

TABLE 5 | Independent evaluation of the clinical performance of the SARS-CoV-2 Rapid Diagnosis Antigen Test (RDAGT) from Certest Biotec at two different laboratories in Ecuador (total samples: number of samples included in the evaluation; positive samples: number of SARS-CoV-2 positive samples included in the evaluation for RDAGTs or RT-qPCR; negative samples: number of SARS-CoV-2 negative samples included in the evaluation for RDAGTs or RT-qPCR; PPV, positive predictive value; NPV, negative predictive value; parenthesis includes IC95%).

| Clinical Lab | Total samples | Positive samples (RDAGT/RT-qPCR) | Negative samples (RDAGT/RT-qPCR) | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|--------------|---------------|----------------------------------|----------------------------------|--------------------|--------------------|--------------------|--------------------|
| UDLA | 653 | 52/72 | 581/605 | 72.2 (61.9 – 82.5) | 95.9 (94.3 – 97.5) | 68.4 (57.9 – 78.8) | 96.5 (95 – 97.57) |
| OneLabt | 570 | 50/87 | 483/483 | 57.5 (47.1–67.9) | 100 | 100 | 92.9 (90.7 – 95.1) |

TABLE 6 | Comparative analysis of the clinical performance for several SARS-CoV-2 Rapid Diagnosis Antigen Test (RDAgTs) with evaluation studies published in peer review journals (* for Abbott, results from two different commercial RDAgTs are included).

| RDAgT Brand | Sensitivity (%) | Specificity (%) | Reference |
|-----------------------------------|-----------------|-----------------|------------------------|
| CerTest (Spain) | 53.5-79.7 | 97.7-100 | 14,21,27, our study. |
| Rapigen (South Korea) | 28.6-62 | 100 | 14,20,25, our study. |
| SD-Biosensor (South Korea) | 43.6-79 | 96.2-100 | 14,21,26,27, our study |
| Abbott (USA)* | 20-79.6 | 100 | 14,17,18,21,27 |

that the two labs involved in the study were located in a tropical latitude (Ecuador) but were two environmentally different settings: Quito is in the Andean Region of Ecuador at 2800 meters above sea level and Ballenita is at sea level in the Santa Elena province in the coastal region of Ecuador. As the weather conditions are different among these two locations, cross reactivity with a respiratory virus circulating at the time of this study is a plausible explanation for the differences observed between Quito and Ballenita. A similar phenomenon has been described for anti-SARS-CoV-2 serological tests, particularly in developing countries and tropical regions, due to the higher prevalence of some pathogens compared to high-income countries, where most of the COVID-19 diagnosis tools evaluations are conducted (Cota et al., 2020; Tso et al., 2021; Echeverría et al., 2021). Our results endorse the need for locally assessed evaluation studies in middle- and low-income settings to guarantee a reliable specificity for SARS-CoV-2 detection with RDAgTs.

This clinical performance evaluation has some limitations. For instance, no viral cultures were used to assess the LoDs as no BSL3 facility was available. However, the viral load calculations made by using a titrating of the CDC-designed SARS-CoV-2 positive control, described in the methods, were in agreement with other reports analyzing the same commercial brand RDAgTs (Corman et al., 2021; Lee et al., 2021; Mina et al., 2020; Pérez-García et al., 2021; Weitzel et al., 2021). Another limitation is that SD-Biosensor only included symptomatic patients, although this commercial brand is among the most used worldwide and several evaluation reports have already been published (Cubas-Atienzar et al., 2021; Lee et al., 2021). Moreover, the two laboratories were only involved in Certest RDAgT evaluation as the Ecuadorian representatives for the other two brands could not provide as many tests as requested.

In conclusion, the clinical performance and analytical sensitivity of Certest and SD-Biosensor RDAgT brands tested were within the WHO requirements. These results support the

use of RDAgTs as a fast, cheap, and reliable point of care tool for SARS-CoV-2 detection for most COVID-19 contagious individuals. The massive use of RDAgTs would have a tremendous impact on COVID-19 pandemic control in developing countries where SARS-CoV-2 remains at a high level of transmission.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB certified by Ministry of Health from Ecuador (code 008-2020). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DM-J and MG-B wrote the manuscript. All the authors contributed to data collection and analysis, and also to manuscript revision and approval prior to submission.

