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Evolution of SARS-CoV-2: Impact of variants on hosts, COVID-19 vaccines and countermeasures

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Evolution of SARS-CoV-2: Impact of variants on hosts, COVID-19 vaccines and countermeasures

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PERSPECTIVE
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Saliva for COVID-19 Testing: Simple but Useless or an Undervalued Resource?

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During the COVID-19 pandemic, countries with robust population-based asymptomatic testing were generally successful in controlling virus spread, hence reducing hospitalizations and deaths. This effectiveness inspired widespread asymptomatic surveillance for COVID-19/SARS-CoV-2 globally. Polarized vaccination programs, coupled with the relatively short-lived immunity vaccines provide, mean that reciprocal cross-border exchanges of each new variant are likely, as evidenced by Delta and Gamma, and asymptomatic testing will be required for the foreseeable future. Reliance on nasopharyngeal swabs contributes to "testing fatigue" arising due to difficulties in standardizing administration, unpleasantness, and inappropriateness of use in younger people or individuals with special needs. There has also been erosion in confidence of testing due to variable and/or poor accuracy of lateral flow devices to detect COVID-19. Here, we question why saliva-based PCR assays are not being used more widely, given that standardization is easy and this non-invasive test is suitable for everyone, providing high sensitivity and accuracy. We reflect on our experience with the University of Nottingham COVID-19 Asymptomatic Testing, where (as of October 2021) 96,317 samples have been processed by RT-qPCR from 23,740 repeat saliva donors, yielding 465 positive cases. We challenge myths that saliva is difficult to process, concluding that it is an undervalued resource for both asymptomatic and symptomatic detection of SARS-CoV-2 genomes to an accuracy of >99% and a sensitivity of 1–10 viral copies/μl. In July 2021, our data enabled Nottingham to become the first UK University to gain accreditation and the first UK institute to gain this accolade for saliva.

Keywords: SARS-CoV-2, lateral flow, polymerase chain reaction, COVID-19, nasopharyngeal swab, saliva, asymptomatic testing

INTRODUCTION

Since the first reports of SARS-CoV-2 infections in late 2019, there has been an emerging acceptance of the need to co-exist with the virus in our communities. Outbreak control will be critical, requiring large-scale testing for the foreseeable future, well beyond mass vaccination programs. General population surveillance provides valuable real-time data on infection rates, spread, and demographics (1). The importance of monitoring new variants, prevalence, and capacity for evasion of immunity is highlighted by Delta and Gamma variants (2), and now Delta+. By August 2021, 70% of the 3 billion vaccines produced were delivered in just 10 countries vs. 1% in the developing world. New variants will emerge from what the World Health Organization has dubbed a "two-tier pandemic", hence perpetuating cycles of reinfection[1].

For SARS-CoV-2 detection, public testing schemes typically rely on nasopharyngeal swabs for lateral flow or polymerase chain reaction tests (LFTs or PCR, respectively). With high specificity and sensitivity (*circa* 95–99%), PCR approaches are the mainstay of COVID-19 tests, employing extraction of RNA followed by reverse transcriptase quantitative PCR (RT-qPCR) or loop mediated isothermal amplification (LAMP). LFTs detect epitopes in the viral spike protein, giving sensitivities of 5%—70% relative to RT-qPCR detection of the SARS-CoV-2 genome (3, 4). Detection limit of LFTs is *circa* 100 viral copies/μl (5), 10- to 100-fold less sensitive than PCR approaches.

Discussion continues on the relative merits of each approach regarding cost, labor, route of deployment, and result turnaround. However, a common issue is the use of nasopharyngeal swabs (6). A pervasive error exists in the failure to reach the correct nasopharynx target site, even when performed by trained medical experts (7, 8). Inexperienced or self-administered operators have LFT sensitivities of sub-50%, with uncertainty on how far "up" or "back" the swab should go or at what "angle" and for "how long". At best, this causes discomfort because the swab is wedged against the middle turbinate (7), dissuading regular repeat testing. At worst, false-negative results lead to relaxed behaviors that amplify virus transmission. Indeed, numerous people with overt COVID-19 symptoms report that they have tested negative *via* multiple LFTs over consecutive days but positive by RT-qPCR approaches.

OVERLOOKED BENEFITS OF SALIVA FOR SARS-COV-2 DETECTION

The purpose of this Perspective is to prompt discussion and highlight saliva-based direct RT-qPCR detection of SARS-CoV-2 as an alternative method, thus far overlooked for mainstream testing. Saliva samples avoid the issues of invasive, qualitative nasopharyngeal swabs by easy provision, less variability and more reliability because volumes of 100 μl are acceptable. Anecdotally, samples provided in the morning before eating food, brushing teeth, or using oral hygiene products, such as mouth wash, provide high-quality samples. Our Asymptomatic Testing Service[2]. (University ethics approval committee approval FMHS 96-0920) is currently evaluating if simplified collection routes benefit sensitive communities, such as special education, dementia patients, homeless hostels, or victims of sexual abuse, where any perceived penetration is unwelcome.

Other benefits of direct saliva-based RT-qPCR detection are as follows: (i) Reduced risk of infection to the staff conducting the sampling, since self-harvesting is easy. (ii) Sample stability; at least 20 days at 4°C without viral transport medium (see **Figure 1**). (iii) No RNA-extraction step, decreasing testing time and cost. (iv) Possibility of sample pooling to increase throughput and reduce cost. (v) Not competing with other diagnostic schemes, hence increasing capacity without additional demand on manufacturers. (vi) Fewer components, reducing supply chain issues, the importance of which was highlighted during the worldwide shortage of nasopharyngeal swabs (9). (vii) High specificity/sensitivity comparable with nasopharyngeal swab-based detection (3, 6, 10–12).

Underscoring these benefits, from November 2020 to February 2021, the University of Nottingham provided free access to undergraduate students (typically 18–22-year-olds) of LFTs using nasopharyngeal swabs (provided by the UK Government) and RT-qPCR testing using saliva collection aids (provided by our Service). *Circa* 20,000 tests were completed, with evidence citing ease, reliability, and confidence in the result for why there was a preference toward saliva tests (13).

Why saliva is underutilized is unclear, though it may be due to historical reasons and paucity of data in the literature on accuracy. Indeed, nearly a year into the pandemic (late 2020), the Infectious Diseases Society of America guidelines stated that *"saliva as the sole sample source for COVID-19 diagnosis cannot be recommended due to a paucity of studies"* (14). However, other studies have shown high sensitivity of SARS-CoV-2 detection using saliva, wherein there was a higher correlation with care worker-collected nasopharyngeal swabs than self-sampled anterior nasal swabs (15–17). Another factor is the small sample size and unclear saliva sampling methodology, no doubt being an underlying cause of the conflict on the level of correlation with nasopharyngeal swab[3].

Within our own service, the issues associated with saliva as a diagnostic sample are modest relative to benefits. For example, while we overcame potential background fluorescence issues by using double-quenched probes from IDT (internal ZEN quencher at 9 base pairs from 5' end; IowaBlack Quencher at 3' end), the level of complementarity between the N1 and E primer/probe sets (and/or amplicons) caused aberrant amplification curves, which could only be overcome switching to a combination of N2 and E. We have also found that sequencing the viral genome from saliva can be a challenge, possibly due to fragmented viral RNA.

Impurities and inhibitors within saliva may be problematic but can be overcome if processed in conjunction with compatible RT-qPCR reaction mixes, as explained below. It is true that

[1]https://www.nature.com/articles/d41586-021-01390-4

[2]http://www.nottingham.ac.uk/coronavirus/university-testing-service/index.aspx

[3]https://www.ecdc.europa.eu/sites/default/files/documents/covid-19-use-saliva-sample-material-testing.pdf

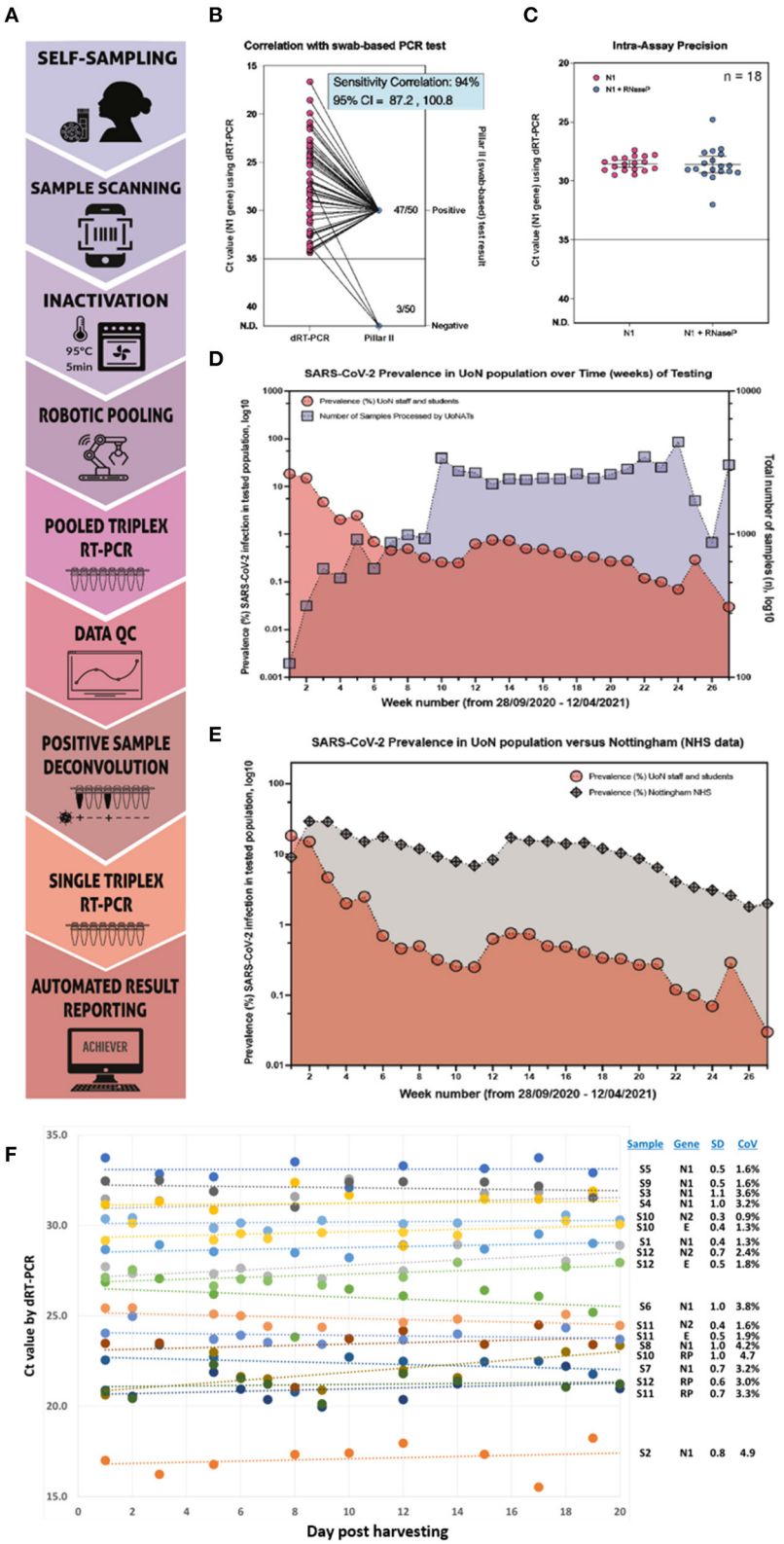

FIGURE 1 | Saliva-based RT-qPCR detection of SARS-CoV-2 is a reliable diagnostic method. **(A)** A Stepwise testing process: (i) Self-sampling of saliva into a barcoded vial; (ii) Donor scans barcode using their personal devices (e.g., smartphone) and enter information to secure database; (iii) Batch inactivation and lysis for

(Continued)

FIGURE 1 | 5 min at 95°C in an oven; (iv) Robot-assisted two-way pooling into 96- or 384-well plates; (v) Direct RT-qPCR to target genes; (vi) Data analysis; (vii) Deconvolution of positive samples to allow (viii) re-testing of original saliva as single samples in a new RT-qPCR cycle; (ix) Automated reporting *via* Achiever Medical LIMS, Interactive software. **(B)** Correlation of positive results from our saliva test vs. a UK government-approved swab-based testing service known as a "Pillar II" test. Of 50 patients identified as positive using our testing method, 94% tested positive *via* Pillar II. **(C)** Assay reproducibility shown as the plotted Ct values for a positive sample tested repeatedly over 14 days using four different qPCR machines by four different operators ($n = 18$). **(D)** Plotted prevalence (% of detected positive samples over total samples tested) over time (weeks, red circles) against the number of total samples processed each week (blue squares). **(E)** Plotted prevalence (% of detected positive samples over total samples tested) in our cohort over time (weeks, red circles) and the prevalence (%) of positive samples detected by the local public healthcare system (gray diamonds). The data for the prevalence (%) of positive SARS-CoV-2 diagnostic tests were sourced from a UK government site. **(F)** Stability of saliva was evaluated *via* regular repeat analysis of 12 samples stored at 4°C for up to 20 days *via* RT-qPCR amplifying viral genes N1 ($n = 9$), N2 ($n = 3$), or E ($n = 3$), or the internal control human gene, RP (RNAseP; $n = 3$). High stability was observed over the time course, with all standard deviations (SD) being ≤1.1 and coefficients of variation (CoV) being <5%. Dataset consistency was confirmed using Kolmogorov–Smirnov normality test (normality test passed, $p < 0.05$).

pooling presents the greatest challenge to sensitivity due to a skewed ratio of impurities vs. viral genomes per unit volume. While we have found the limit of detection in single saliva samples is <1 viral genome/μl, the maximum sensitivity in eight samples combined in two-way pools reduced to 4 viral genomes/μl. Impact on samples of samples with medium to low viral loads is negligible, but when Ct values exceed 30, the error rate of detection in pooled samples reduces accuracy to <99%. Although this level is required by UK regulatory agencies for diagnosis of SARS-CoV-2, saliva is an attractive sample, especially when mass surveillance is needed during easing of social distancing and travel bans.

AN EVIDENCE-BASED PIPELINE FOR SARS-COV-2 DETECTION USING SALIVA

The simple, streamlined pipeline we use is in **Figure 1A**, while **Figures 1B–E** show data generated between September 2020 and July 2021 from regular testing among University of Nottingham staff, students, and support services. At time of writing (October 2021), *circa* 96,317 samples were processed from 23,740 unique donors, yielding 465 positive test results. Key aspects are as follows.

Harvesting

Donors are provided with a Ziploc bag containing a collection tube (dual linear and QR bar codes; Brooks Ltd, product [65-7643]), tissue, paper straw (cut to lengths of ∼5 cm; purchased *via* Amazon from IntrinsicPaperStraws.com, item Black 6 × 140 mm), and a stepwise guide[4]. To avoid exogenous contaminants, donors are requested to provide a saliva sample in the morning before they have eaten, brushed their teeth, or used oral hygiene products, consistent with the guidelines from the European Center for Disease Prevention and Control (ECDC) (see footnote 3).

Inactivation

Saliva samples are oven-baked to a target temperature of 95°C/5 min to inactivate and lyse virus, hence simplifying safety procedures and bypassing the need for toxic chemicals and/or RNA extraction. While, at least in our experience, heating causes swabs and/or viral transport medium to become more viscous, with saliva, the effect is to increase sample fluidity. This is critical, yet often overlooked because of compatibility with downstream liquid handling processes.

Pooling, Then RT-qPCR

When prevalence of infection is low (<6%), samples are configured into two-way pools of six to eight samples per pool for one-step RT-qPCR with Center for Disease Control (CDC) primer-probes for the N and/or E genes. Critical points are as follows: (i) Quantabio UltraPlex 1-Step ToughMix RT-qPCR Reagents, designed for use with samples containing high levels of potential inhibitory factors. (ii) Positive pooled samples are deconvoluted and confirmed *via* single, unpooled tests. If prevalence exceeds 6%, the complexity of deconvolution becomes prohibitive and the process pipeline defaults to single, non-pooled testing.

In support of saliva in surveillance and diagnosis of SARS-CoV-2 infection, we provide illustrative data from the University of Nottingham Asymptomatic Testing Service. **Figure 1B** shows that of samples identified as positive in our assay, 94% agreement correlation (95% CI 87.2–100.8) existed with a hospital-accredited swab-based qPCR service. The 6% differential might be explained by saliva being a more consistent sample to harvest, as explored above. Also, 1–5 days elapsed between positive saliva result and the swab provision; hence, viral load may have reduced.

Saliva tests showed high intra-assay precision after repeat testing of the same positive samples over 14 days between four different operators and four different qPCR machines (**Figure 1C**). The high concordance required to satisfy the regulators and achieve accreditation (see below) suggests that heat inactivation has little or no negative effect on sensitivity and, anecdotally, may increase sensitivity in some cases. This is possibly because there is no loss of viral RNA, which occurs to varying degrees when using extraction procedures. In **Figures 1D,E**, an increased number of samples were tested from people on campus associates with reducing prevalence rates in staff and students (1D) and with 10–100-fold lower infection rates than the surrounding geographical area of Nottingham (1E, data from UK government). These data suggest that early detection is breaking transmission chains, even in high-population zones such as student halls of residence.

[4]https://www.nottingham.ac.uk/coronavirus/university-testing-service/how-to-provide-your-sample/how-to-get-tested.aspx

DISCUSSION

Combined with the few data available in the literature, our work on SARS-CoV-2 RT-qPCR detection points to saliva as an undervalued resource. *Via* this Perspective, we seek to promote discussion around the potential for a missed opportunity to achieve COVID-19 surveillance and outbreak control. The perception that saliva is difficult to work with can be overcome by simple modifications, such as by heating and using one-step inhibitor-resistant RT-qPCR. To assist with appropriate harvesting approaches, the European Center for Disease Prevention and Control (ECDC) recently published (17) a technical report "Considerations for the use of saliva as sample material for COVID-19 testing". Although concluding that saliva sample collection is easy, non-invasive, acceptable for repeat testing, and can be performed by non-healthcare professionals, ECDC noted that performance of RT-qPCR tests has variously reported both higher and lower sensitivity for saliva samples compared with nasopharyngeal swabs. In part, heterogeneity is likely to reflect differences in sampling techniques, sampling times, and the type of population being tested, which the ECDC technical report explores (17).

Within the guidance from ECDC is the need to provide a sample into a "collection container, upon waking up, before brushing teeth and eating". We came to the same conclusions early on in the UoN Testing Service because saliva samples of various consistencies and viscosities slowed down testing and processing time. Sample provision before eating is likely to be one factor in the high accuracy, sensitivity, and consistency observed within the UoN Testing Service and in other laboratories, which have reported that saliva has offered greater sensitivity than nasopharyngeal swabs for diagnosis of asymptomatic and mild COVID-19 infection (11). Retrospective studies have provided similar findings. Guillaume and colleagues (12) surveyed 385 references, which yielded 16 unique studies that were identified for quantitative synthesis. Eight peer-reviewed studies and eight preprints were included in the meta-analyses (5,922 unique patients), with a conclusion that diagnostic accuracy of saliva is similar to that of nasopharyngeal swabs.

Other benefits include ease of donation, minimal invasiveness, high-sensitivity testing, and accurate reporting. Stability of saliva as a source material is also high (**Figure 1F**), wherein regular analysis *via* RT-qPCR to N1, N2, E, and/or RNAseP of the same samples stored at 4°C for up to 20 days showed standard deviations (SD) of $\leq$1.1 and coefficients of variation (CoV) of <5%. These attributes meant that saliva samples were preferred over nasopharyngeal swabs within our cohorts, and we expect the same to be true for communities with special considerations; hence, the assay will increase inclusivity.

In July 2021, the University of Nottingham became the first university in the UK and the first institution in the UK to gain accreditation status from the oversight body, UKAS (UK Accreditation Service). This permitted results from our testing service to be reported directly to the government organization, Public Health England, thereby requiring donors who are positive for SARS-CoV-2 to follow national laws. At the time of writing (October 2021), all datasets in the form of research and protocols manuscripts are being prepared to give detailed information on the use of a triplex testing *via* CDC primers N2 and E, along with an internal control of RNAseP, in SARS-CoV-2 detection in saliva following direct heat inactivation. In these articles, we will draw on evidence from *circa* 100,000 samples tested. This will include data required for UKAS accreditation showing >99% concordance of 400 samples (250 negative and 150 positive), most of which were twinned swab and saliva hospital samples. We will provide evidence for analytical specificity, analytical sensitivity (limit of detection), limit of quantification, diagnostic specificity, diagnostic sensitivity, precision, sample stability, repeatability, reproducibility, range/linearity/accuracy, robustness (control of known interference), and low coefficients of variation of $\leq$5.3% even in the most viscous saliva samples.