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Taking the Path Into the Dark Woods – Initial Industry Impact of the COVID-19 Pandemic

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During the initial onset of the COVID-19 pandemic, each industry experienced hardships. One area that has not been explored in great detail was how the diagnostic industry managed while bringing SARS-CoV-2 tests to the market. This perspective piece provides a sample view of what went on behind the walls of a diagnostic manufacturer that released one of the initial SARS-CoV-2 testing options, some of the barriers that were encountered, and how they could be overcome.

Keywords: molecular diagnostics, COVID-19, SARS-CoV-2 testing, industry, virology

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Writing on a topic that needs no introduction can be difficult. It goes without saying that there are plenty of facts that can be strewn about here with regard to COVID-19, like the number of cases, hospitalizations, deaths, etc. Entering year 3 of the pandemic, each individual across the globe has likely had considerable speedbumps in some capacity or another due to the pandemic, provided said individual is in the camp that believes that we are in a global pandemic. The amount of time that has passed since this all began is a bit ludicrous to conceptualize still to this day, but one thing that may be more incredible is the amount of effort that has been deployed worldwide to combat this pandemic, in so many ways, in order to keep people safe.

As we enter the next wave of the Greek alphabet, Omicron and its variants stirring up the most recent excitement (which could easily change by the time this is published), it is prudent to remember that before Omicron, Delta, Alpha and all the others, there was a non-descript pneumonia of unknown origin that caused severe respiratory distress in a patient in China. It seems like almost a lifetime ago, but from the month of December 2019 on, day by day, a virus swept through the human population like wildfire. Spreading from city to city, town to town, country to country and eventually continent to continent. Severe Acute Respiratory Syndrome Coronavirus 2, or SARS-CoV-2, made haste with an impression of impending doom everywhere it surfaced.

There are multiple different vantage points of the COVID-19 pandemic. One that maybe was underrepresented during the earlier days was that of the diagnostic manufacturer. No one can deny what the frontline healthcare professionals were facing. Most of the healthcare industry saw it and even still, it is probably understated. Past the frontline workers, visibility started to dilute out layer by layer, so that by the time it got to the diagnostic manufacturers of SARS-CoV-2 tests, there was not much insight on how the industry was managing to the outside world. This perspective provides a unique view from a diagnostic manufacturer during the initial onset of the pandemic.

Firstly, the decision to make a SARS-CoV-2 test had to be brought to the table. In the beginning, around January 2020, no one really knew which direction the pandemic was going to go. From the

perspective of a smaller company (at the time), this leads to one very important decision to make: Do we drop all other projects and allocate resources and funds to develop a very specific niche test that may not have long-term demand or any return on investment? The final answer to this question for GenMark Diagnostics, said smaller company, focusing on multiplex molecular diagnostics for infectious diseases, was yes. The decision was made in January 2020 when the cases were just beginning to make their way outside of China. At that point, it was clear this would be an all hands on deck approach. With only a small number of test manufacturers going down the same very dark road that had very limited visibility, the Research and Development (R&D) team quickly pivoted to researching the SARS-CoV-2 genome to find conservative regions to target for PCR assays. At this point, it is pivotal to remember there were a limited number of sequences available in the GSAID database to work with, alongside minimal knowledge of how well this virus could and would mutate. In fact, there were only about 40 sequences available to be analyzed for assay development purposes. Luckily the CDC-developed test targeting the N gene had given a little runway on what might be a good location to potentially look at.

With this information on hand, R&D located two conserved regions that could be targeted in the N gene, the development could move from *in silico* analyses to wet benching, followed by manufacturing and into full scale launch. Luckily for GenMark (and likely other manufacturers), a universal cartridge design allows for flexibility in assay development, making it straightforward to place primers and probes on an already FDA-cleared diagnostic test cartridge and platform. Despite this, R&D faced many unforeseen hurdles during the month long development phase. During the initial phases of the pandemic, oligonucleotides were in short supply due to the demand from other companies also embarking on the same journey, but this was far from the only limiting factor. In addition to limited available sequences, commercialized controls were not obtainable to both vendors and laboratories because no manufacturer had started making them yet. Clinical samples were in scarce supply as well and only a few designated labs in the country had them, leading to a very high demand for a very small allocation of samples. This did not distract the team from the goal though. Controls were made through plasmid constructs to confirm primer designs, limit of detection (LoD) studies were completed with *in vitro* transcribed target material and sensitivity and specificity was outsourced to a clinical laboratory that had access to some patient samples. For summation on how quickly the process happened, the clock started on day 1 with checking primers on the bench and kits were shipped out by day 19 for clinical validation. R&D worked tirelessly around the clock and the necessary studies were completed in order to launch as a Research Use Only (RUO) test on March 2, 2020.

R&D achieved a herculean effort in the development of this test and now the next important facets needed to fall into place to mobilize these efforts. In a diagnostic company of any size, there are a lot of individuals that are required to launch a product and generally these people will have ample time for meetings and coordination before a product is brought to market. One department that was heavily leaned on during this time was

Regulatory Affairs (RA), comprised of team members that were consistently navigating an ever-changing and sparsely guided regulatory outlook for Emergency Use Authorization (EUA) for a SARS-CoV-2 test. At the time, there was limited guidance on what the FDA was going to accept for a test to be authorized, and the test could not be marketed or sold until authorization came through. The FDA wanted LoD, inclusivity, specificity/cross reactivity and clinical performance. Albeit with limited accessible resources, the RA team diligently researched the needs for submission and kept as in touch as possible with FDA while compiling data from internal and external studies to ensure that all possible aspects dotting i's and crossing t's were complete before submission to ensure a speedy review. Once the qualifications for submission were met and the test was submitted for EUA for full review, prior to when the FDA changed their guidance which allowed a test to be marketed and sold prior to authorization if all required components were submitted to FDA. After 8 days on high alert to return any questions to FDA if prompted, on March 19, 2020, the EUA for the GenMark Dx® ePlex® SARS-CoV-2 Test was granted and the test was delivered to the market. However at the time, 8 days felt more like 8 months.

Delivering a test with such rapidity creates its own series of bottlenecks in many other areas of the business. First and foremost, as many will likely recall, that timeframe was during the lockdown in the United States when very little information was available to anyone on how to stop and/or control the spread of COVID-19. This made working in any setting with other people challenging. Manufacturing lines across any industry could be problematic given the proximity of workers for long periods of times. Couple this with a crumbling supply chain, sick time, exposures, and many individuals having to pivot from office to a work-from-home environment, things went wayward quickly. Every manufacturer had to deal with these issues in some capacity, which in turn meant that instruments and test kits could not be manufactured and pushed out the door as fast as they were being sold. The constant question of for labs and manufacturers alike at the time was "How do we scale up?". It's hard to scale up in an environment where the world is shutting down and hard to produce more when scaling up rapidly is difficult, which ultimately meant that industry-wide, there were not enough test kits or instruments to meet demand. Noting these challenges, there needed to be a considerable amount of evolution that needed to take place. Internally, companywide policies for health screening (temperature checks, health questionnaires, etc.), masking, special cleaning processes and distancing (including implementation of working from home for non-essential employees) helped to keep cases down internally allowing the manufacturing teams to continue working onsite to provide keep the lines going for product. Over time, additional shifts and positions were opened to try and help bulk up the manufacturing team to meet demands. Externally, teams working from home quickly adapted to Zoom and Microsoft Teams meetings and a more electronically focused work environment, while Supply teams canvassed and qualified different manufacturers to see where additional materials could be sourced when necessary.