Receiving accreditation from UKAS means that we can assist other institutions to gain this accolade and both accelerate and broaden their own testing programs. In parallel, the US Food & Drug Administration (FDA) approved Emergency Use Authorization (EUA) for "SalivaDirect™" (18). Various saliva RT-qPCR tests are in development or in the process of regulatory approval through the FDA EUA process or the CE Marking process in the European Union, including Rutgers University, OraSure Technologies/DNA Genotek, University of Illinois Champagne, and others. In instances where community surveillance requires pooling of large numbers of samples (10 or more per pool), there is the potential that viscosity may cause pipetting errors or reduce the sensitivity of SARS-CoV-2 detection, especially when using direct RT-qPCR approaches on samples with low viral loads (17). These issues can be overcome by extracting viral RNA prior to pooling and analysis but may not be needed due to differences in regulatory bodies. In the UK, regulations permit only a maximum of four samples to be pooled for diagnostic purposes for SARS-CoV-2.

Saliva may not be a one-size-fits-all solution. While various companies now offer saliva-based antigen or antibody tests, the ECDC suggests that the current limited evidence does not support the use of this sample material in this way and further clinical validation studies are needed on the different available tests (17). Nevertheless, even if this stance does not change for protein-based testing, the acceptability and ease of saliva as a donor sample coupled with approval as a diagnostic for SARS-CoV-2 genomes by multiple regulatory bodies, including the FDA, ECDC, and UKAS, is positive. This is likely to assist with sustained regular repeat testing over long periods, which will be essential to detect emergence of new variants during this two-tier pandemic. Thus, to conclude, saliva is presented as a suitable first-line diagnostic test to survey and control infection rates among populations in a more efficient and less invasive manner, complementing other testing strategies and improving our ability to control infectious events in the future.

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 University of Nottingham Ethics Committee, reference number FMHS 96-0920. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CD is lead of the programme and SP-G lead on data harvesting and first draft of the manuscript. All authors were involved in writing and contributing to the manuscript, and running the asymptomatic testing service.

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Alpha, Beta, Delta, Omicron, and SARS-CoV-2 Breakthrough Cases: Defining Immunological Mechanisms for Vaccine Waning and Vaccine-Variant Mismatch

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The COVID-19 pandemic, caused by the SARS-CoV-2 coronavirus, is responsible for over 400 million cases and over 5. 5 million deaths worldwide. In response to widespread SARS-CoV-2 infection, immunization of the global population has approached 60% one dose and 54% full dose vaccination status. Emerging data indicates decreasing circulating antibody levels as well as decreases in other immune correlates in vaccinated individuals. Complicating the determination of vaccine effectiveness is the concomitant emergence of novel SARS-CoV-2 variants with substantial antigenic differences from the ancestral D614G strain. The Omicron variant (B.1.1.529) spike protein has over 30 mutations compared with the D614G spike protein, which was used to design most SARS-CoV-2 vaccines in use today. Therefore, breakthrough cases of SARS-CoV-2 infections or severe disease in fully vaccinated individuals must be interpreted with caution taking into consideration vaccine waning and the degree of vaccine variant-mismatch resulting in adaptive immune evasion by novel emerging SARS-CoV-2 variants.

Keywords: SARS-CoV-2, breakthrough cases, immune evasion, vaccine mismatch, variants of concern (VoCs)

INTRODUCTION

In December 2019, a pneumonia-like disease of unknown cause was discovered in Wuhan, Hubei Province, China (1). The causative virus for this outbreak was identified as a new coronavirus, which was later named severe acute respiratory syndrome coronavirus 2019 (SARS-CoV-2). The novel coronavirus 2019 (COVID-19) is responsible for over 400 million cases and over five million deaths worldwide. Messenger RNA (mRNA) based vaccines for the prevention of SARS-CoV-2, such as those from Pfizer-BioNTech or Moderna, and the urgency associated with the global pandemic have redefined the timeline for vaccine approval and rollout. Although

disparities in global vaccine distribution remain central to the COVID-19 pandemic, roughly 60% of the world population has been vaccinated; that is, having received a first dose, while 54% are fully vaccinated (both doses), and over 10 billion doses have been administered in total. Although full vaccination significantly reduces the probability of being infected by SARS-CoV-2, a certain risk for viral transmission remains. However, the risk of acquiring a SARS-CoV-2 infection from a partially or fully vaccinated individual is relatively low (<1%) (2, 3). When a fully vaccinated individual contracts SARS-CoV-2, it is known as a vaccine breakthrough infection. Breakthrough cases are likely explained by a combination of immunological phenomena, including the failure to generate an immune response to viral components following vaccination, wanning vaccine immunity caused by a decline in immunological correlates of protection, or a shift in antigenicity of the circulating virus resulting in vaccine mismatch, where those vaccinated with an early vaccine iteration (such as those developed against the alpha strain) are not protected against emerging virus variants. Breakthrough cases are also influenced by a vaccine's capacity to generate sterilizing immunity, which is a form of innate immunity present in mucosal tissues (such as in the nose, throat, and upper respiratory tract) that fully prevents disease by an invading pathogen, such as SARS-CoV-2 (4). Additionally, high-risk groups such as immunosuppressed individuals and the elderly do not respond to vaccination as well as young, healthy individuals.

In the United States (US), the Centers for Disease Control and Prevention (CDC) reported that weekly case numbers and deaths remain highest for unvaccinated individuals. However, among the three major vaccine manufacturers in the US, Johnson and Johnson (J&J) has the highest rate of morbidity and mortality among fully vaccinated individuals, followed by Pfizer and Moderna. Interestingly, Moderna showed the lowest number of breakthrough cases among the three manufacturers. Those individuals 18 years of age or older who received J&J or AstraZeneca vaccines were subsequently recommended to receive a booster dose (5). Further data obtained from the CDC COVID-NET surveillance program showed that, in a three-month time span (Jan 2021–Apr 2021), over 10,000 SARS-CoV-2 breakthrough infections had been reported across 46 US states; a plausible explanation for this was the shift from the alpha variant (B.1.1.7) to more virulent forms of the virus, such as the beta (B.1.351) and delta (B.1.617.2) variants. Within these 10,000 cases, the median age was 58 years old; 63% of cases occurred in females (despite higher SARS-CoV-2 disease severity and mortality in males); 27% were asymptomatic; 10% of patients were hospitalized; and a further 2% died while in hospital (6). Among the 2% of reported deaths, the median age was 82 years. Sequence data also revealed that breakthrough cases were caused by novel variants, such as alpha (B.1.1.7, 56%), epsilon (B.1.429, 25%), B.1.427 (8%), gamma (P.1, 8%), and beta (B.1.351, 4%) (3). The constant emergence of novel variants creates the need to reassess vaccinated immune protection, waning vaccine protection, and immune status of each individual.

Comparable to the situation in the US, instances of COVID-19 breakthrough cases are becoming more globally recognized as a serious health threat. For example, a recent cohort study (unpublished data, referring to findings prior to peer review) in Israel found that in a sample of 1497 healthcare workers (HCWs) fully vaccinated with the Pfizer-BioNTech Comirnaty® mRNA (BNT162b2) vaccine, 39 workers tested positive for COVID-19 following RT-PCR testing. (3) Among these 39 healthcare workers, the majority of individuals were female (64%), nursing staff members (46%), and had an average age of 42 years old. The most common symptoms experienced among infected individuals included upper respiratory congestion, myalgia, and loss of smell or taste. At 6 weeks following infection, 19% of infected individuals reported experiencing ongoing loss of smell, cough, fatigue, and weakness, a phenomenon otherwise known as "long COVID-19". Interestingly, in both the CDC and Israeli data, women appear to be at an increased risk (63 and 64%, respectively) of contracting a breakthrough COVID-19 infection. These results counter the fact that males have been widely reported as having a disproportionately higher rate of infection, disease severity, and mortality when compared to females (7). This female bias in breakthrough cases may be explained by where these cases are most likely to occur. For example, HCWs, including those donned with full personal protective equipment (PPE), are at a high risk of COVID-19 exposure. These risks may be heightened for frontline and triaging HCWs such as nurses and emergency physicians, who are the first line of medical treatment for potentially infected individuals. In a case study in Italy, one partially vaccinated and two fully vaccinated healthcare professionals (two doctors and one nurse) were infected by the same SARS-CoV-2 (B.1.1.7) positive patient (8). The patient was a 50-year-old male who reported to the emergency service with respiratory failure and pulmonary oedema, which required immediate endotracheal intubation. The patient died 2 h following admission to the ICU and during intubation all procedures were performed with full PPE, including: particulate filter respirators (P3), two pairs of gloves, face shields, and a single-use coverall (8). Noteworthy is the fact that the HCWs in this case study were not wearing goggles under their face shields, which is a recommended safety measure from the CDC. Given that the majority of frontline HCWs are females (9), possible explanations for the greater number of female breakthrough COVID-19 cases is the increased exposure, non-universal PPE standards, and sub-optimized implementation of PPE in hospitals and testing centers, such as the lack of additional eye protection in the Italian case study. In a separate study in the United States, the majority of breakthrough cases (54%) were reported in women, which further challenges the notion that men routinely exhibit disproportionately high rates of SARS-CoV-2 infection, disease severity, and mortality compared to women (7, 10). This female bias in breakthrough infections should be investigated in further detail to delineate the underlying immunological mechanisms at play, in addition to the social and behavioral factors placing women at greater risk of infection.

IMMUNE AND VIROLOGICAL MECHANISMS OF BREAKTHROUGH CASES

Potential causes for COVID-19 breakthrough cases may be explained via a myriad of mechanisms such as PPE failure; individual immune status; age; sex; variant infectivity or pathogenicity; relaxed isolation and masking measures; waning vaccine induced immune protection; and mismatch between vaccine and circulating SARS-CoV-2 variants. One retrospective cohort study found that the incidence of breakthrough cases (alpha or beta variant) among patients fully vaccinated (≥14 days after second dose) with the Comirnaty® vaccine was three times higher for immunocompromised individuals than for those with normal immune system function (11). In addition to impaired immune function, old age also places vaccinated individuals at a greater risk of a severe breakthrough infection. In a data report from the CDC from January to April 2021, the median age from 10,000 reported breakthrough cases was 52 years old, while the two percent of patients who died had a median age of 82 years old (6). An additional CDC report in July 2021 found that in a cluster of 469 COVID-19 breakthrough cases (with 346; 74% fully vaccinated) in Massachusetts, the median age was 40 years old (12). These data show that those <70 years of age are at a greater risk of death following a breakthrough case and that breakthrough cases occur across a wide range of ages. The CDC also found that the majority of individuals infected with breakthrough cases experienced mild symptoms, such as headache, cough, and sore throat, while others were asymptomatic (6, 12). Although these symptoms are indicative of moderate viral load and limited ability to infect others, studies have found that viral load levels can be as high in individuals with a breakthrough infection as those who are unvaccinated against SARS-CoV-2 (2, 12). The phenomenon of viral shedding in breakthrough cases places the elderly and immunocompromised at a higher risk of encountering a breakthrough infected individual who is unaware of their COVID-19 disease status. The ability of these vaccinated individuals to actually transmit the virus remains to be elucidated, since their mild symptoms could reflect a limited viral replication in the upper respiratory tract.

VACCINE WANING AND VACCINE EFFECTIVENESS

As the large-scale, global vaccination campaign against SARS-CoV-2 continues, a void remains in our understanding of vaccine effectiveness. Clinical trial data have found the Comirnaty® mRNA vaccine to be up to 91.3% effective against COVID-19 through 6 months of follow-up and a further 96·7% effective against severe disease (13). The statistics look promising; however, given the rapid and reactionary nature of the SARS-CoV-2 vaccine response, there is little known about the durability of vaccines beyond 6 months. Early vaccine data (**Table 1**) show that at 3 months after the second dose of the Comirnaty® vaccine, IgG antibodies and neutralizing antibody titers decreased at a consistent rate in all individuals, while at 6 months after receipt of the second dose, neutralizing antibody titers were substantially lower among men than women, lower among persons aged 65 years or older, and lower among immunosuppressed or immunocompromised individuals (15). If not "rescued" by booster vaccine doses, specific subsets of patients such as the elderly or the immunosuppressed could face the greatest exposure to breakthrough infections due to reduced circulating antibody levels.

SILENT SPREADERS

Here we refer to fully vaccinated individuals suffering from breakthrough infections as potential "silent spreaders" of SARS-CoV-2. Silent spreaders are unsuspecting candidates for spreading the SARS-CoV-2 virus. These individuals are those who have experienced a COVID-19 breakthrough case but due to the asymptomatic or mild nature of symptoms experienced during the disease course, have no knowledge they are infected carriers and potential spreaders of the SARS-CoV-2 virus. This unawareness is in part due to a false sense of security following vaccination, where individuals with both doses of a reputably manufactured vaccine operate assuming they can no longer contract the virus and therefore no longer need to be tested. It is also likely that those infected with a COVID-19 breakthrough case are harboring the SARS-CoV-2 delta variant (B.1.617.2). A recent study from India's deadly second SARS-CoV-2 wave in June 2021 found that among 592 fully vaccinated (Covishield and/or Covaxin) individuals with breakthrough cases, 86.7% (n = 443) were infected with the delta virus variant (22). The delta virus variant of SARS-CoV-2 is highly transmissible and is still overwhelmingly spread by unvaccinated individuals, who are themselves at high risk of serious disease. With that, vaccines are rendered most effective at combatting severe disease and due to gaps in our understanding regarding long-term protection, necessitate further clinical trials to delineate their efficacy in combatting asymptomatic disease (23). On a more intimate and domestic scale, those fully vaccinated individuals who are positive for a breakthrough case, and who display mild symptoms or none at all, are potentially endangering their family members (elderly and children), immunocompromised, partners, or colleagues. These examples emphasize the timely need for equipping the public with the knowledge required to mitigate the spread of COVID-19 from fully vaccinated individuals.

CORRELATES OF PROTECTION (COP)

Perhaps the defining characteristic of long-term protection offered by a particular vaccine is its capacity to stimulate, produce, and retain key correlates of protection (CoP). CoPs are the vaccine-induced biomarkers associated with a lower risk of infection or severe disease (24). For example, among Comirnaty® -vaccinated individuals, the defining CoP was shown to be neutralizing antibody titers (3). Predictive models support neutralizing antibody titers as being highly correlated to immune protection. One model found that among seven current vaccines, the neutralization level for 50% protection against

TABLE 1 | Summary of current literature detailing the durability of available SARS-CoV-2 mRNA vaccines and their efficacy offered over time for fully vaccinated individuals.

Vaccine	References	Country	Study type	Sample size (n)	Vaccine effectiveness (VE)
Comirnaty® (Pfizer-BioNTech mRNA)	Chemaitelly et al. (14)	Qatar	Case-control	494,859*; public	Negligible for first 2 weeks following first dose and reached peak at 77·5% (95% CI) in the first month after the second dose. Effectiveness declined 4 months after second dose, reaching a low of 20% in months 5 through 7 after the second dose.
	Levin et al. (15)	Israel	Longitudinal prospective study	4,868; healthcare workers	Peak neutralizing antibody titers reported during days 4 through 30 following second dose. At 6 months after second dose, neutralizing antibody titers were substantially lower.
	Shrotri et al. (16)	England and Wales	Cross-sectional	605; public	Significant reduction in S-antibody levels at 70 days following both doses with a peak noted at 21–41 days after second dose. Women had higher initial antibody levels than men as did those aged 18-64 years old, vs. those 65 years or older.
	Naaber et al. (17)	Estonia	Longitudinal prospective study	122; Controlled group	Elevated IgG antibody levels from serum samples 3 weeks after first dose. IgG levels declined 45% between 1 and 6 weeks after second dose. At 6 months, IgG levels were 7% of their peak levels detected at 1 week post second dose.
	Goldberg et al. (18)	Israel	Retrospective cohort study	4·79 million; public	Immunity against the SARS-CoV-2 delta variant (B.1.617.2) waned in all age groups 3 months after receipt of the second dose.
	Tartof et al. (19)	USA	Retrospective cohort study	4·7 million; public	Effectiveness against infections declined from 88% during the first month of both doses to 47% after 5 months of both doses.
Spikevax® (Moderna mRNA-1273)	Doria-Rose (20)	Not disclosed	Phase 3 clinical trial	33; Public (healthy individuals)	Antibodies persisted for 6 months after second dose and remained high in all age groups. All patients also showed a detectable neutralization response 6 months after second dose (interim results)
	Tré-Hardy et al. (21)	Belgium	Prospective	201; healthcare workers	Although IgG antibodies were present up to 3 months after vaccination, significant IgG antibody decrease was observed between 3 and 6 months following vaccination.

**Variable sample size reported depending on post-dose timepoint.*

severe disease is roughly 3% of the mean convalescent titer, while 50% protection against detectable SARS-CoV-2 infection requires roughly 20% of the mean convalescent titer (25). These findings reaffirm that current SARS-CoV-2 vaccines offer greater protection against severe infection (i.e., a lower percentage of mean convalescent titer required for protection) than against mild or asymptomatic infection.

The capacity of a given vaccine to produce CoP biomarkers can vary based on factors such as the type of vaccine, the immune status of the individual receiving the dose, and whether the vaccine matches the variant (i.e., an alpha vaccine protects against the alpha variant). A 2021 study on CoP found an association between risk of disease and levels of anti-spike IgG, anti-RBD IgG, and neutralizing antibody titers, where those with higher levels of anti-spike IgG and neutralizing antibody titers exhibited a lower risk of symptomatic disease against the alpha variant (B.1.1.7) in individuals fully vaccinated with the ChAdOx1 NCoV-19 (AZD1222) vaccine. (26) A study from Gilbert et al. (2022) showed similar results, where Moderna's Spikevax® vaccine (mRNA-1273) elicited spike IgG, RBD IgG, cID50, and cID80 neutralization levels which were inversely correlated with COVID-19 risk. (27) Additional findings suggest that individuals vaccinated with both doses of Spikevax® exhibited higher antibody titer levels than those vaccinated with Comirnaty® (28).

Current literature is sparse and demonstrates the timely need for coordinated, clinical trials to further identify correlates of vaccine efficacy. One approach would be comparing banked serum samples from confirmed breakthrough individuals against fully vaccinated, non-breakthrough individuals at key follow-up timepoints. An additional approach would be a monitoring of the mucosal immune response and level of sterilizing immunity by using biofluids, such as nasopharyngeal swab isolates or salivary samples. Findings from such studies would allow more focussed

efforts in vaccine development as well as a tailored approach to patient-specific management (29).

MEMORY B AND T-SPECIFIC IMMUNITY

Although much of this article has focussed on research efforts aimed at understanding the durability and dynamics of circulating, neutralizing antibody levels following vaccination, it is important to acknowledge recent contributions surrounding the SARS-CoV-2 adaptive immune response. A recent study found that a 3rd (booster) dose of an mRNA vaccine causes an initial increase in circulating anti-omicron neutralizing antibodies; however, these levels were 10-20 fold lower than against the original (Wuhan-Hu-1) strain (30). Despite this, a separate (pre-print) study examined the memory B cell repertoire following the 3rd mRNA vaccine dose and found an increased expansion of anti-receptor binding domain specific memory B cells (31). These memory B cells encoded antibodies exhibiting significantly increased potency compared to antibodies produced by the 2nd dose; furthermore, greater than 50% of the neutralizing antibodies produced by the memory B cells in individuals with three mRNA vaccine doses neutralized the omicron variant (31).

Recent findings suggest that the adaptive immune response also contributes to robust, long-term SARS-CoV-2 protection while also playing an important 'second-line' defensive role following escape of omicron from neutralizing antibodies (32, 33). One study found that most vaccine- induced T cell responses were capable of recognizing all known SARS-CoV-2 variants (with average preservation >80% for omicron) following T cell repertoire analysis (33). Similarly, a study investigating T cell cross reactivity to the omicron spike protein in vaccinated (Comirnaty®, Janssen) and unvaccinated convalescent COVID-19 patients found 70–80% of the CD4+ and CD8+ T cell responses to spike were maintained across study groups (34). The same study also found similar levels of omicron cross-reactive T cells to delta and beta variants for all groups. These data submit that T cell populations are capable of cross-recognizing all SARS-CoV-2 variants, including the highly mutated omicron variant. Importantly, such findings suggest that cellular immunity is highly conserved to the omicron spike protein and that omicron spike-specific CD8+ and CD4+ T cell responses elicited by current mRNA vaccines contribute to robust protection against severe disease caused by the omicron variant, despite declining neutralizing capacity of free-circulating antibodies (34, 35).

MUCOSAL IMMUNITY

For full protection against viral infections, such as SARS-CoV-2, sterilizing immunity may be required. Sterilizing immunity is achieved via the presence and secretion of neutralizing antibodies at the mucosal site of infection. For SARS-CoV-2, this refers to the secretion of IgA from mucosal tissues, including the nose, throat, and upper respiratory tract (4). Early emphasis has been placed on the maintenance of circulating levels of neutralizing antibodies in sera following intramuscular (IM) vaccination. With time, these circulating antibody levels begin to wane, therefore necessitating further vaccine "boost" doses (36). When waning levels of circulating neutralizing antibodies are coupled with emerging variants of concern, such as the omicron variant, breakthrough infections will continue to occur (37). Additionally, without sterilizing immunity, the risk of transmission of SARS-CoV-2 by vaccinated individuals cannot be overlooked.

To overcome this, vaccines are being developed to target and stimulate mucosal sites, rather than solely inducing systemic responses. To date, the only approved intranasal (IN) vaccines are live-attenuated influenza vaccines (LAIV), although 14 mucosal IN SARS-CoV-2 vaccines have progressed to the first phase of clinical trials. (36–38) A 2021 study demonstrated that similar efficacy could be achieved between a heterologous mRNA IM prime and IN Ad5 boost and two intramuscular mRNA immunizations, with the IN vaccination providing an elevated mucosal immune response to SARS-CoV-2 (37). Mucosal immunization via IN vaccination is also efficient at inducing adaptive immune responses, including secretory IgA (sIgA) antibodies and resident memory T (T_{RM}) cells (38). Early data suggests that IN vaccines may be most effective when combined with an intramuscular mRNA vaccine, such as Comirnaty® or Spikevax® (39, 40). Additional pre-print findings (awaiting peer review) have demonstrated a correlation between anti-Spike/RBD IgA levels and breakthrough infection, where those with lower levels of anti-Spike/RBD IgA 2–4 weeks post second IM (Comirnaty® or Spikevax®) dose exhibited significantly greater rates of reinfection ($p < 0.01$) (40). Therefore, IN vaccination poses as a promising preventative measure against the SARS-CoV-2 virus; however, future trials are needed to determine whether IN vaccination is effective at preventing severe disease.

VACCINE BOOSTING

Homologous Boosting

As more long-term SARS-CoV-2 vaccine waning data becomes available, it is important to better understand the underlying implications of vaccine durability, effectiveness, and potential side effects. Although the initial vaccine boosting campaign was targeted toward older individuals (>60 years of age) and the immunocompromised, the eligibility criteria have been expanded to include those 16 years of age and older (41). Early data from a large SARS-CoV-2 vaccine booster trial in Israel shows that the rate of infection decreased by a factor of 11.3 for the booster group, vs. those who had not yet received a third dose; the same study found that the rate of severe illness was lower by a factor of 19.5 (95% CI) (42). Booster shots for SARS-CoV-2 inactivated vaccines have also shown to be beneficial for increasing levels of circulating neutralizing antibodies. For example, a 2021 cohort trial compared both levels of neutralizing antibody titers and positive antibody conversion rate among 67 individuals who had received a third booster shot, to those who had received two doses. Interestingly, they found that those who received a booster had a higher positive antibody conversion rate in the first month than those at 8 months after their second dose; the

study also showed higher antibody levels at 1 month after the third dose than at 1 month after the second dose (43). In addition to greater positive antibody conversion, those on a three-dose vaccine regimen demonstrated greater neutralizing antibody levels than those on the conventional two dose regimen These results were regardless of age, sex, or vaccine procedure and demonstrate that a third dose can reverse declining neutralizing antibody levels. Although more studies are needed to support these early findings, vaccine boosting is a promising addition to the universally adopted two-dose program. It is not unlikely that, moving forward, at-risk individuals will be advised to continue receiving yearly COVID-19 booster doses, similar to influenza.

Many high- and middle-income countries including China, Canada, Israel, UAE, and the US have begun administering booster doses. The UK has procured a further 30 million Comirnaty® vaccines to be administered as boost doses to at-risk individuals. Canada's health agency has recommended and authorized Pfizer-BioNTech or Moderna booster shots for older individuals (70 and above), healthcare workers, and immunocompromised individuals (44). In the USA, health officials are encouraging all adults to seek boosters. Booster eligibility criteria is more relaxed in some densely populated areas within the US as well. For example, New York City states that any individual 18 years or older should not be turned away when seeking a booster shot, contingent on being 6 months or longer since their second shot of the Pfizer-BioNTech or Moderna mRNA vaccines (45).

Hetero Boosting

Current countries administering booster doses have opted to administer both the Comirnaty® and Spikevax® mRNA vaccines, regardless of which vaccine type was administered for the prime (dose 1) and boost (dose 2) vaccine of a given individual. Reaching a consensus as to whether mixing and matching with different vaccine brands and types has been fraught with uncertainty. Due to the halting of vaccination of AstraZeneca's ChAdOx1 nCov-19 (ChAd) vaccine in many countries, individuals were left partially vaccinated with the option of receiving a second dose with an mRNA vaccine, such as Comirnaty® or Spikevax®. Data from mixed-dose individuals suggested that receiving a heterologous ChAd-Comirnaty® dosing resulted in a greater IgG and IgA response to the SARS-CoV-2 spike protein with increased neutralizing antibody titer levels than that seen in individuals who received homologous ChAd-ChAd dosing (46, 47). In addition, the neutralizing antibody titer levels were roughly threefold higher in the serum of the ChAd-Comirnaty® than the serum of the homologous Comirnaty® group. A separate study from Nordström et al. (2021) found that a heterologous prime-boost vaccine schedule consisting of ChAd-Comirnaty® was 67% effective while ChAd-Spikevax® prime-boost offered 79% vaccine efficacy, compared to a homologous prime-boost regiment of ChAd-ChAd, offering only 50% efficacy (48). These findings suggest that further prospective clinical trials are needed to compare long-term vaccine efficacy following a third booster dose of either homologous or heterologous vaccine dosing. The data also suggests that the Spikevax® vaccine may be equipped to offer similar or exceeding protection to that of the Comirnaty® vaccine, potentially expediting the global rollout for individuals who require a third dose in a timely manner. Additionally, Moderna's booster dose (3rd dose) is to be administered at half the volume (0.25 mL, 50 mcg) of the first two doses (0.5 mL, 100 mcg). The decision to administer the Spikevax® booster at half the volume of the first two doses was to reduce adverse side effects consistent with a full dose, such as fatigue, body aches, and fever, while also helping address global vaccine shortages. A half dose (50 mcg) of Spikevax® was found to increase neutralizing antibody levels 37-fold higher than pre-boost levels and a full dose booster (100 mcg) was found to increase these levels further (83-fold higher than pre-boost levels) (49). It is important to highlight individuals at risk who would most benefit from a booster dose, such as people living in care homes, the elderly, front line healthcare workers, and people with underlying health conditions or impaired immune function (50). With greater priority assigned to identifying individuals who require a third dose, the already limited global COVID-19 vaccine supply can be more equitably distributed to poorer countries who have not yet received a first dose.

BREAKTHROUGH AND VARIANTS OF CONCERN

As an increasing portion of the global population is vaccinated against SARS-CoV-2, a growing concern is the emergence of additional, potentially more virulent, variants of concern (VoCs). In late November 2021, a novel SARS-CoV-2 variant termed the omicron (o) variant (B.1.1.529) was discovered in Botswana and a few days later in an individual in Hong Kong who had traveled from South Africa (51). The omicron variant has since been listed as a variant of concern largely due to its rapid transmission and highly mutated spike protein (**Figure 1**) with 50 genetic mutations and more than 30 spike protein mutations (52). Confirmed cases of the omicron variant have been reported in over 80 countries, including Canada, Hong Kong, Australia, Botswana, France, Germany, Portugal, Italy, and the Netherlands. The emergence of the fifth VoC in April 2021, the delta SARS-CoV-2 variant, was associated with global surges in cases, higher viral loads, longer duration of infectiousness, and high rates of reinfection (53). Therefore, it remains of great concern to rapidly elucidate the underlying immunological mechanisms that dictate disease course for individuals infected with emerging novel variants.

Due to the recency of the omicron variant emergence, little is known about its virulence and whether currently vaccinated individuals are protected. Available data does, however, lend itself to interpretation. Due to the large number of mutations (>30) observed in omicron (many of which overlap with those observed in alpha, beta, gamma, and delta VoCs), it is not unreasonable to predict that the omicron variant may be associated with higher viral binding affinity, greater antibody escape, and increased transmissibility (53, 54). Recent modeling using S-gene target failure (SDFT) suggests that the rate of infection of the omicron

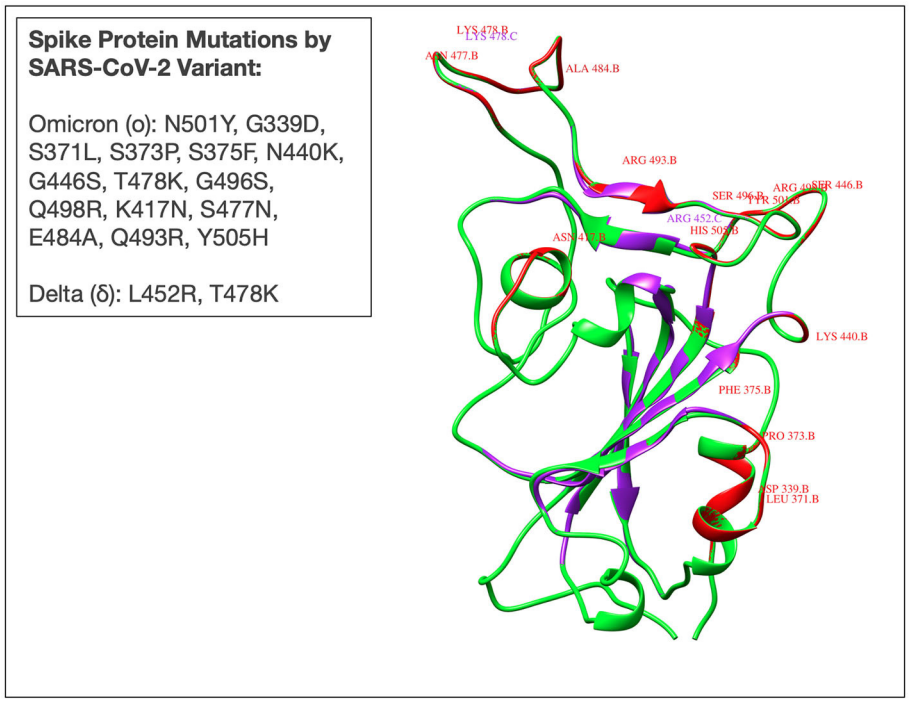

FIGURE 1 | Superimposed three-dimensional (3D) structure of the receptor binding domain (RBD) of spike protein derived from Omicron (B.1.1.529), Delta (B.1.617), and Wuhan (ancestral- D614G) SARS-CoV-2 variants. Red and purple colours reflect the omicron and delta RBD, respectively. The Wuhan RBD is represented by a green colour. Mutations in delta (L452R, T478K), and omicron (N501Y, G339D, S371L, S373P, S375F, N440K, G446S, T478K, G496S, Q498R, K417N, S477N, E484A, Q493R, Y505H) are also represented by corresponding colours. Mutant 3D models for delta and omicron variants were generated using UCSF Chimera software from the resource of Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01,081). The PDB ID: 7T9J was used as a template for the mutant model's prediction.

variant will be 100-fold higher compared to that of the delta variant of SARS-CoV-2 (55). There is limited data detailing whether the immune protection for fully vaccinated individuals is effective at preventing omicron infection; however, vaccine manufacturers remain optimistic that immunization will offer protection against severe infection caused by the omicron variant (56). Due to the large number of mutations present in the omicron variant, the delineation of the efficacy of current SARS-CoV-2 vaccines in preventing mild infections caused by the omicron variant remains of high importance.

A recent study (pre-print) from South Africa found that individuals fully vaccinated with the Comirnaty® mRNA vaccine may still be vulnerable to breakthrough infections with omicron. The study compared neutralization levels for the ancestral SARS-CoV-2 variant (D614G) and the novel omicron variant (B.1.1.529) in cells from individuals vaccinated with the Comirnaty® vaccine (57). Their results showed that the geometric mean titer (GMT) FRNT50 (inverse of the plasma dilution required for 50% reduction in infection foci number) was 1,321 for D614G, but dropped to 32 for the omicron variant, a 41-fold decrease. Additional studies are required to fully understand the level of protection offered by current vaccines against the omicron variant, such as the breadth of vaccine-induced cellular immune protection against the variant. Current emphasis has been placed on determining whether a third vaccine (booster) dose will restore protection and prevent or reduce breakthrough infections, especially in at-risk groups, such as the elderly, who typically mount less robust and coordinated cellular immune responses. For these individuals, a bolstered level of circulating antibodies from a booster shot may be required for adequate protection from the omicron variant and additional emerging VoCs.

Another recent study demonstrated that individuals fully vaccinated with the Comirnaty® mRNA vaccine with additional booster shots remain at risk of developing an omicron infection (58). Among the seven participants in the study, six were fully vaccinated with the Comirnaty® vaccine, and five of these individuals received a third (booster dose) of Comirnaty®, while the sixth individual received an additional full dose (100 mcg) of the Spikevax® vaccine. Five of these individuals tested positive for the omicron variant and had mean viral loads of 4.16 x 10E7 RNA copies per mL of swab eluate, with a mean age of 27.7 years. These findings suggest that omicron can produce a breakthrough infection in individuals who have received a booster dose, who are of young age, and who are otherwise healthy. Additionally, this data indicates that greater than three vaccine doses may be required for protection and a wider age range is at risk of developing an omicron breakthrough infection. Additional

(pre-print) findings from the UK suggest that two doses with Comirnaty® or ChAdOx1 offer insufficient levels of protection against infection and mild disease caused by the omicron variant (59). Furthermore, a 2022 study found that the omicron variant has roughly an 88% likelihood of escaping neutralizing antibodies produced by current vaccines, some 14 times as high as that of the delta variant (60). Although there have been limited reports of severe disease caused by the omicron variant, its rapid spread has caused surges in hospitalizations worldwide, where the United States is reporting >2,000 omicron related deaths per day (61). As increasingly virulent, heavily mutated variants of SARS-CoV-2 emerge, further investigation and development surrounding second-generation, multivalent vaccines is required. The design of such vaccines should be focussed on addressing the deficiencies of existing vaccines, including: (i) reducing reliance on booster doses for long-term protection; (ii) increasing robust memory responses; and (iii) are preventative in nature (62).

CONCLUSION

Perhaps the most robust SARS-CoV-2 transmission mitigation measure remains striving for a universally vaccinated population. Curbing the spread relies on both the individual and the scientific community, the latter being ultimately responsible for equipping the public with the knowledge required to make informed decisions about vaccination. Those resisting vaccination are directly endangering others while also jeopardizing the efforts of many global health leaders. This results in the emergence of potentially harmful, highly mutated variants, such as the delta and omicron variants, which will continue to persist in the population further saturating health care systems and intensive care units.

Additional efforts should be focussed on assessing the efficacy and durability of current SARS-CoV-2 vaccines. Highly mutated viral variants are demonstrating an increased ability to evade the vaccine-induced immune response especially due to shifts in antigenicity, which creates instances of vaccine mismatch where individuals fully vaccinated with early vaccine iterations fail to mount an immune response to mutated viral components. The omicron (B.1.1.529) variant is an example of this phenomena, where many individuals remain at risk of a breakthrough infection despite being fully vaccinated (with a booster) due to the many genetic and protein-level mutations. The risk of breakthrough infection underscores the need for further clinical trials designed to evaluate not only the durability of protection offered by a given vaccine, but also the efficacy of multivalent vaccines and heterologous prime-boost vaccination regimens. As variants of concern emerge, vaccine manufacturers will need to shift vaccine development toward novel and circulating virus stains.

Central to mitigating SARS-CoV-2 transmission is the continued testing of fully vaccinated individuals, which may be achieved by increased mobile testing sites, dedicated regional testing centers, and greater access to self-administered testing kits, such as those from Maple™, Bio-Rad™, or Abbott™. Particular attention should be paid to individuals who have traveled (domestic or international), experienced symptoms (mild or otherwise), or have come in contact with a known infected individual. The CDC also recommends that, in indoor areas with high-transmission potential, individuals wear masks regardless of vaccination status (63). Tracing databases and case logging applications should be modified to incorporate confirmed COVID-19 breakthrough cases. Additionally, increased serosurveillance and monitoring of major vaccine manufacturers and their respective rates of breakthrough cases should also be tracked, given the discrepancies reported by the CDC. At the level of the individual, precautionary measures such as isolation, personal hand hygiene, limited social gatherings, and masking policies should be followed according to local or federal guidelines. Breakthrough cases present a new global challenge for managing the spread of COVID-19; however, with adequate resources, appropriate scientific dissemination, and a dedicated multi-disciplinary workforce, COVID-19 breakthrough cases can be closely managed and tightly regulated.

The authors of this paper acknowledge that special efforts are needed to elucidate the degree to which those who have breakthrough infections are also able to effectively transmit the virus to other individuals. Further epidemiological studies specifically designed to address this issue should be focussed on specific groups such as the immunocompromised individuals, those suffering from chronic diseases, and the elderly, as well as to the influence of each given type/modality of vaccine. Results from such studies will help shape the design of future public health policies aimed at disseminating risk-related information pertinent to the COVID-19 pandemic.

AUTHOR CONTRIBUTIONS

BH: writing (original draft), investigation, editing, conceptualization, and data curation. MR, JB-M, AAK, and PN: editing, review, and conceptualization. CR: editing, review, conceptualization, and writing. SR, AT, AM-A-R: conceptualization and review. AK: figure creation/visualization. DK: conceptualization, editing, review, supervision, methodology, and investigation writing. All authors contributed to the article and approved the submitted version.

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Booster Vaccination Strategies for "Living With COVID-19"

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Although the primary and secondary vaccination rates in Korea account for over 75% of the total population, confirmed cases of COVID-19 are dramatically increasing due to immune waning and the Omicron variant. Therefore, it is urgent to evaluate the effectiveness of booster vaccination strategies for living with COVID-19. In this work, we have developed an age-specific mathematical model with eight age groups and included age-specific comorbidities to evaluate the effectiveness of age-specific vaccination prioritization strategies to minimize morbidity and mortality. Furthermore, we have investigated the impacts of age-specific vaccination strategies for different vaccine supplies and non-pharmaceutical intervention levels during two periods: (1) when vaccine supply was insufficient and (2) after the emergence of the omicron variant. During the first period, the best option was to vaccinate the 30–49 year age group and the group with comorbidities to minimize morbidity and mortality, respectively. However, a booster vaccination should prioritize the 30–49 year age group to promote both minimal morbidity and mortality. Critical factors, such as vaccination speed, vaccine efficacy, and non-pharmaceutical interventions (NPIs), should be considered for effective vaccination prioritization as well. Primary, secondary vaccinations, and a booster shot vaccinations require different age prioritization strategies under different vaccination rates, vaccine efficacies, and NPI levels.

Keywords: SARS-CoV-2, COVID-19, age-specific vaccination, booster shot strategies, comorbid-group priority vaccination, non-pharmaceutical intervention

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1. INTRODUCTION

SARS-CoV-2 infection has increased dramatically worldwide since the Omicron variant has become dominant. As of February 26, 2022, approximately 433 million cases and 6 million deaths had been reported worldwide (1). In Korea, the cumulative number of confirmed cases exceeded 2,800,000, and the death toll exceeded 7,000, causing a public health and economic crisis (2). Before the development of a coronavirus vaccine, most countries relied on non-pharmaceutical intervention strategies (NPIs), such as social distancing, isolation, contact tracing, and quarantine, as preventive measures against the spread of COVID-19.

Nonetheless, NPIs alone cannot end the epidemic, although they can slow the spread of the disease and prevent larger outbreaks to ensure that the rate of hospitalizations and deaths are manageable (3). However, lifting the NPIs could trigger a sharp rise in infection rate at any time without the majority of the population being immune to COVID-19, while the implementation of NPIs can cause economic damage and various adverse health effects (4, 5). Therefore, high vaccine

coverage and NPI adherence are essential to control the COVID-19 pandemic. There is no clear evidence of which vaccination strategies are most effective in reducing the number of deaths and infections and enabling the safe lifting of NPIs without rebounding the infection.

There are two distinct situations in which South Korea must choose a vaccination strategy. The first is the period when the quantity of vaccine is insufficient at the initial stage of supply, and the second is the period when booster doses are recommended owing to waning of vaccine-induced immunity and the surge of the Omicron variant. In these two periods, when supply is insufficient to form herd immunity and the vaccination rate is low owing to vaccine hesitancy (6, 7), evidence is needed as to which population should be vaccinated first to effectively reduce both morbidity and mortality. Vaccination prioritization strategies for COVID-19 in Korea has involved vaccination of workers in high-risk medical institutions and epidemiological investigators, followed by vaccination of the high-risk group and the rest of the population, which was then expanded to target adolescents over 12 years of age with relatively low serious risks (8).