Customer service roles were also tasked with a different set of responsibilities than usual. Account Representatives along with Customer Service and Product Management had to manage the anticipated supply output and do their best to deliver as many kits or instruments to labs as possible. Being one of the few first tests released, the demand far exceeded the inventory. This created frustration both with laboratories that desperately needed kits and capacity to test them, but also internally when there was not enough material to go around due to the manufacturing and supply chain constraints. All functions had to align daily to make sure that every single kit that was made was shipped immediately under allocation efforts to make sure that all kits got to as many customers as possible. Not to be forgotten, the courageous Molecular Applications Specialists (MAS) were still traveling around the country to install instrumentation and train laboratories not knowing the level of safety around them at any time. The dedication to keep laboratories up and running during this time was undoubtedly a challenge for each person to face every week as they stepped out into a variety of unknowns. This team ensured that customers had their instruments up and running and their staff trained in order to start or keep testing samples for SARS-CoV-2 while also maintaining an outlet for the customer to the company and vice versa, being the only team allowed for in person contact at the time. Without the MAS team, many instruments may have gone uninstalled and a multitude of customers may not have been able to offer our testing solution.

While the in-house Technical Support groups did not have to travel, they had to deal with a different set of challenges like managing limit of detection questions, assay verification to new or unknown comparator methods (in which labs usually had more than 1-2 comparator methods at any given time), ever-changing sample types and discrepant results. By now, it is widely known that many manufacturers took different routes to make a product to detect SARS-CoV-2, but at the time it was very difficult to predict how different chemistries, primer/probe gene locations, number of genes targeted, LoD, sample to answer versus traditional molecular approaches and/or how different primary sample types were going to perform for each company's assay and all the while, each company is only allowed to support its own assays. Supporting a new product is always a new endeavor for the Technical Support teams but doing it on the fly with so many confounding variables and comparator tests, along with the clinical ramifications for both false negatives and false positives became a new challenge that was not predicted by anyone. The Technical Support groups still assisted customers in any way they could by relying on the instructions for use (IFU) not only for ePlex but other tests/instruments as well, FDA guidance, internally performed studies and engaging different functions within the organization, like R&D, Marketing, MAS's and Scientific and Medical Affairs, to help provide as much information as possible, while noting that they could only support issues that arose with the ePlex instrument and the ePlex SARS-CoV-2 Test.

The data, experiments, publications, pre-prints and any other outlet of information being released had to be digested on an almost immediate basis by Scientific and Medical Affairs to

ensure that any customer had an outlet to ask questions, although many times there was not a clear answer. Even though the data were desperately needed, staying on top of the swirling tornado of data coming in from so many places across the world remained a challenge because it was hard to know what was reliable and what wouldn't stick, particularly for the pre-prints still pending peer-review. With only two people, the work had to be split evenly to make sure there was efficiency while trying to avoid overlap. Constant data and information exchange was critical as was fully discussing the meaning and outcomes of the research that was surfacing all while also utilizing different working time zones internally and externally to get the most out of each day for GenMark and customers alike.

Bioinformatics had a similar challenge trying to deal with sequences that were constantly mutating and with an extremely limited database of information to help provide definitive clarity on the ability for a test to detect a particular newly emerging strain. The team set up specific cadences to mine and search for new information as it surfaced, passing it on to all internal stakeholders in R&D, Marketing, Scientific and Medical Affairs and Regulatory, while the aforementioned teams would also feed in any information of new strains back to the Bioinformatics team for review and perspective.

All this said, there are so many things that can be elaborated on here and other efforts that can be detailed, but the main point is to highlight the tremendous effort made by the diagnostic community rising to the occasion and releasing high quality testing materials which now stretch across many manufacturers. It was a very difficult and uncertain time early on and given the opportunity, it is relevant to give kudos to the diagnostic industry and all of the internal teams and individuals who made testing possible in the early days in the face of so many challenges and roadblocks. It is key to consider that every function in an organization played a critical role during this pandemic. For some functions like R&D and Scientific and Medical Affairs, all the answers may not always be point blank and may have required scientific intel and fortitude to guide the way while others like Regulatory, Manufacturing and Customer Support teams relied on industry experience and tactile response management to situations as they arose. While it is clear that COVID-19 is not going away anytime soon, it is good to know that there are ways to adequately test for it in so many different capacities now than ever before and hopefully the experiences learned in this round prepare us in case of future rapid onset pandemics.

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

The author confirms being the sole contributor of this work and has approved it for publication.

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The author declares 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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