However, unlike the vaccination strategy in Korea, the World Health Organization's recommendations include immunocompromised persons, adults with comorbidities, and pregnant women in higher-priority use groups (9). Therefore, there is an urgent need to evaluate the effectiveness of prioritization vaccination for populations with comorbidities. In November 2021, when the domestic vaccination completion rate reached 75% and the primary vaccination rate reached 80%, the NPIs began to decrease. However, owing to the earlier-than-expected decline in the effectiveness of vaccines in the elderly (immunity waning), many patient deaths occurred that the medical system could not afford.

In addition, the Omicron variant that was announced on November 25, 2021, is expected to spread worldwide and become the dominant species in Korea during February 2022 (10). Caution is needed as the Omicron-variant virus may evade vaccine-induced or natural immunity to COVID-19 (11). A booster shot appears to counteract the waning protection of delta variants and can maintain the vaccine effect against Omicron variants (12). However, the effectiveness of booster vaccination may vary depending on the primary vaccination status of the entire population and current epidemic status. In addition, the effect of booster vaccination on the epidemic situation is determined by the proportion of Omicron in the total infection rate and the extent to which Omicron evades immunity (13).

In Korea, Omicron is expected to dominate in February 2022, but the booster shot rates remain relatively low at approximately 50% owing to safety concerns. Hence, we developed an age-specific mathematical model with eight age groups and included age-specific comorbidities to evaluate the effectiveness of age-specific vaccination prioritization strategies. In this study, we focused on the population age structure and underlying diseases of Chungcheongbuk-do (CB) province. We estimated age-specific transmission rates using age-specific demographics and confirmed case data of COVID-19 in CB. Furthermore, we investigated the impacts of age-specific vaccination strategies for different vaccine supplies and NPI levels during two periods: the first period was when vaccination began with insufficient supply, and the second period was after the emergence of the Omicron variant.

2. METHODS

2.1. Epidemiological Data

As of February 26, 2022, South Korea had 2,831,283 confirmed COVID-19 cases and 7,895 deaths. Daily confirmed COVID-19 cases and deaths from April 1, 2020, to February 26, 2022, were obtained from the Korea Centers for Disease Control and Prevention (KCDC) and the CB provincial website (2, 14). The clinical and epidemiological characteristics of COVID-19 are heavily dependent on age; therefore, we incorporated age-specific features in our model, and age-specific cases were divided into eight groups, as shown in **Table 1**. The CB province comprises approximately 3% of the total Korean population, and COVID-19 cases in CB constituted approximately 2% of the total COVID-19 cases in South Korea. CB constitutes 7.4% of Korea; this implies that the population density per area is lower than the average in South Korea and, therefore, provides a rationale for the lower number of confirmed cases of COVID-19 in CB. However, the proportion of the elderly population (age $>$ 50 years) in CB was higher than the overall proportion of the elderly population in South Korea (42 vs. 40%, respectively), whereas the proportion of younger age groups (age $<$ 30 years) was lower. Furthermore, the age-specific comorbidities in the CB province are presented in the last row of **Table 1**. Note that the population with comorbidities had at least one human immunodeficiency virus infection, tuberculosis, cancer, cardiovascular disease, chronic respiratory disease, chronic liver disease, diabetes, and chronic neurological disease (**Table 1**). The severity and case fatality rates are high in patients with comorbidities (15, 16).

Between January 2020 and January 2022, there were five large waves of COVID-19 in South Korea. **Figure 1A** compares the levels of NPI (social distancing) implemented by the Korean government in metropolitan and non-metropolitan areas. Note that a high level of social distancing was implemented at the end of November 2020 in metropolitan areas, and COVID-19 vaccination began on February 26, 2021. **Figure 1B** presents the weekly age-specific data of confirmed COVID-19 cases across eight age groups from April 1, 2020, to February 6, 2022. In **Figure 1B**, the top and bottom panels show the weekly number of COVID-19 cases in South Korea and the CB Province, respectively. In both panels, the weekly number of COVID-19 cases showed a similar age-specific temporal pattern. Lastly, **Figure 1C** shows the first, second, and third vaccination doses per week for each age group in Korea.

2.2. Mathematical Model

We developed an age-structured mathematical model to investigate the impact of age-specific vaccinations on COVID-19 transmission dynamics. The age-specific classes were composed of the following eight groups: $0-9$, $10-19$, $20-29$, $30-39$, $40-49$, $50-59$, $60-69$, and $70-$. The population was separated into eight compartments based on the epidemiological characteristics

TABLE 1 | Age-specific population size and number of confirmed COVID-19 cases are compared for CB and South Korea.

		0–9	10–19	20–29	30–39	40–49	50–59	60–69	70 -
	Korea	3,874,174	4,746,103	6,754,283	6,788,072	8,220,344	8,606,589	6,957,802	5,735,658
Population	(51,683,025)	(7.50%)	(9.18%)	(13.07%)	(13.13%)	(15.91%)	(16.65%)	(13.46%)	(11.10%)
	CB	120,558	147,489	197,119	192,514	238,517	270,172	233,727	196,859
	(1,596,955)	(7.55%)	(9.24%)	(12.34%)	(12.06%)	(14.94%)	(16.92%)	(14.64%)	(12.33%)
	Korea	7,189	11,422	24,314	22,204	24,363	28,874	23,120	16,237
Confirmed	157,723	(4.56%)	(7.24%)	(15.42%)	(14.08%)	(15.45%)	(18.31%)	(14.66%)	(10.29%)
Cases	CB	114	199	464	482	498	650	475	341
	(3,222)	(3.54%)	(6.18%)	(14.4%)	(14.96%)	(15.46%)	(20.17%)	(14.74%)	(10.58%)
Population	CB	17,261	13,820	27,144	39,720	72,073	133,439	142,468	157,749
w/ comorbidities		(14.32%)	(9.37%)	(13.77%)	(20.63%)	(30.22%)	(40.39%)	(60.95%)	(80.13%)

The percentage indicates the ratio of the population (confirmed cases) of each age group to the total population (confirmed cases). The age-specific population with comorbidities in the CB Province. The percentage indicates the ratio of population with comorbidities in each age group.

of each age group i. $S_i(t)$ is susceptible, $E_i(t)$ is exposed, $A_i(t)$ is unconfirmed infectious, $I_i(t)$ is confirmed infectious, $H_i^m(t)$ is quarantined or hospitalized with mild symptoms, $H_i^s(t)$ is hospitalized with severe symptoms, $R_i(t)$ is recovered, and $D_i(t)$ is dead. Moreover, $V_i^F(t)$ is the first dose vaccinated, $V_i^S(t)$ is the second dose vaccinated, $V_i^B(t)$ is the third dose (or booster) vaccinated, $R_i^{V^F}(t)$ is recovered and first-dose vaccinated, $R_i^{V^S}(t)$ is recovered and second-dose vaccinated, and we have the epidemiological status for vaccinated classes $X_i^v(t)$ at the same status as $X_i(t)$ for $X = E, A, I, H^m, H^s, R$.

Furthermore, we divided the total population into groups under normal conditions and groups with comorbidities. $X_i^n(t)$ and $X_i^c(t)$ are populations with normal conditions and comorbidities of the same status as $X_i(t)$ for $X = S, E, A, I, H^m, H^s, V^F, V^S, V^B, E^v, A^v, I^v, H^{mv}, H^{sv}$, respectively.

A schematic diagram of this model is shown in **Figure 2**. The model is presented as a system of ordinary differential equations, which are provided in **Supplementary Section 1**. The parameters used and the baseline values are shown in **Supplementary Section 1**. We computed the effective reproduction number, R_t, which involves the fraction of susceptible population and potentially infectious vaccinated population. It measures the average number of secondary cases per infectious individual at time t, which is obtained by calculating the spectral radius of the next-generation matrix. The details of the derivation of R_t of the model are provided in **Supplementary Section 2**.

2.3. Age-Specific Vaccination Prioritization Strategies

In this subsection, we present four age-specific vaccination prioritization strategies. We have proposed four prioritization strategies, because there are critical age-specific characteristics, such as a higher activity level (aged 30–49 years) and a higher mortality rate (over 60 years and people with comorbidities). In addition, we considered a uniform vaccination strategy: all age groups to be vaccinated (over 20 years old) due to Korea's vaccination policy (only for people aged 19 years or older by October, 2021). These four strategies were applied to the primary dose and booster vaccination, except for the second dose vaccination. The second dose of vaccination was implemented after ψ days, the mean period between the first and the second dose of vaccination, as recommended by the Korean government.

- Strategy 1 $(30 - 49)$: priority vaccination for those aged between 30 and 49 years.
- Strategy 2 $(60+)$: priority vaccination for those above 60 years old.
- Strategy 3 (*Comorb.*) : priority vaccination for those with comorbidities.
- Strategy 4 $(20+)$: uniform vaccination for those above 20 years old.

It was assumed that when the vaccination rate of the priority group reached 80%, vaccination was switched to the uniform vaccination strategy. Vaccination was stopped when the vaccinated population reached 80% of the total Korean population. At the beginning of the vaccination, Korea's policies were limited to 19 years or older; however, in October 2021, vaccination was extended to individuals older than 12 years. Therefore, for the first and second dose vaccination, people above 20 years of age were vaccinated before October 2021. The current vaccination data in Korea, according to which about 60% of those aged between 12 and 18 years were vaccinated, is reflected for the population V_i^{Fn}, V_i^{Fc}, V_i^{Sn}, and V_i^{Sc}. Currently, in Korea, the booster vaccination is implemented for aged 20s or older; hence, the booster shot is applied to people above 20 years of age. Then, the results are compared to the case when people above 10 years of age are vaccinated.

2.4. Estimation of Age-Specific Transmission Probability

In this subsection, we estimate the age-specific transmission probability β_i for each age group i per contact by fitting the age-specific confirmed case data in the CB. The contact matrix involving home, school, workplace, and other factors for each age group in Korea (17) was used and adjusted using the population in CB (18). The contact matrices M^L, M^M, and M^H, for low, moderate, and high NPI levels, respectively, were constructed by the linear combination of

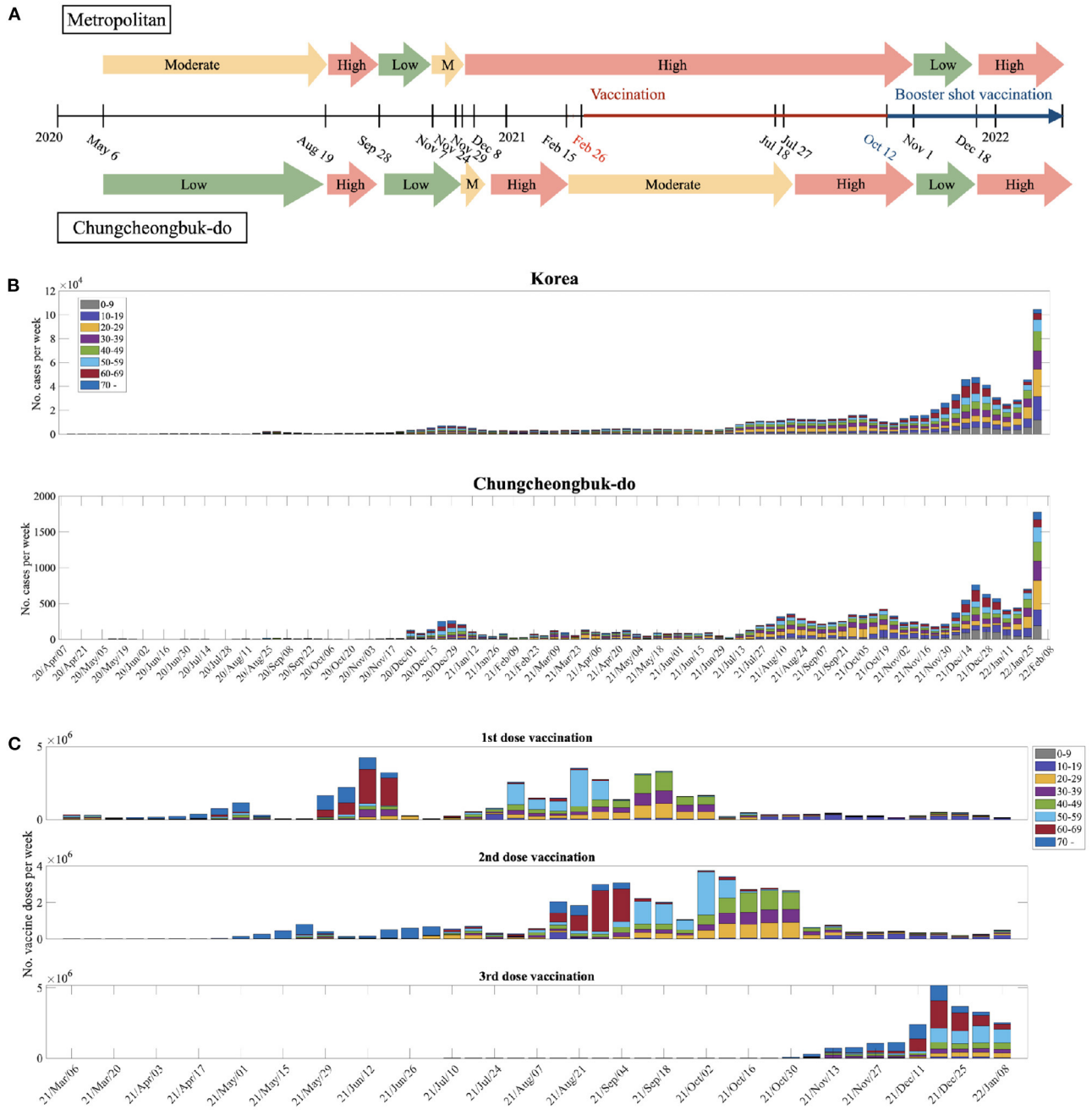

FIGURE 1 | (A) Levels of NPI implemented by the Korean government. **(B)** Weekly age-specific data of confirmed COVID-19 cases across eight age groups are shown from April 1, 2020, to February 6, 2022. The top panel shows the weekly data of COVID-19 cases in South Korea, whereas the bottom panel shows the weekly data of COVID-19 cases in the CB province. **(C)** The 1st, 2nd, and 3rd vaccination doses per week for each age group in Korea.

location-specific matrices of home, school, workplace, and others and by multiplying the weights given in **Table 2** based on the level of NPI implementation. The contact matrices are presented in **Supplementary Section 3**. The mobility of the vaccinated population was assumed to be higher than that of the unvaccinated population. Therefore, for the contact matrix of the vaccinated group, we used M^L for all NPI levels. The transmission probability β_i^v of those vaccinated but without antibody in age group i per contact was assumed to be the same as that for β_i.

Furthermore, we estimated the age-specific transmission probability under various NPI levels, as shown in **Figure 1A**. For the first and second dose vaccinations, $\{\beta_i\}_{i=1,\ldots,8}$ values were obtained under three different NPI levels: low, moderate, and high. For the booster shot, $\{\beta_i\}_i$ values were estimated based on the data from November 1 to December 5, 2021.

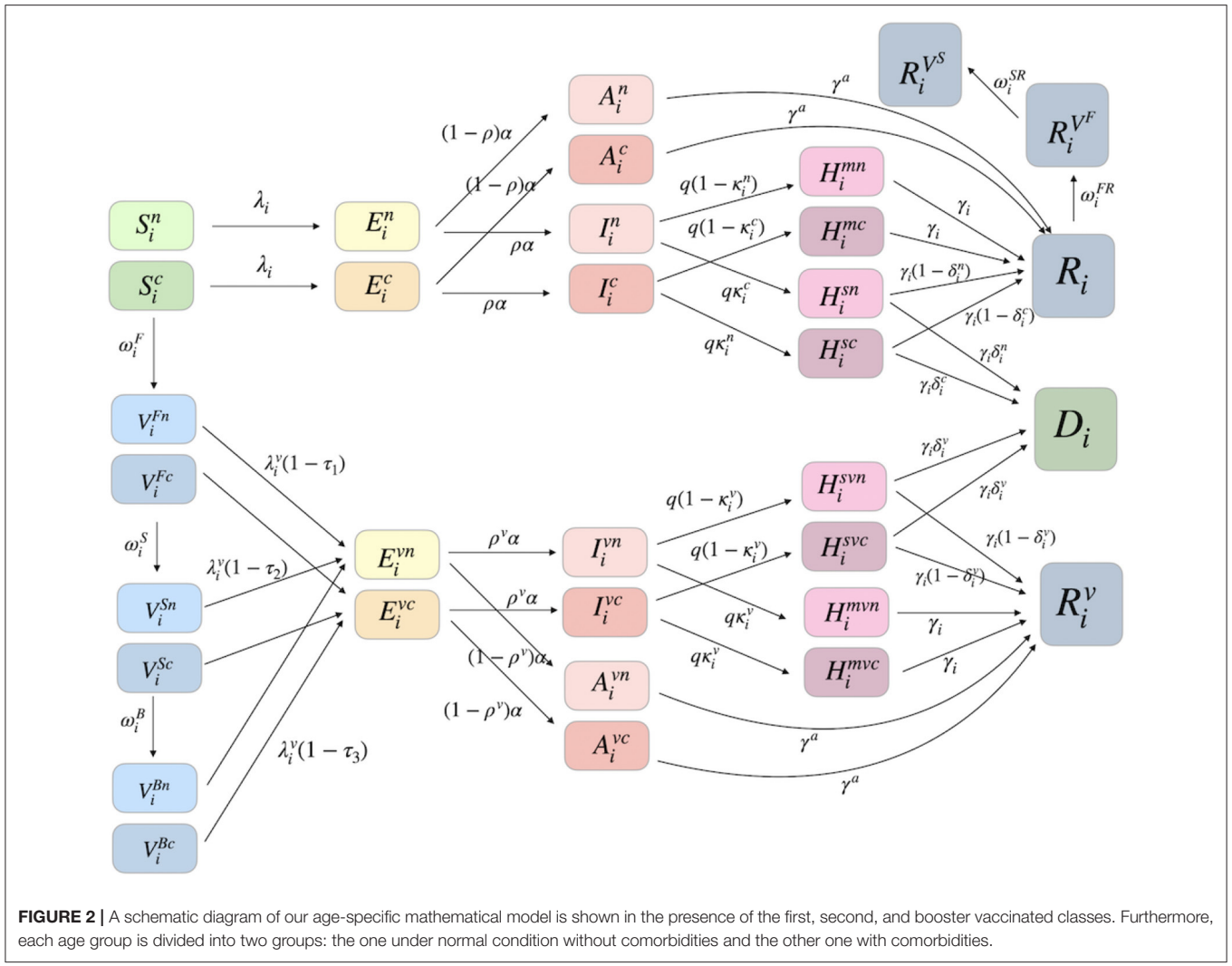

FIGURE 2 | A schematic diagram of our age-specific mathematical model is shown in the presence of the first, second, and booster vaccinated classes. Furthermore, each age group is divided into two groups: the one under normal condition without comorbidities and the other one with comorbidities.

TABLE 2 | Age-specific transmission probability is estimated under different time periods for corresponding NPI levels.

Vaccine dose	NPI level	Time interval	Contact matrix	Coefficient [home, school, work, others]	$\{\beta_i\}_{i=1,\ldots,8}$	R_t
First	Low	10/31/2020–11/19/2020	M^L	[1.1, 1, 1, 0.9]	0.1006, 0.0253, 0.0235, 0.0131, 0.0142, 0.0298, 0.1506, 0.0501	1.9220
Second	Moderate	2/25/2021–3/21/2021	M^M	[1.2, 0.5, 0.6, 0.8]	0.0242, 0.0159, 0.0380, 0.0228, 0.0118, 0.0159, 0.0747, 0.0859	1.2027
	High	1/5/2021–1/30/2021	M^H	[1.5, 0.2, 0.8, 0.6]	0.0338, 0.0135, 0.0183, 0.0202, 0.0147, 0.0218, 0.0470, 0.0375	1.0808
Third	Low	11/1/2021–12/1/2021	M^L	[1.1, 1, 1, 0.9]	0.0667, 0.0234, 0.1066, 0.0621, 0.0525, 0.0860, 0.6399, 0.5892,	1.2848

The mean of R_t for each period is shown.

This is due to the step-by-step recovery for the first major reorganization of the quarantine rules. The estimated $\{\beta_i\}_i$ values are presented in **Table 2**. Details of the age-specific estimation results are provided in **Supplementary Section 4**. We also carried out sensitivity analyses on parameters related to vaccination: β_i, β_i^v, ρ, ρ^v, τ_1, τ_2, ν_0, and $1/\psi$ (see **Supplementary Section 7**).

3. RESULTS

3.1. The Impacts of Age-Specific Vaccination Prioritization

In this subsection, we investigate the impacts of age-specific vaccination prioritization for the primary and second doses from March 11, 2021 [14 days, duration for antibodies to be detectible

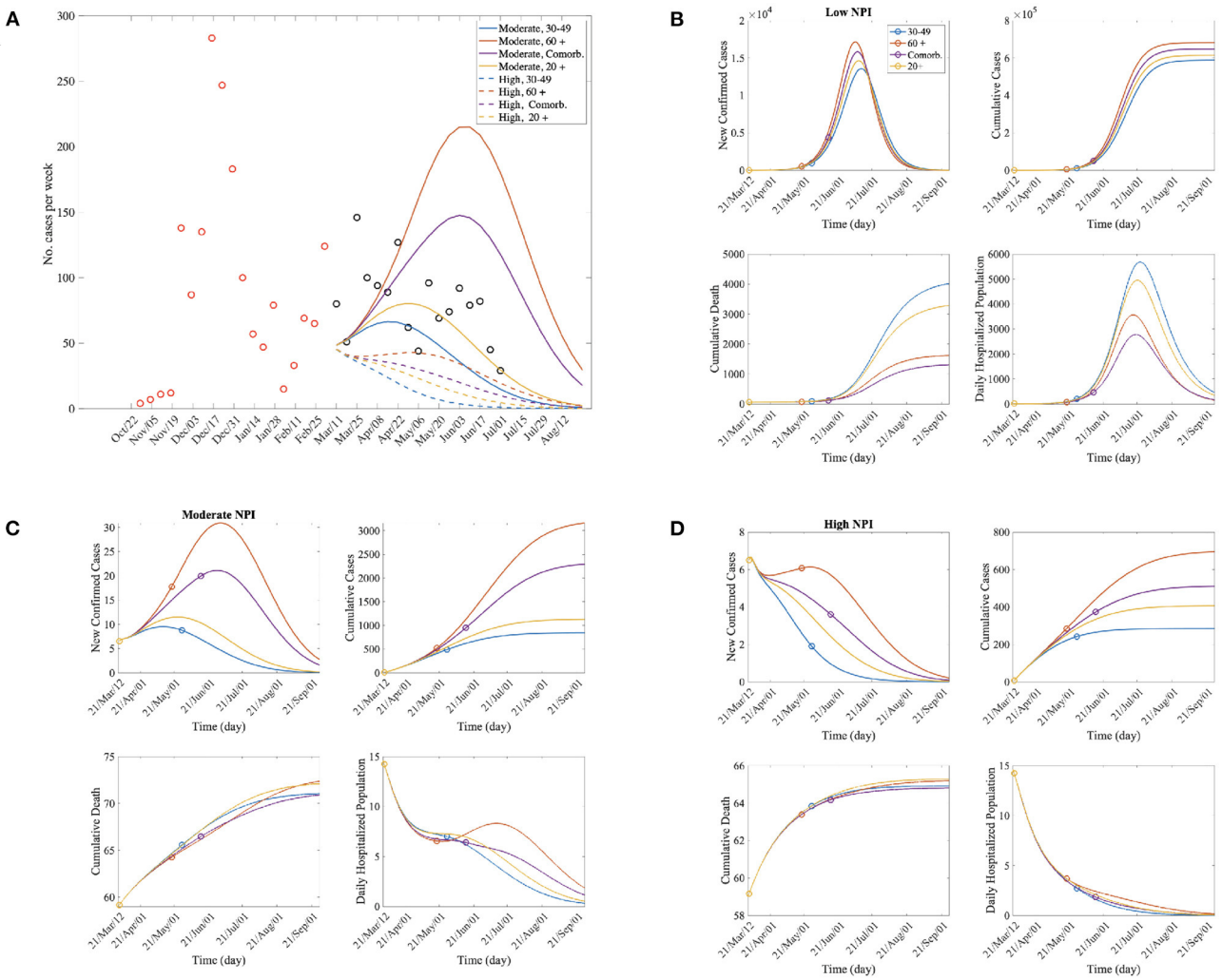

FIGURE 3 | (A) The red and black circle represent the number of confirmed cases per week before and after vaccination, respectively. The number of confirmed cases is similar to the results obtained for the moderate NPI-level simulation. **(B–D)** Time series of new confirmed cases, cumulative confirmed cases, cumulative death, and daily hospitalized population for each vaccination priority scenarios; For each curve, the circle represents the end point of priority vaccination for a specific group according to the vaccination scenarios.

(19), after the first vaccination began which is on February 26, 2021] to 6 months thereafter. The initial conditions were determined by reflecting the confirmed case data in CB, Korea at the start date of the simulation. **Figure 3** shows the impact of the four age-specific vaccination prioritization strategies on daily confirmed cases and deaths. In **Figure 3A**, weekly age-specific confirmed COVID-19 cases of CB (circled) are compared with the model outputs under moderate and high NPI levels. The confirmed case data was most similar to the simulation results with a moderate NPI among the low, moderate, and high NPI scenarios (see the solid curves in **Figure 3A**). Indeed, according to **Figure 1A**, the level of NPI implemented in the CB region during that period was moderate.

The panels in **Figures 3B–D** show the time series of confirmed cases; cumulative confirmed cases; cumulative deaths; and the hospitalized population with severe symptoms at the low, moderate, and high NPI implementation levels, respectively. The number of confirmed cases was lowest when the 30 − 49 years age group was vaccinated first for all the NPI levels; however, the cumulative death was lowest when the comorbidity group was vaccinated first for all the NPI levels. When the comorbidity group was vaccinated first, both of the number of deaths and the number of confirmed cases were lower than when those over 60 were vaccinated first. Therefore, it can be said that it is an effective policy to inoculate the comorbidity group first in order to reduce the number of deaths and the patients with severe symptoms.

Epidemic outputs under the four age-specific vaccination prioritizations are summarized in **Supplementary Tables 2–6**. The number of confirmed cases decreased the most under Strategy 1 (30 − 49 years old) combined with a moderate NPI level. This implies that age-specific vaccination strategies with appropriate NPI policies are needed to maximize the benefits. In addition, we calculated the average R_t for 60 days under the vaccination priority scenarios in **Supplementary Table 3**. R_t

TABLE 3 | The impacts of rollout speeds (daily doses of vaccine) on cumulative age-specific infected cases and deaths are shown under three different NPI levels.

	NPI level	No vaccine (# of cases)	Daily doses	30 – 49	60+	Comorb.	20+
					(# of cases)		
Cumulative cases	Low	1.1290×10^6	5,000	**834,970**	937,740	886,590	862,340
			7,500	**705,260**	812,590	768,470	733,410
			1,0000	**588,080**	681,520	648,010	613,990
			15,000	**399,660**	456,130	437,110	418,330
	Moderate	46,113	5,000	**2524.8**	17643	9337	3907.7
			7,500	**1287.9**	6953.1	4410.7	1847.2
			1,0000	**845.18**	3159.9	2289.9	1130.7
			15,000	**511.83**	1196.7	1012.4	631.51
	High	1876.7	5,000	**437.98**	1314.1	844.11	659.42
			7,500	**337.93**	934.99	639	496.66
			1,0000	**284.95**	694.3	510.53	406.17
			15,000	**228.32**	458.03	372.1	309.34
Death	Low	8655.6	5,000	6725.9	**3330.7**	3790.8	5709.8
			7,500	5432.7	2211.8	**2141.8**	4418.3
			1,0000	4019.6	1620.9	**1304.4**	3286.9
			15,000	1860.4	806.95	**596.75**	1556.4
	Moderate	320.28	5,000	**85.597**	106.88	96.668	92.241
			7,500	**75.142**	82.058	77.459	77.694
			1,0000	71.043	72.366	**70.9**	72.112
			15,000	67.811	66.863	**66.645**	67.996
	High	72.915	5,000	**65.93**	67.323	66.481	66.781
			7,500	**65.281**	65.985	65.385	65.835
			1,0000	64.922	65.195	**64.811**	65.288
			15,000	64.522	64.435	**64.236**	64.687

Bold texts indicate the largest reduction.

decreased the most under Strategy 1 ($30-49$ years old). This is consistent with the results that the reduction in the number of confirmed cases.

Finally, we present the effects of the daily vaccination doses. **Table 3** shows the number of cumulative confirmed cases and deaths under different NPI levels, daily dose of vaccination, and vaccination priority policies. **Supplementary Table 2** shows percentage reduction of the estimated confirmed cases and deaths for each case. It can bee seen that at the Mod NPI level, the number of confirmed cases and the number of deaths due to vaccination were significantly reduced than at the low and high NPI levels. Therefore, if appropriate level of NPI policies such as social distancing are implemented, vaccine effectiveness can be increased. In all cases, the number of confirmed cases was the lowest when the $30-49$ year olds were vaccinated first. When the daily dose is high ($\nu_0 = 10,000, 15,000$), that is, when the vaccine supply is sufficient, priority should be given to the comorbidity group to reduce the number of deaths. On the other hand, when a moderate or high NPI level with relatively small vaccination doses ($\nu_0 = 5,000, 7,500$), was implemented, the cumulative death was the lowest when the $30-49$ year olds were first vaccinated. Moreover, when ν is small, the change in the number of cumulative confirmed cases and deaths according to the vaccination strategy is large. Therefore, it is important to apply an effective vaccination strategy when the vaccine supply is limited.

3.2. The Impacts of Age-Specific Booster Vaccination Prioritization

In this subsection, we investigate the impact of age-specific booster vaccination prioritization during the second period. As reported in recent studies, vaccination efficacy is decreasing due to the reduction in neutralizing antibodies (20) or the prevalence of the Omicron variant (21). Hence, we investigated the impact of the reduction in vaccine efficacy of the second dose (τ_2), priority vaccination policy, and rollout speed for booster shots.

Figure 4A presents model outputs of daily confirmed cases (left) and cumulative death (right) according to the priority vaccination strategies with daily confirmed data where black and red circles represent the number of confirmed cases in CB before and after the Omicron is dominant in Korea (January 24th, 2021). The confirmed case increases rapidly after the Omicron variant becomes dominant. According to (20), the vaccination efficacy against infection is reduced to about 0.4 after 5 months of vaccination. Therefore, we assume that the vaccine efficiency is further reduced.

Figure 4A shows the effects of the priority vaccination policies ($30-49$, 60+, Comorb., 20+) under $\tau_2 = 0.4$, and daily third vaccine dose ($\nu^B = 10,000$). When those aged 30–49 years were vaccinated first, the number of confirmed cases and deaths decreased the most. The infection prevention rate decreases significantly while the death prevention rate does not decrease

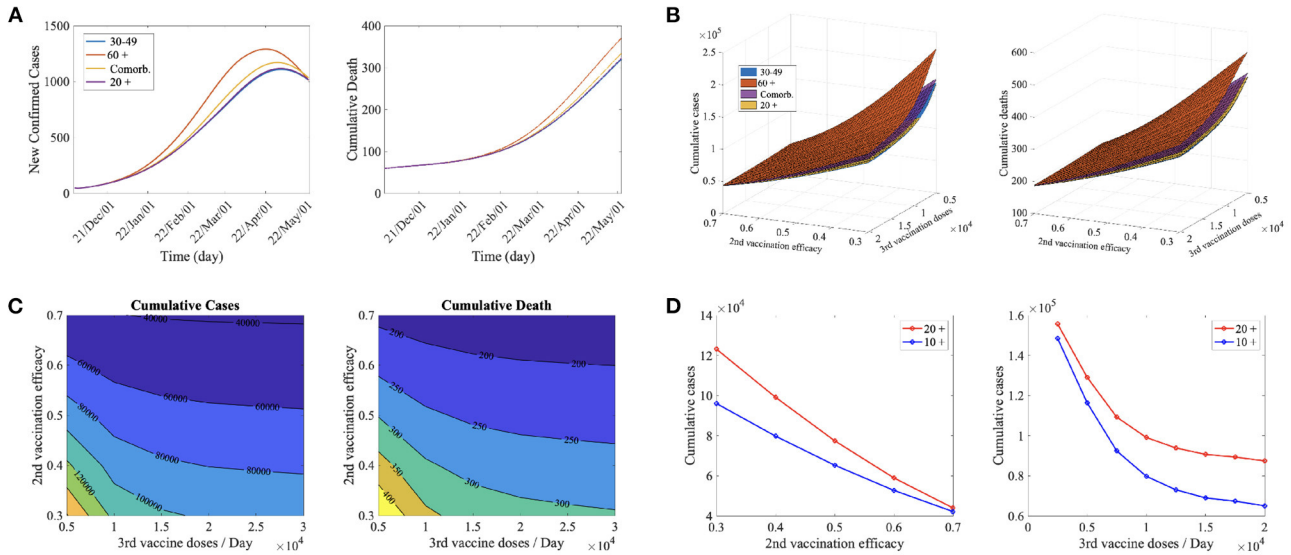

FIGURE 4 | (A) Time series of confirmed cased and cumulative deaths for the priority vaccination policies (30–49, 60+, Comorb., 20 +) for the second vaccine efficacy, $\tau_2 = 0.4$, and daily the third vaccine dose, $\nu^B = 10,000$. **(B)** Cumulative confirmed cases and cumulative deaths for the priority vaccination policies (30–49, 60+, Comorb., 20 +) for $\nu^B = 5,000, \ldots, 20,000$, and $\tau_2 = 0.3, \ldots, 0.7$. **(C)** Cumulative confirmed cases and cumulative deaths for the priority vaccination on those aged 30–49 years for $\nu^B = 5,000, \ldots, 20,000$, $\tau_2 = 0.3, \ldots, 0.7$. **(D)** Comparison of cumulative confirmed cases for vaccination on 20 years and older (20 +) and 10 years and older (10 +) for (left) $\tau_2 = 0.3, \ldots, 0.7$, $\nu^B = 10,000$ and (right) $\tau_2 = 0.4$, $\nu^B = 2,500, \ldots, 20,000$.

significantly (20), so prioritizing the high-activity group rather than the high-risk group seems to be effective in decreasing both confirmed cases and deaths.

Figures 4B,C shows the cumulative confirmed cases (left) and cumulative deaths (right) for 180 days in the variation of second vaccination efficacy (τ_2) and daily third vaccine doses (ν^B) for (**Figure 4C**) the priority vaccination strategies policies ($30-49$, 60+, Comorb., 20+) and (**Figure 4D**) for those aged $30-49$ years. In **Figure 4B**, priority vaccination on $30-49$ years groups and on 60+ years group are the most and the least effective strategies reducing cumulative cases and deaths in almost all cases. **Figure 4C** shows that as the second vaccination efficacy is smaller, the cumulative cases and deaths decrease more when the daily third vaccine doses increase. Therefore, it is necessary to accelerate the third vaccination as the efficacy of the second vaccination decreases.

In Korea, the first and second vaccinations are currently administered to teenagers, but booster shot vaccinations are not implemented in this subpopulation. We also studied the effects of booster shot inoculation on teenagers. **Figures 4C,D** show the difference in the number of confirmed cases when those aged 20 or older and those aged 10 or older were vaccinated according to the variation in second vaccination efficacy (**Figure 4C**) and the rollout speed of third vaccination (**Figure 4D**). For all cases, the cumulative confirmed cases were smaller when 10 years of age and over were vaccinated. It was shown that when the second vaccination efficacy was lower (**Figure 4C**) and the daily third vaccine dose was greater (**Figure 4D**), vaccination of teenagers reduced the number of confirmed cases. Currently, Korea's secondary vaccine efficiency is decreasing owing to the prevalence of Omicron, and the vaccine supply is sufficient. Therefore, it is recommended for teenagers to be vaccinated with a booster shot.

3.3. The Impacts of Different NPI Levels

In this subsection, we illustrate the impact of the mitigation of NPI levels combined with age-specific vaccination for the primary and second doses (before booster shots). As the vaccination rate increased rapidly, the government planned to relax the NPI policy, and recently, the number of confirmed COVID-19 cases has increased significantly. Hence, we performed simulations of NPI relaxation from moderate to low NPI levels. We compared the effectiveness of the NPI-level mitigation policy for a population of individuals older than 10 and 20 years.

Figure 5A shows the time series of the number of new confirmed cases when the NPI level is mitigated based on the given secondary vaccination rates when the vaccination is implemented on those aged 20 years and older (top panels) and on those aged 10 years and older (bottom panels). The increase in the cumulative confirmed cases under the mitigation of NPI level when the vaccination is implemented on individuals aged 20 years and older (in red curves) and 10 years and older (in blue curves) are compared in **Figure 5B**.

It has been shown that too early NPI-level mitigation with low vaccination coverage could bring about another COVID-19 outbreak wave. **Figure 5** shows that the increase in the confirmed cases was lower when those aged 10 years and older were vaccinated than when those aged 20 years and older were vaccinated, and the difference in the confirmed cases was greater

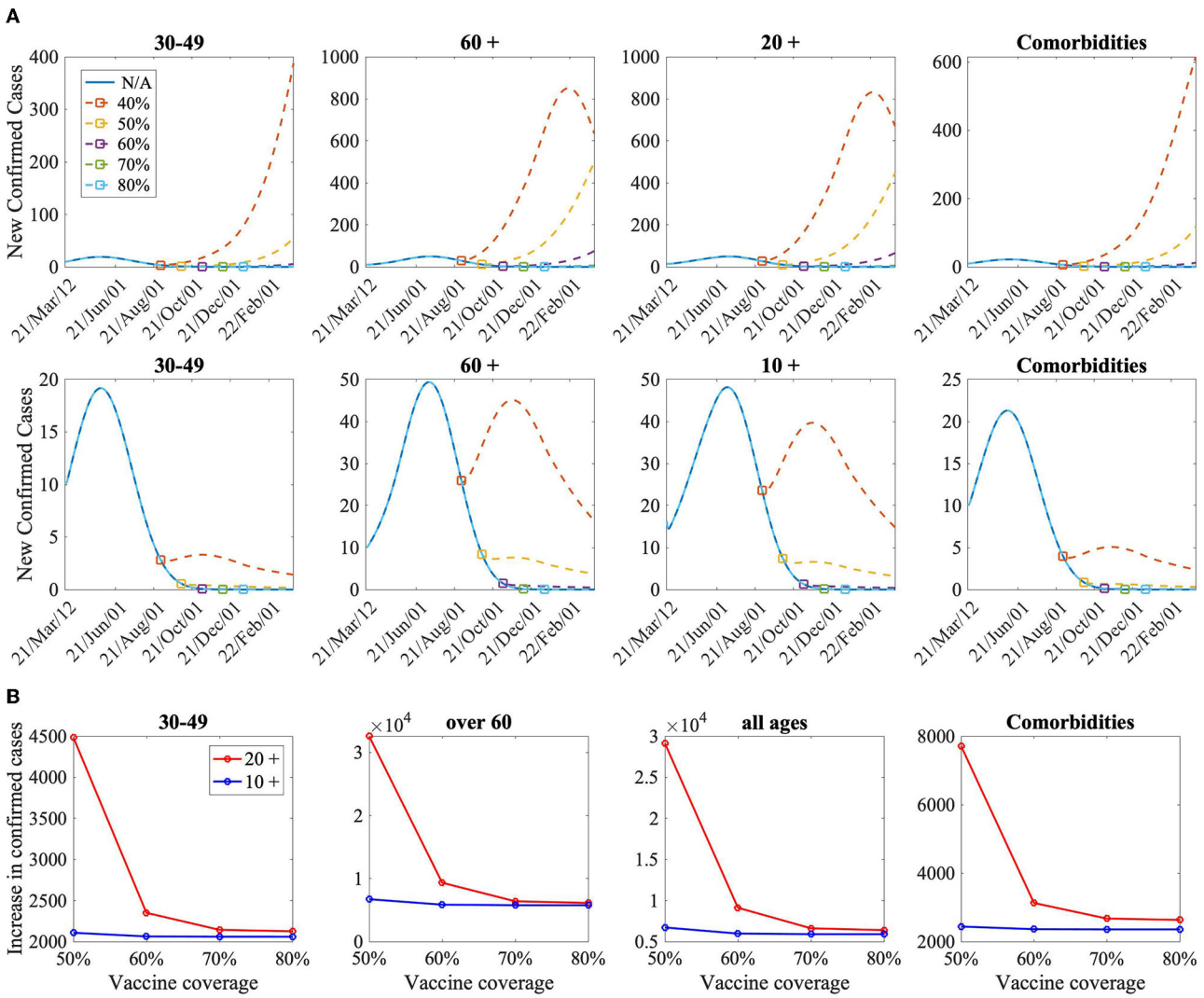

FIGURE 5 | (A) The time series of the number of new confirmed cases according to the reduction of the NPI level for the secondary vaccination rates (50–80%). N/A indicates no mitigation of NPI level for the simulation duration. The top and bottom panels represent the case of vaccination-prioritization strategies for individuals aged 20 years and more and aged 10 years and more, respectively. **(B)** Comparison of increase of cumulative confirmed cases when vaccination is implemented for aged 20 and older (20 +), and for aged 10 and older (10+).

when the vaccination coverage was low. Therefore, to mitigate NPIs effectively, it is necessary to vaccinate various groups of the population, not only to consider the vaccination rate.

4. DISCUSSION

In this study, we developed an age-specific mathematical model to investigate the impact of vaccination allocation in combination with NPIs on COVID-19 transmission dynamics in two periods: when vaccination begins and when booster shots are needed owing to waning of vaccine-induced immunity. Our results indicate that at the initial stage of vaccination, a priority vaccination strategy for those with comorbidities was most effective for reducing mortality, regardless of the NPI level. However, a priority vaccination strategy for individuals aged 30 − 49 years was most effective in reducing morbidity.

Previous studies have revealed that COVID-19 patients with pre-existing comorbidities, such as hypertension, diabetes mellitus, chronic respiratory disease, malignancies, and HIV, could develop a life-threatening situation (22). Our data showed that a vaccine plan prioritizing a population with comorbidities is an effective alternative because, it can lower both mortality and morbidity compared with that for those aged 60 years and older. A modeling study evaluating the performance of the Centers for Disease Control in the United States showed that a higher prioritization of individuals with comorbidities led to better outcomes compared to the current vaccine allocation strategies (23). In Korea, to prevent the collapse of the medical system and minimize the number of deaths, the first vaccination

was planned for medical personnel, workers, and the elderly in nursing homes (8). In our study, it was suggested that if the inoculation of the high-activity group is delayed, the increase in the number of infections may place a burden on the medical system. However, the vaccine's transmission block effect is not as effective as its mortality reduction effect (24). Therefore, it is necessary to maintain a policy to reduce mortality by prioritizing the vaccination of those with comorbidities and the elderly, rather than younger age groups.

In our study, when NPI was relieved early in all vaccination scenarios, the number of infections increased, resulting in a shortage of medical resources. These results are consistent with studies suggesting that maintenance of NPIs is necessary to increase the effectiveness of vaccines and reduce the number of infected people (25, 26). By November 1, 2021, 75% of the population in Korea had been vaccinated. Such high vaccine coverage was expected to reduce mortality and severity of infection, and the government decided to ease the NPI. However, this period coincided with the waning of vaccine-induced immunity period of the vaccine, and the infection spread rapidly among the elderly in nursing homes and communities. As a result, the number of critically ill patients rapidly increased, and NPI relief was canceled 46 days after starting owing to an insufficient medical system for critically ill patients. According to a study in the UK, the timing of NPI mitigation should be decided according to the vaccine coverage. In addition, when a mutant virus appears, NPI mitigation should be performed to evaluate the effectiveness of the vaccine and vaccination rate (27).

Comorbidities are known to be independent risk factors for severe progression of COVID-19 infection (28). Although comorbidities associated with severe progression are predominantly evident in the elderly, the presence of comorbidities might worsen the prognosis of young and middle-aged COVID 19 patients (29). In a study evaluating the vaccination strategy of the US CDC, higher prioritization of individuals with comorbidities in all age groups improved outcomes compared to the CDC allocation (23). It is consistent with the results of this study that vaccination of people with comorbidities at any age can be an effective alternative to prevent infection and severe progression.

The importance of booster shot vaccination is increasing, because vaccine efficiency is reduced due to the waning of secondary vaccines coupled with the emergence of variants of concern, such as Omicron. Our results show that booster shots are effective when applied preferentially to high-activity groups rather than to high-risk groups. This is supported by a retrospective cohort study showing consistently high effectiveness of BNT162b2 against COVID-19-related hospital admissions and severe death (20, 30, 31). However, a recent report suggested that booster shots are effective in reducing severe COVID-19 related outcomes (32). Thus, long-term vaccine effectiveness data against severe COVID-19-related outcomes must be continuously monitored in Korea. It can be seen that the rollout speed of the booster shots becomes more important as the secondary vaccine efficiency decreases. Previous studies on various vaccine rollout scenarios also emphasize the importance of rapid vaccine rollout to vulnerable populations and increasing coverage to avoid future surges (33, 34). In countries with limited infection-induced immunity, such as Korea, a rapid vaccine rollout strategy is needed to overcome the reduced vaccine efficacy against Omicron combined with its high transmissibility (13). It is also necessary to closely check whether the effectiveness of the vaccine is maintained in the population while mitigating NPIs.

In May 2021, the Pfizer-BioNTech COVID-19 vaccine was approved for expanded vaccination of individuals older than 12 years in the United States (35). On July 16, 2021, in Korea, the vaccine was approved for use in individuals older than 12 years of age as well. Based on the results of evidence-based research demonstrating the efficacy and safety of vaccination among adolescents, several countries are planning to vaccinate adolescents. However, as most adolescents are known to experience mild symptoms from COVID-19 infection and have few sequelae, questions remain as to whether the vaccine should be administered to children or should be provided to areas where the majority of at-risk groups have not been vaccinated. Nonetheless, as the rate of COVID-19 infection among adolescents is increasing owing to the Omicron variant, an increased vaccination rate among the elderly, in combination with the mitigation of the NPI level, is necessary to plan an effective vaccination strategy for adolescents (36). In our study, model-based analysis predicted that a vaccination strategy for teenagers would decrease the number of COVID-19 patients. Therefore, studies on the efficacy and safety of vaccines in domestic adolescents are needed.

This study has some limitations. First, for a decrease in vaccine efficacy due to waning of vaccine-induced immunity, sequential decrease in vaccine efficacy by age based on the priority vaccination strategy was not considered. In other words, the same vaccine efficacy was applied to all age groups. Second, the transmission rate parameter β was estimated using data from the period before the prevalence of Omicron variants, so there may be a difference from the current transmission rate. Also, different initial conditions and simulation duration might affect our results. Lastly, waning of vaccine-induced immunity is not explicitly modeled in study, therefore, this assumption may affect our results.

In conclusion, at the initial stage of vaccination, when the amount of vaccine is insufficient, the policy of vaccinating those with comorbidities or the elderly who are at a high risk of death will help reduce the number of deaths. Second, because sudden alleviation of NPI can cause a surge in infected patients, the level of NPI should be determined while closely evaluating whether vaccine coverage and effectiveness of the vaccine are maintained. Third, when vaccine immunity wanes, faster vaccination rollouts may reduce mortality rates. As Korea has limited infection-induced immunity across populations, it is necessary to closely monitor infection-induced and vaccine-induced SARS-CoV-2 immunity while deciding to open up and "live with COVID-19".

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: the daily case data are publicly available from Ministry of Health and Welfare, South Korea (http://ncov.mohw.go.kr/).

AUTHOR CONTRIBUTIONS

Conceptualization and validation: H-SK and SL. Data curation: H-SK. Formal analysis, visualization, and Methodology: JK. Funding acquisition and writing—original draft: JK and H-SK. Writing—review and editing: JK, H-SK, and SL. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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BRIEF RESEARCH REPORT
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SARS-CoV-2 Genomic Surveillance Enables the Identification of Delta/Omicron Co-Infections in Argentina

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Molecular surveillance of SARS-CoV-2 is crucial for the early detection of new variants and lineages. In addition, detection of co-infections with more than one SARS-CoV-2 lineage has been sporadically reported. In this work, surveillance of SARS-CoV-2 variants was performed on 2,067 RNA samples (Ct > 30) obtained during December 2021 and January 2022 from Córdoba province, Argentina, by real-time RT-PCR specific for variants of concern (VOCs) and variants of interest (VOIs) relevant mutations (TaqMan™ SARS-CoV-2 Mutation Panel, Applied Biosystems). The following distribution of variants was obtained: Omicron (54.9%), Delta (44.2%), and Lambda (0.8%). Three samples (0.1%), from the last week of December, were compatible with a Delta/Omicron co-infection. One of them was sequenced by NGS-Illumina, obtaining reads for both VOCs. One of the co-infected patients presented with severe symptoms, was not vaccinated, and had risk factors (older than 60 years and arterial hypertension). We describe for the first time in Argentina the identification of cases of co-infection with two SARS-CoV-2 lineages, VOCs Delta and Omicron, during the third COVID-19 wave in the country (a high viral circulation period), when Delta and Omicron co-circulated. Our findings highlight the importance of continuing molecular surveillance, in order to elucidate possible recombination events and the emergence of new variants.

Keywords: SARS-CoV-2, co-infection, Delta, Omicron, Argentina, molecular surveillance

INTRODUCTION

During the 2 years of the COVID-19 pandemic, the original SARS-CoV-2 that was identified at the end of 2019 has evolved into various lineages (1), presenting characteristic mutations. Among them, variants that posed an increased risk to global public health have been identified as variants of interest (VOIs) and variants of concern (VOCs), which present a defined pattern of mutations (2).

Five VOCs—Alpha (lineage B.1.1.7), Beta (lineage B.1.351), Gamma (lineage P.1), Delta (lineage B.1.617.2), and Omicron (lineage B.1.1.529)—and two VOIs—Lambda (C.37) and Mu (B.1.621)—have been reported to date (2).

Whole-genome sequencing (WGS) has been widely used since the beginning of the pandemic to monitor virus variants, to obtain a better understanding of the virus biology and epidemiology (3). However, it is a time-consuming and expensive technique that requires trained staff and specific equipment, restricting its access in resource-limited settings (4). As an alternative, reverse transcription real-time polymerase chain reaction (real-time RT-PCR) assays for the detection of relevant mutations associated with SARS-CoV-2 variants have been developed, to typify circulating variants, as a more accessible tool for the monitoring of VOCs (4, 5).

Molecular SARS-CoV-2 surveillance has allowed the identification of the simultaneous infection (co-infection) of a single individual by two distinct SARS-CoV-2 lineages, an event that has been sporadically reported (3, 6–8). These cases constitute an opportunity for viral genetic recombination and the emergence of new lineages with different phenotypes (6), which may cause more severe clinical symptoms (7). The frequency of co-infected patients and their role in promoting recombination-driven SARS-CoV-2 evolution is still unknown and poorly understood (6).

In Argentina, the profile of circulating lineages and variants has been changing throughout the pandemic, as has happened in the rest of the world (9). Molecular surveillance in the country started with WGS carried out by the Ministries of Science and Technology, and Health, at the national level (10, 11), but then, given the increase in the number of COVID-19 cases, the appearance of VOCs/VOIs, and the need for rapid results that enable public health decision-making, some provinces implemented different strategies based on real-time RT-PCRs for the detection of VOC/VOI relevant mutations, as additional techniques to WGS. This was the case of the province of Córdoba, in the central region of the country, where a strategy that combined detection of point mutations was developed (12). This strategy was used for the molecular surveillance of SARS-CoV-2 variants during 2021, enabling the typing of a greater number of samples with less processing time (12, 13). Moreover, this strategy allowed the detection of the Omicron variant for the first time in Cordoba on December 2021, when the third wave of COVID-19 started in the country, displacing the VOC Delta, the major variant circulating at that time (13).

In this report, we describe for the first time in Argentina cases of co-infection with the Delta and Omicron variants of SARS-CoV-2 that were detected by molecular surveillance in December 2021 during the third COVID-19 wave in the country.

METHODS

Samples Obtained During SARS-CoV-2 Genomic Surveillance

A total of 2,067 SARS-CoV-2 RNA-positive samples obtained from oropharyngeal swabs collected during December 2021 and January 2022 in the province of Córdoba (central area of Argentina) were analyzed for VOC/VOI detection as part of the molecular surveillance program of the local government of the province. The samples had originally been extracted with the MegaBio plus Virus RNA Purification Kit II (BioFlux) on the GenePure Pro Nucleic Acid Purification System NPA-32P and amplified by real-time RT-PCR using the DisCoVery SARS-CoV-2 Nucleic Acid Detection Kit.

Detection of VOC/VOIs by Real-Time RT-PCR

Detection of the relevant mutations L452R, P681R, P681H, K417N, and L452Q (within the spike protein) was carried out by real-time RT-PCR, using the TaqMan™ SARS-CoV-2 Mutation Panel (Applied Biosystems), following the strategy described by Castro et al. (12). Each reaction was performed as a multiplex, including probes simultaneously detecting the wild type (wt) and the mutant nucleotide sequences. Briefly, 7 µl of RNA was added to 8 µl of a mixture containing TaqPath™ 1-Step RT-qPCR Master Mix, CG (4×), TaqMan™ SARS-CoV-2 Mutation Panel Assay (40×), and nuclease-free water.

Whole-Genome Sequencing

Samples that were compatible with a co-infection profile in the real-time RT-PCRs for VOC/VOI screening were subjected to WGS by the Illumina platform, using the Illumina COVIDSeq RUO kit, version COVIDSeq Test Kit. Manual inspection of variant-specific mutation sites was accessed using the program Tablet (14). The sequenced sample was submitted to the GISAID database under the accession number EPI_ISL_8938300.

RESULTS

From the 2,067 samples analyzed using the mutation-specific real-time PCR strategy for detection of VOCs/VOIs, 913 (44.2%) belonged to VOC Delta, 1,135 (54.9%) belonged to VOC Omicron, 16 (0.8%) belonged to VOI Lambda, and 3 (0.1%) presented profiles compatible with co-infections. The distribution of variants abruptly changed during this 2-month study, with Delta comprising the majority of detections in early December, Omicron comprising the majority of detections in mid-December 2021 to mid-January 2022, and Omicron comprising all detections by late January 2022 (**Figure 1**).

The main features of the 3 co-infected patients are shown in **Table 1**. **Figure 2A** shows the mutation profile detected by real-time RT-PCRs on samples obtained from these patients. Wild-type and mutant RNA was simultaneously detected for L452R and K417N. Only mutant RNA was detected for P681R and P681H, without amplification of wild-type RNA, indicating the presence of a mutation in that position (**Figure 2A**, **Supplementary Table 1**). We ruled out cross-contamination by repeating nucleic acid extraction and VOC/VOI-specific qRT-PCR on samples from all 3 co-infected patients and arriving at the same results.

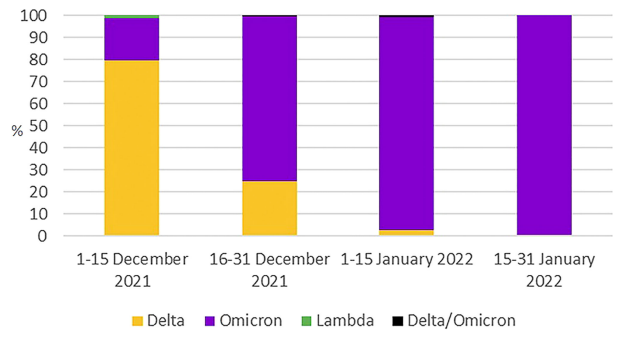

FIGURE 1 | Distribution of circulating VOCs/VOIs during December 2021 and January 2022 in the province of Cordoba, Argentina.

Sample no. 1 contained a sufficient viral load and RNA quantity to perform WGS for further investigation. The sequence obtained from this sample was 29,867 nucleotides in length. Compared to the WIV04 reference sequence (EPI_ISL_402124), it included 0.87% unidentified nucleotides (Ns) and 0.34% nucleotide mutations. The average percentage of reads matching the Omicron variant was higher than the average percentage of reads matching the Delta variant (**Figure 2B**). Positions in which the average percentage of reads matching the Delta variant was higher had lower coverage. Pangolin COVID-19 Lineage Assigner (Pangolin v3.1.19) could not assign a lineage to this sequence.

DISCUSSION

Co-infection with distinct SARS-CoV-2 lineages is considered a rare phenomenon. However, its likelihood increases as infection prevalence increases and is thought to be underestimated (6).

In this work, we report the co-infection with Delta and Omicron VOCs. This is the first description of co-infected individuals carrying two distinct lineages of SARS-CoV-2 in Argentina. VOC Delta was first described in our province in July 2021, when it was detected in a traveler and his close contacts. Due to the efforts carried out by the health authorities of the province, which included tracking and isolating Delta-positive cases and their close contacts, the spread of this VOC was delayed, so its increase was gradual, until reaching its highest proportion of circulation (85%) in November 2021 (12), but without a substantial increase in the number of cases (13).

VOC Omicron was detected in Argentina—and particularly in Córdoba province—during the first few days of December 2021, in a traveler from Dubai, and it quickly spread throughout the province (13). The sharp increase in Omicron frequency was accompanied by an increase in the number of cases, giving rise to the third wave of COVID-19 in the province and the entire country (11, 13).

In this context of co-circulation of variants, 3 samples with Delta/Omicron SARS-CoV-2 co-infection were identified, all of them detected the last 2 weeks of December, when co-circulation of Delta and Omicron was registered (13). Co-infection events between dominant SARS-CoV-2 lineages have been previously reported, also in a very low proportion of the tested samples (3, 6–8). Although these events are rare, they are believed to be quite common during periods of high viral prevalence (15) and are believed to be underreported (6), as they are not easy to identify. Generally, one of the lineages is present in a greater proportion (3), which sometimes causes only one lineage to be detected, which has been registered for Delta/Omicron mixed infection (8). In addition, specialized personnel are required for the interpretation of variant-specific real-time PCR, which is not always capable of detecting subtleties in the reaction results. In our study, co-infections were detected during the third wave of COVID-19 that took place in our country, with very high levels of viral circulation, in accordance with previous reports (6, 15).

The report of co-infections of SARS-CoV-2, both locally and globally, becomes relevant in a context of changing circulation of variants and emergence of new ones. In this sense, recombination, already reported for other coronaviruses and also recently for SARS-CoV-2, is a possibility in individuals simultaneously infected with more than one lineage (3, 15). In turn, the emergence of newly recombined viruses might result in increased transmissibility or immune evasion (3), as recombination permits the combination of advantageous mutations from distinct variants (15). Since recombination is only possible with co-infection, decreasing the prevalence and circulation of SARS-CoV-2 will minimize the chance of forming recombinant lineages with genetic combinations that could potentially increase virus fitness (15).

Until now, no major clinical implications have been described in patients with co-infection with more than one SARS-CoV-2 lineage (3). In this study, only one of the patients presented severe symptoms (pneumonia and dyspnea), although they were probably due to the lack of vaccination and to the presence of risk factors (over 60 years of age and arterial hypertension) rather than the co-infection. However, more clinical research is needed and should be carried out on these patients.

TABLE 1 | Main characteristics of the patients with SARS-CoV-2 Delta/Omicron co-infections.

Sample ID	Date of swab collection/onset of symptoms	Sex	Age (years)	Vaccine	Co-morbidities	Clinical manifestations	Hospitalization
1	December 27, 2021	M	18	Yes[a]	No	Fever, throat pain	No
2	January 7, 2022	F	5	Yes[b]	No	Fever, dyspnea	Yes
3	December 29, 2021	M	64	No	Arterial hypertension	Asthenia, dyspnea, pneumonia	Yes

[a]*One dose of Recombinant Novel Coronavirus Vaccine (Adenovirus Type 5 Vector).*
[b]*One dose of SARS-CoV-2 inactivated (Vero cells) vaccine.*

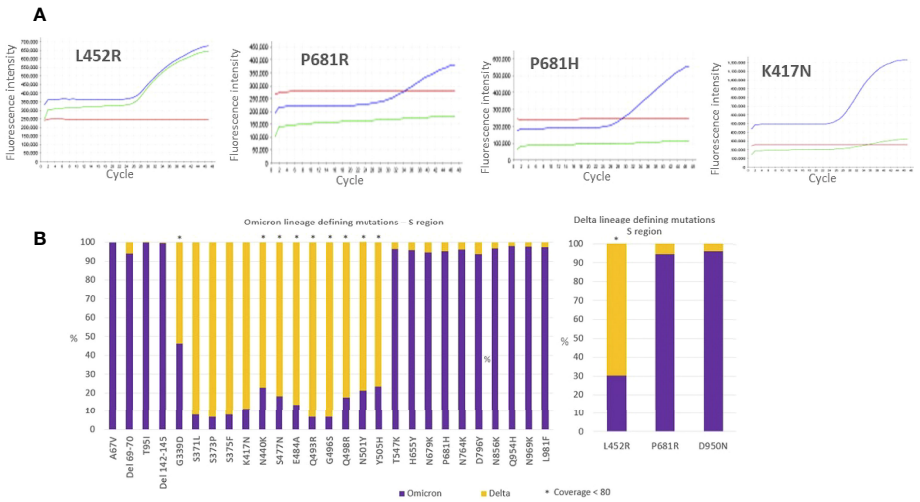

FIGURE 2 | **(A)** Real-time RT-PCR for VOC typing. Blue curve: presence of mutation; green curve: absence of mutation (presence of wild type); red curve: negative control. **(B)** Proportion of sequencing reads obtained for the S region by the whole-genome sequencing technique matching with VOCs Omicron or Delta for the analyzed mutations that define the lineage. *Positions in which the number of reads were less than 80.

In conclusion, we found, for the first time in Argentina, co-infections by two SARS-CoV-2 lineages (Delta/Omicron) during the third wave of COVID-19, the largest in our country (11). This highlights the importance of continuing molecular surveillance, especially in moments of high viral circulation, to detect both co-infections and recombinations. It is important to continue studying co-infection cases to determine if co-infection is associated with more severe disease and/or outcomes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.gisaid.org/, EPI_ISL_8938300.

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MP performed the conceptualization, analyzed the data, and wrote the original draft. PS carried out molecular detections and analyzed the results. MZ, AL, FF, and GC carried out molecular detections. SG, MV, and LL contributed to the analysis of the data. MB supervised the work and revised and edited the manuscript. VR performed conceptualization and supervision, and revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fviro.2022.910839/full#supplementary-material

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Changes in Symptoms Experienced by SARS-CoV-2-Infected Individuals – From the First Wave to the Omicron Variant

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The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a global pandemic and public health crisis since the beginning of 2020. First recognized for the induction of severe disease, the virus also causes asymptomatic infections or infections with mild symptoms that can resemble common colds. To provide better understanding of these mild SARS-CoV-2 infections and to monitor the development of symptoms over time, we performed a detailed analysis of self-reported symptoms of SARS-CoV-2 positive and SARS-CoV-2 negative individuals. In an online-based survey, a total of 2117 individuals provided information on symptoms associated with an acute respiratory infection, 1925 of the participants had tested positive for SARS-CoV-2 infection, and 192 had tested negative. The symptoms reported most frequently during the early phases of the pandemic by SARS-CoV-2 infected individuals were tiredness, headache, impairment of smell or taste and dry cough. With the spread of the alpha and delta variants, the frequency of nose symptoms such as blocked or runny nose and sneezing increased to being reported by almost 60% of infected individuals. Interestingly, the spread of the omicron variant brought a sharp decrease in the incidence of impaired sense of smell or taste, which was reported by only 24% in this phase of the pandemic. The constellation of symptoms should be monitored closely in the months ahead, since future SARS-CoV-2 variants are likely to bring about more changes.

Keywords: SARS-CoV-2, omicron, COVID-19, variants, coronavirus

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INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus associated with the coronavirus infectious disease 19 (COVID-19), was first detected in Wuhan, China, at the end of 2019 (1), has quickly spread globally and has caused a pandemic and public health crisis since the beginning of 2020. The first infections in Europe occurred in Italy and Germany in late January 2020 (2). By March 2020, numbers of SARS-CoV-2 infection rose rapidly in many countries, and have done so again in multiple waves; as of May 18th 2022, more than 519 million people have been infected by SARS-CoV-2, and more than 6.2 million SARS-CoV-2 related deaths have been reported (3).

From the first cases in China, the typical symptoms of SARS-CoV-2 infection were described as dry cough, fever, and pneumonia (4, 5). These first reports of symptoms associated with SARS-CoV-2 infection/COVID-19 were based on symptoms found in hospitalized patients with severe disease. Notably, in the first reports on COVID-19, disease course was considered mild if patients did not require mechanical ventilation. It soon became obvious though that many SARS-CoV-2 infected individuals experienced asymptomatic or non-severe disease course that resembled common colds and did not require hospitalization (6). Interestingly, it was found that many SARS-CoV-2 infected patients experienced altered or completely lost sense of smell and/or taste, which has since been regarded as a key indicator of SARS-CoV-2 infection as summarized in multiple meta-analyses (7).

The high frequency of lost sense of smell and taste observed in many studies has been convincingly shown to be associated with a virus variant carrying the mutation D614G in the SARS-CoV-2 spike protein that replaced the original Wuhan Hu-1 strain as the prevalent pandemic SARS-CoV-2 variant in early 2020 (8). The variant was shown to be more infectious due to improved structural stability of the spike protein resulting in increased binding affinity to the SARS-CoV-2 receptor angiotensin-converting enzyme 2 (ACE2) (9). The improved binding to ACE2 facilitates effective infection of and replication in the olfactory epithelium, and in particular in sustentacular cells and Bowman's gland cells as demonstrated both in the mouse model (10) and in tissue samples of deceased COVID-19 patients (11), and is regarded as mechanistically underlying the loss of smell. Similarly, enhanced ACE2 binding leads to higher infection rates of cells in the taste buds that express high levels of ACE2, leading to a disruption of normal cellular turnover from the stem cell layer and thereby to an impaired sense of taste (12). The importance of the D614G mutation for viral fitness is also demonstrated by the fact that so far, all subsequent variants of concern and variants of interest retained this mutation [reviewed in (13)]. Following variants that have spread regionally or globally all exhibited further mutations in the spike protein and in other proteins that gave the variants advantages such as increased transmissibility or infectivity, and also antibody escape to some degree (13). The most successful SARS-CoV-2 variants to date, which spread globally and largely or completely replaced other variants have been at first the G614 variant carrying the hallmark spike protein mutation D614G [Pango lineage name B.1 (14)], the alpha variant [B.1.1.7; defining mutations: Spike: N501Y, A570D, P681H, T716I, S982A, D1118H; other proteins: ORF1ab: T1001I, A1708D, I2230T; ORF8: A27*, R52I, Y73C; N: D3L, S235F; deletions: 11288:9, 21765:6, 21991:3; (15, 16)] the delta variant [B.1.617.2; defining mutations: Spike: T19R, L452R, T478K, P681R, D950N; ORF3a:S26L; M:I82T; ORRF7a: V82A, T120I; N: D63G, R203M, F377Y; (17)] and the currently dominating variant omicron [B.1.1.529; defining mutations: Spike: A67V, T95I, G339D, S371L, S373P, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N510Y, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K; ORF1a: K856R, A2710T, T3255I, P3395H, I3758V; ORF1b: P314L, I1566V; E:T9I; M: D3G, Q19E, A63T; N:RG203KR; (18)].

To get a detailed picture of the symptoms of SARS-CoV-2 infection, we created an online questionnaire and starting from April 2020 invited both positive and negative tested individuals to report their symptoms. The symptoms included in the survey comprised general symptoms such as fever, fatigue, headache and joint or muscle pains, and a range of specific eye, nose, throat, respiratory and gastrointestinal symptoms. We were especially interested in the symptoms of infected individuals with a mild disease course, who would not be likely to seek medical care and therefore would not be easily reached in a clinical study. Our results show that over time, the pattern of symptoms reported by SARS-CoV-2 positive individuals changed significantly, with nose symptoms becoming more common since the rise of the alpha and delta variants, and the typical loss of smell and taste becoming far less prevalent with the rise of the omicron variant. Our data show that continued close monitoring of SARS-CoV-2 symptoms in the future is clearly warranted.

MATERIALS AND METHODS

Ethics Approval

The study was approved by the local Ethics Committee of the Medical Faculty of the University Duisburg-Essen (approval number 20-9233-BO) and was carried out in accordance with the ethical guidelines and regulations. The study participants gave their informed consent to their voluntary participation in this study and to the subsequent use of the data for publication.

Data Collection

Data were collected in an online questionnaire based on LimeSurvey software hosted on the servers of the University of Duisburg-Essen. Participants of the online survey were recruited *via* public health offices of the city of Hamm (North Rhine-Westphalia, Germany), of the administrative district Soest (North Rhine-Westphalia, Germany), of the administrative district Hochsauerland (North Rhine-Westphalia, Germany) and the SARS-CoV-2 testing center in Lünen, administrative district Unna (North Rhine-Westphalia, Germany), as well as *via* social media. Participants were invited to complete the survey in case of a positive as well as negative SARS-CoV-2 test result. The data presented in this manuscript were collected between April 6th 2020 and May 17th 2022.

Data Analysis

Data were analyzed using R (version 3.6.3) and RStudio software and fsmb, plyr, splyr, tidyverse, ggplot2 and viridis packages. Only data from individuals who had completed the survey were included in the analysis.

For graphic representation of the survey results of tested individuals, data were sorted by the date of survey completion and the category "fever higher than 38.0°C" was calculated from

the questionnaire responses using the tidyverse package, and a heatmap of the survey data was generated using ggplot2 package.

Odds ratios (OR) for individual symptoms were calculated as the conditional maximum likelihood estimate with 90% confidence interval using the function fisher.test of R stats package. OR and frequency of symptoms were visualized using ggplot2.

Statistical significance in symptom frequency in vaccinated and unvaccinated individuals was calculated by Fisher's exact test using the function fisher.test of the R stats package.

RESULTS

To obtain an overview of the subjective symptoms reported by SARS-CoV-2 infected individuals, especially those with a mild disease course, an online survey was performed. Invitations to the survey were published on social media, and SARS-CoV-2 tested individuals were also directly invited to participate when they received notification of their test results, or by subsequent invitation, by their local public health offices. Participants included individuals who had tested positive or negative for SARS-CoV-2 by PCR. Since the survey was performed in German language, participants from social media are expected to be mainly from Germany, or from neighbouring German-speaking countries.

In total, responses of 2117 participants who had been tested for SARS-CoV-2 infection were included in the survey, 1925 (90.1%) had tested positive for SARS-CoV-2 infection, while 192 (9.1%) of those participants had tested negative. The characteristics of the whole study population are represented in **Table 1**. The SARS-CoV-2 positive-tested individuals were grouped according to the time of infection, with the time period April 2020 to January 2021 being dominated by the G614 variant SARS-CoV-2 in Germany, the SARS-CoV-2 alpha variant being the dominant circulating virus strain from February 2021 to June 2021, the SARS-CoV-2 delta variant from July 2021 until the first week of January 2022, and the SARS-CoV-2 omicron variant from the second week of January until the end of data collection on May 17th 2022 (19). The number and baseline characteristics of the participants from these four variant phases were comparable, with roughly two third of participants being female and the majority of participants between 18 and 69 years of age, although there was a higher frequency of participants in the 50 – 59 years of age group in the G614 dominated early phase of the pandemic compared to the alpha, delta and omicron dominated phases (**Table 1**).

Of the SARS-CoV-2 positive-tested individuals, only a minority (G614: 7.6%, alpha: 5.8%, delta: 1.2%, omicron: 5.1%) reported no symptoms, whereas the majority reported mild (G614: 43.5%, alpha: 48.7%, delta: 50.4%, omicron: 55.4%) or moderate symptoms (G614: 30.5%, alpha: 33.9%, delta: 34.6%, omicron: 32.0%). A small number of the participants had been hospitalized (G614: 4.9%, alpha: 2.5%, delta: 0.5%, omicron: 0.5%) or had required intensive care treatment (G614: 1.2%, alpha: 0.0%, delta: 0.0%, omicron: 0.0%) due to the SARS-CoV-2 infection.

The symptoms reported by the tested study population are shown as a heatmap in **Figure 1**, and a direct comparison of the frequencies of symptoms in the different phases of the pandemic is facilitated by the bar graphs in **Figure 2**. Clearly, a majority in both SARS-CoV-2 positive and negative individuals reported general symptoms such as tiredness, lethargy and headaches. The frequency of fever higher than 38.0°C decreased from 35.7% of

TABLE 1 | Characteristics of the study population.

	positive tested participants, n (% of total)				negative tested participantsn, (% of total)
subgroup	**G614**	**Alpha**	**Delta**	**Omicron**	
number	**485**	**448**	**564**	**428**	**192**
age (years)					
18-29	89 (18.3%)	121 (27.0%)	113 (20.0%)	82 (19.2%)	32 (16.8%)
30-39	64 (13.1%)	99 (22.1%)	123 (21.8%)	112 (26.2%)	53 (27.9%)
40-49	90 (18.6%)	73 (16.3%)	128 (22.7%)	97 (22.7%)	44 (23.2%)
50-59	158 (32.6%)	86 (19.2%)	133 (23.6%)	76 (17.8%)	40 (20.8%)
60-69	63 (12.9%)	55 (12.3%)	50 (8.9%)	54 (12.6%)	20 (10.5%)
70-79	12 (2.5%)	13 (2.9%)	13 (2.3%)	7 (1.6%)	2 (1.1%)
> 80	9 (1.9%)	1 (0.2%)	4 (0.7%)	0 (0%)	1 (0.5%)
gender					
female	283 (58.4%)	278 (62.1%)	330 (58.5%)	311 (72.7%)	120 (62.5%)
male	202 (41.6%)	170 (37.9%)	233 (41.3%)	116 (27.1%)	70 (36.5%)
diverse	0 (0%)	0 (0%)	1 (0.2%)	1 (0.2%)	2 (1.0%)
smoking	63 (13.0%)	60 (13.4%)	103 (18.3%)	63 (14.7%)	57 (30.0%)
hayfever[1]	82 (16.9%)	82 (18.3%)	41 (7.3%)	43 (10.0%)	36 (18.8%)
severity					
asymptomatic	37 (7.6%)	26 (5.8%)	34 (1.2%)	22 (5.1%)	55 (28.9%)
mild	211 (43.5%)	218 (48.7%)	284 (50.4%)	237 (55.4%)	70 (36.8%)
moderate	148 (30.5%)	152 (33.9%)	195 (34.6%)	137 (32.0%)	44 (22.9%)
severe	57 (10.3%)	41 (9.2%)	48 (8.5%)	30 (7.0%)	19 (9.9%)
hospitalization	24 (4.9%)	11 (2.5%)	3 (0.5%)	2 (0.5%)	4 (2.1%)
intensive care	6 (1.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

[1] *hayfever relevant at the time of SARS-CoV-2 infection.*

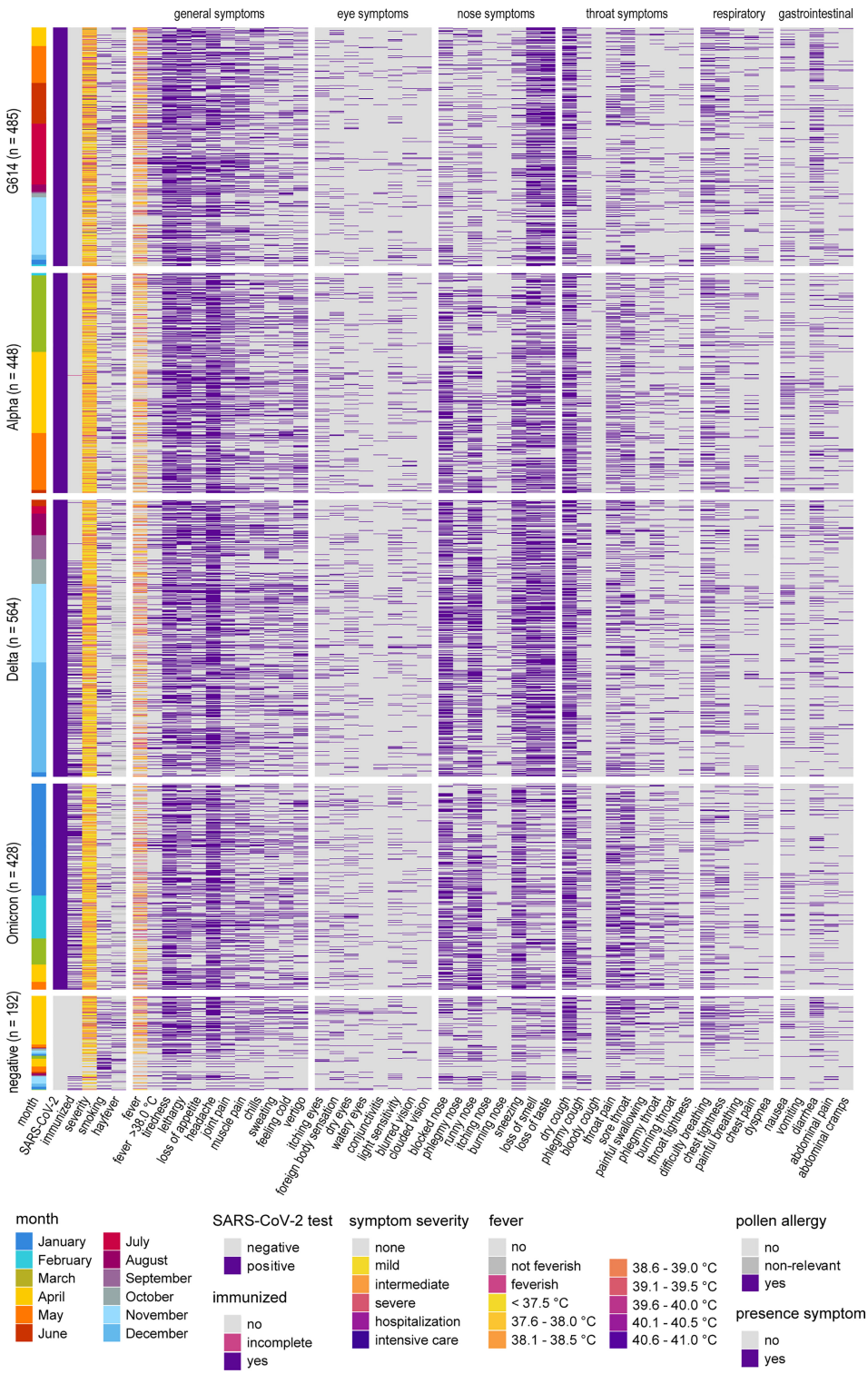

FIGURE 1 | Heatmap of symptoms reported by SARS-CoV-2 positive and negative tested individuals. Symptoms reported by SARS-CoV-2 positive (4 top sections) and SARS-CoV-2 negative individuals (bottom). Subjects were sorted according to SARS-CoV-2 test outcome and date of questionnaire completion; groups of positive subjects are displayed according to periods of dominance of SARS-CoV-2 G614 and the alpha, delta and omicron variants. "Month" indicates the date of completion of the questionnaire. "feverish/not feverish": subjective judgement, participant did not measure their body temperature.

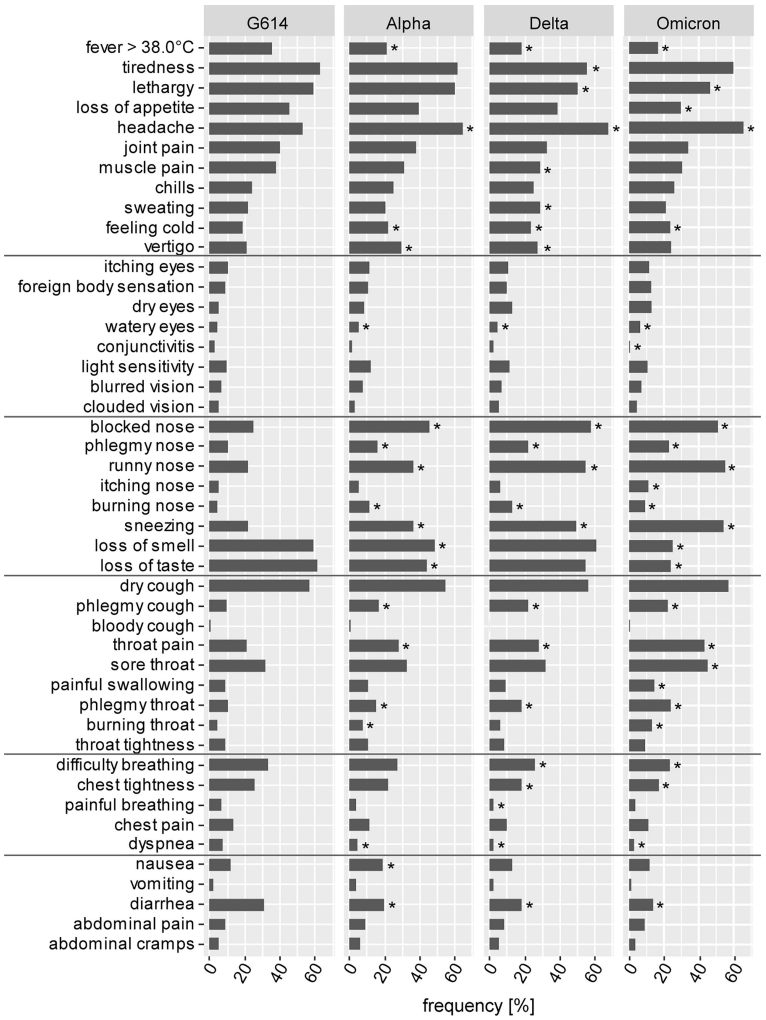

FIGURE 2 | Frequencies of symptoms. Frequencies for each symptom were calculated for SARS-CoV-2 positive individuals from G614, alpha variant, delta variant and omicron variant dominated phases of the SARS-CoV-2 pandemic. Statistically significant differences compared to the frequencies in the G614 phase are indicated by * ($p < 0.05$, Fisher's exact test).

respondents in the G614-dominated phase to 20.7% in the alpha-dominated phase, 17.9% in the delta-dominated and 16.6% in the omicron-dominated phase. On the other hand, during the G614-dominated phase, 52.5% of survey participants reported headache as a symptom, and that increased further to 64.5% for the alpha-, 67.4% for the delta-, and 65.7% for the omicron-dominated phase. Also vertigo saw an increase from 20.7% for G614 to 29.3% and 27.3% for alpha and delta, respectively, but went down to 24.1% for omicron. Lethargy, muscle pain and loss of appetite were reported less frequently in the omicron-dominated phase.

The presence of eye symptoms has been low throughout all phases of the pandemic and there have not been considerable changes over the time of data collection. Interestingly, most nose symptoms were reported only by a minority of participants during the early phase of the SARS-CoV-2 pandemic, but the frequency of respondents reporting a blocked or runny nose or sneezing has increased considerably since the spread of the alpha, delta and omicron variant: while only 24.4% and 21.8% reported a blocked or runny nose for G614 infections, this increased to 45.1% and 36.2% for alpha variant infections, 57.6% and 54.8% for delta variant infections and 50.9% and 55.1% for omicron variant infections. The proportion of sneezing increased from 21.8% for G614 to 36.1% for alpha, 49.3% for delta and 54.2% for omicron. Importantly, there have been changes to the symptoms that have been regarded as highly typical of a SARS-CoV-2 infection since early days of the pandemic, which is loss of smell or taste. About two thirds of SARS-CoV-2 positive individuals reported an impaired or lost sense of smell or taste during the G614, alpha and delta dominated phases, however, these frequencies have dropped considerably to only 25% and 23.8%, respectively, for the current omicron variant dominated phase (**Figure 2**).

Dry cough has been another hallmark symptom of SARS-CoV-2 infection and was reported by almost 60% of SARS-CoV-2 positive individuals, and this frequency remained largely unchanged for the variant-dominated phases. Other throat symptoms had been reported at far lower frequencies during the G614 phase but have increased significantly with the spread of the alpha, delta and omicron variants, most notably throat pain, sore throat and also phlegmy throat and phlegmy cough. Approximately one third of individuals reported difficulty breathing and other respiratory symptoms during the G614-dominated phase of the pandemic (31.3%), and this frequency has also dropped since the spread of the delta and even more so the omicron variant, with the frequency of individuals reporting difficulty breathing now down to 23.4%.

Also gastrointestinal symptoms have been associated with SARS-CoV-2 infections since the beginning, although not reported by a majority of infected individuals. The most often reported gastrointestinal symptom in the G614-dominated early phase of the pandemic was diarrhea (28.2%), which has decreased in frequency in the variant-dominated phases (alpha: 19.4%. delta: 17.9%, omicron: 13.8%).

The changes in symptom frequencies are reflected in the odds ratios for the individual symptoms (**Figure 3**). Since the distribution of many symptoms was similar in SARS-CoV-2 positive and negative individuals, many odds ratios are close to 1. Clearly, the highest odds ratios were observed for impaired or lost sense of smell in the earlier G614, alpha and delta phases (estimate: 3.8; 90% confidence interval (CI): 2.5 – 6.1 for G614; alpha: 2.7, CI 1.7 – 4.3; delta: 4.5, CI 2.9 – 7.0) and impaired or lost sense of taste (G614: 4.3, CI 2.8 – 6.8; alpha: 2.2, CI 1.4 – 3.5; delta: 3.6, CI 2.4 – 5.7), showing only a very slight decline with the appearance of the alpha and delta variants. For the omicron-dominated phase however, these numbers have dropped considerably to only 1.0 (CI 0.6 – 1.6) and 0.9 (CI 0.6 – 1.5), respectively.

Other notable differences were observed for blocked nose (G614: 0.5, CI: 0.3 – 0.8; alpha: 1.7, CI 1.1 – 2.6; delta: 2.3, CI 1.5 – 3.4; omicron 1.7, CI 1.2 – 2.7), runny nose (G614: 0.4, CI: 0.2 – 0.6; alpha: 0.8, CI 0.5 – 1.2; delta: 1.5, CI 1.0 – 2.3; omicron: 1.6, CI 1.1 –

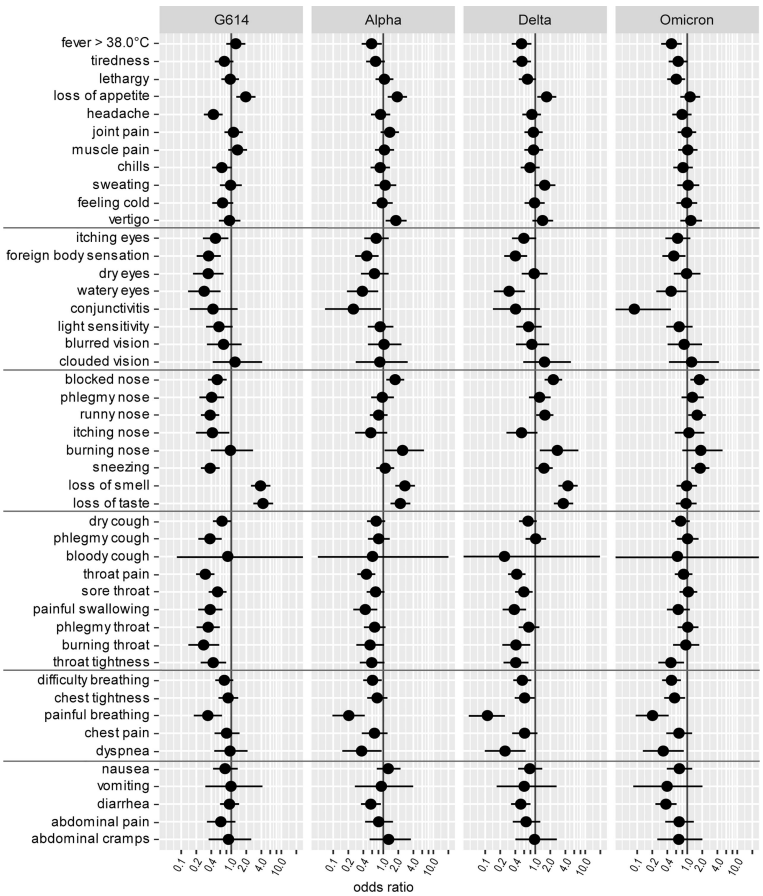

FIGURE 3 | Odds ratios of symptoms. Odds ratios of symptoms and 90% confidence intervals were calculated from frequencies of symptoms in SARS-CoV-2 positive individuals from the G614, alpha variant, delta variant and omicron variant dominated phases of the SARS-CoV-2 pandemic in relation to frequencies of symptoms in symptomatic SARS-CoV-2 negative individuals.

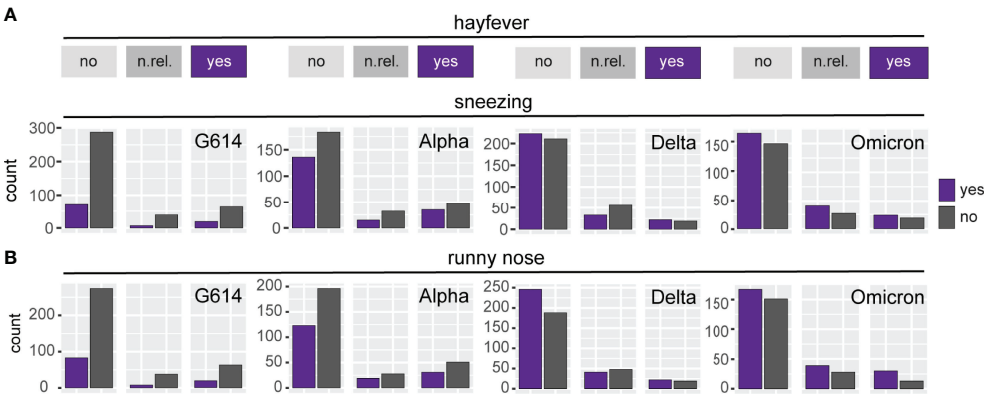

FIGURE 4 | Sneezing and runny nose symptoms stratified by hayfever. Counts of respondents indicating presence or absence of the symptom "sneezing" **(A)** or "runny nose" **(B)**. Respondents were grouped according to their suffering from hayfever ("yes"), not suffering from hayvever ("no"), or suffering from hayfever but not during the time of SARS-CoV-2 infection ("n.rel.": not relevant). The increase in frequency of the symptoms "sneezing" and "runny nose" in the variant dominated phases can be clearly observed in individuals who do not suffer from hayfever.

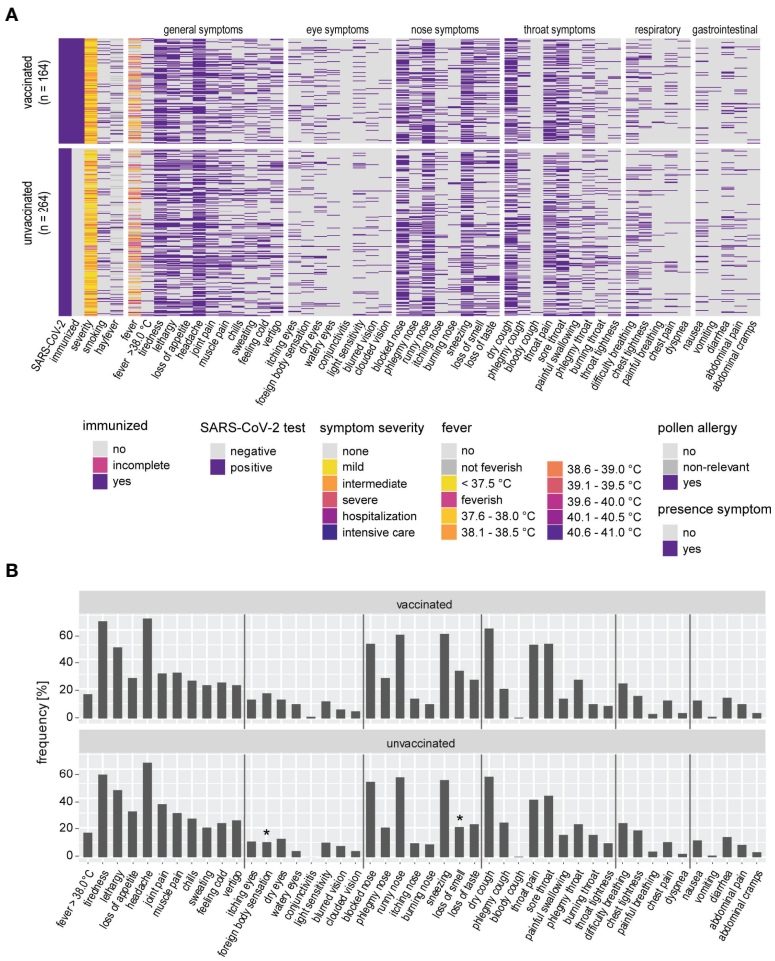

FIGURE 5 | Symptoms reported during the omicron-dominated phase stratified by vaccination status. **(A)** Heatmap of SARS-CoV-2 infected vaccinated (upper section) and unvaccinated (lower section) individuals from the omicron-dominated phase. **(B)** Frequencies for each symptom were calculated for vaccinated and unvaccinated SARS-CoV-2 positive individuals. Statistically significant differences are indicated by * ($p < 0.05$, Fisher's exact test).

2.4) and sneezing (G614: 0.4, CI: 0.2 – 0.6; alpha: 1.1, CI 0.7 – 1.6; delta: 1.5, CI 1.0 – 2.2; omicron: 1.8, CI 1.2 – 2.8), all turning from an OR below 1 for G614 to an OR well above 1 for the variants. The increased frequencies of runny nose and sneezing symptoms observed for the variants are not due to a coincidence with hayfever seasons, as the changes can be clearly observed for participants with no reported hayfever or with hayfever that was not relevant at the time of infection (**Figure 4**).

It is important to note that the differences in symptoms reported by participants in the current, omicron-dominated phase are not due to vaccination: of the 428 participants in the omicron phase, 164 had been fully vaccinated at the time of infection, while 264 had been unvaccinated, and the frequencies of symptoms were largely the same in vaccinated and unvaccinated individuals (**Figure 5**). Of note, the only symptoms where we found a statistically significant difference in frequency between vaccinated and unvaccinated individuals are foreign body sensation in the eyes and loss of smell, where the frequencies were lower in unvaccinated than in vaccinated individuals (foreign body sensation: 4.2% vs. 9.8%; loss of smell 20.5% vs. 32.3%).

A comparison with another detailed symptom study from the delta variant dominated phase by Vihta et al. reveals an

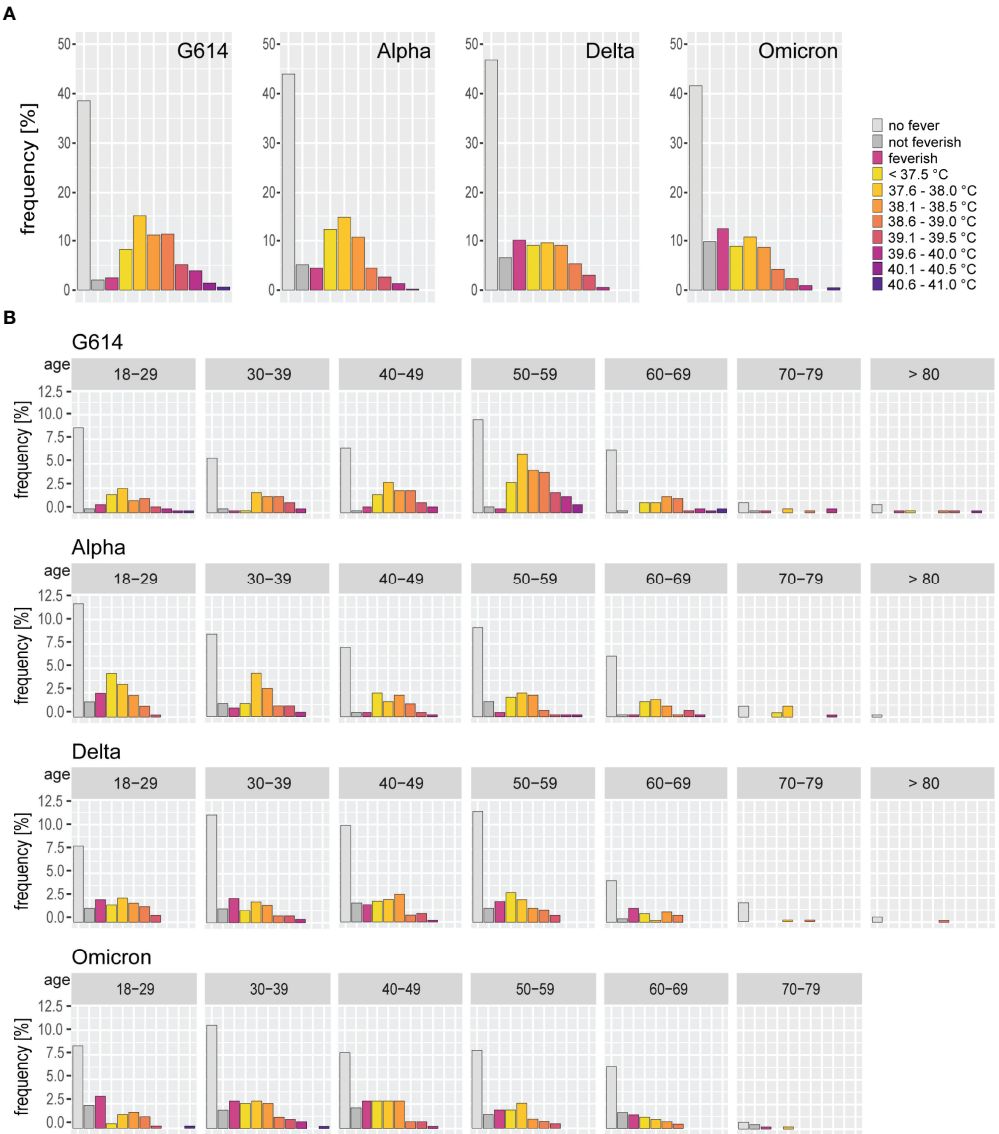

FIGURE 6 | Detailed fever data stratified by SARS-CoV-2 variants and age groups. **(A)** Frequency of the fever levels reported by participants in the SARS-CoV-2 G614, alpha, delta and omicron dominated phases of the pandemic. **(B)** Frequency of fever levels reported by the participants in the SARS-CoV-2 G614, alpha, delta and omicron variant dominated phases stratified by age groups. The frequency of participants replying with "feverish" who had not measured their body temperature increased in the variant phases, introducing more uncertainty into the fever data. However, the frequency of participants reporting no fever or not feeling feverish increased, suggesting a reliable decrease in fever induced by the SARS-CoV-2 variants.

interesting difference in the frequency of fever, which was reported to be increased for delta in comparison to G614 in a large community level cohort in the United Kingdom (20). We therefore subjected our fever data to a more detailed analysis (**Figure 6**). The decrease in frequency can be most strongly observed for the temperature range between 37.6°C and 39.0°C, which were most frequently reported in the G614 dominated phase (**Figure 6A**). It is noteworthy, however, that in the alpha and more so in the delta and omicron dominated phases, the frequency of respondents who did not measure their body temperature and replied by "I felt feverish, but didn't measure my body temperature" or "I didn't feel feverish, but didn't measure my body temperature" increased, introducing higher uncertainty into the fever data for the later phases. Of note, "feverish" was not included in the "fever > 38.0°C" category. This increase in the "feverish" and "not feverish" replies cannot be solely attributed to the fact that a larger proportion of young people were infected in the variant phases – the frequency of infected people below 50 years of age increased from 50.1% in the G614 phase to 65.4%, 64.5% and 68.0% in the alpha, delta and omicron dominated phases, respectively - since these replies increased similarly across all age groups in the variant phases (**Figure 6B**).

DISCUSSION

The data presented here provide a detailed picture of the symptoms experienced by SARS-CoV-2 infected individuals, and of the changes in symptom patterns that have occurred with the spread of the alpha, delta and omicron variants.

The data collected in this survey show that a range of symptoms is observed in a majority of patients, such as tiredness, lethargy, loss of appetite, joint or muscle pains, headache and dry coughing. Since the delta variant became dominant, also sneezing and blocked or runny nose were reported by almost 60% of infected individuals. For some symptoms however, we found surprisingly low frequencies in infected individuals and therefore low odds ratios, including all throat symptoms other than dry cough. Furthermore, only roughly one fifth of SARS-CoV-2 infected individuals experienced throat pain during the earlier phases, which could be expected to be much higher since virus is routinely identified in throat swabs, showing that it is indeed infecting throat tissue. However, the frequency of these throat symptoms has increased in the current omicron-dominated phase. While an impaired or lost sense of smell or taste had been a hallmark symptom of SARS-CoV-2 infection that was identified early in the pandemic (21, 22), the spread of the omicron variant has led to a significant decline in frequency.

For symptoms reported from the first few months of the G614-dominated beginning of the pandemic, a bias may have been introduced into the dataset since some symptoms had been described early in the pandemic as typical for SARS-CoV-2 infection, and had therefore been criteria for testing while testing capacity was still limited. However, there was no apparent change in symptom patterns when testing capacities in Germany had become sufficient in early summer of 2020 to test all patients with respiratory symptoms, therefore, the frequencies reported for the G614 phase can be expected to be accurate. It should be noted that replies were sorted by the date of questionnaire completion, which may have introduced a slight offset in the allocation to the alpha-, delta- and omicron-dominated phases.

Other studies have been performed investigating symptoms of SARS-CoV-2 and their predictive value. Studies in the early days of the COVID-19 pandemic were reporting symptoms of hospitalized patients and thus missed the symptoms that are associated with mild disease courses (4, 5, 23). Since then, multiple other studies have been performed that focused on the symptoms experienced by SARS-CoV-2 infected individuals at a community level (22, 24–29). A large study has been performed during the first phase of the pandemic using a mobile phone app that included data from more than seven thousand participants who tested positive for SARS-CoV-2 in a cohort of more than 2 million people from the United Kingdom and the United States (28). In this study, the authors found frequencies for the symptoms "loss of smell and taste", "fever", "skipped meals" and "diarrhea" that are comparable to the frequencies reported by us and reported positive odds ratios for these symptoms, with the highest odds ratio for "loss of smell and taste". The authors report a similar frequency of "persistent cough" in the SARS-CoV-2 positive individuals as we found for "dry cough"; in contrast to our data, the frequency of "dry cough" in SARS-CoV-2 negative individuals was lower than in our cohort, leading to a low positive odds ratio for the persistent cough, in contrast to our findings for the G614 phase.

A further large-scale study in the United Kingdom revealed similar changes in the symptoms for the delta variant as we have observed in our study, with the remarkable exception that the authors report an increase in fever for the delta variant compared to G614 (30). Our data show that the incidence of fever higher than 38.0°C decreased to below 20% for delta, compared to more than 35% for G614. In contrast to our survey, the UK survey asked participants if they had experienced fever during their SARS-CoV-2 infection, but did not ask for specific temperatures or specify a lower threshold for fever (personal communication). This could partly explain the difference between the survey outcomes, even though we would still see a slight increase in respondents who replied "no fever", or a bit more pronounced in those who replied either "no fever" or "not feverish" from the G614 phase to the variant dominated phases, indicating that there is a true decline in our data. Vihta et al. also suggested that non-white ethnic groups were more likely to report fever than white ethnic groups (30), whether this explains the difference to our findings cannot be estimated since we do not have any information on ethnicity of our participants.

A subsequent large-scale study using the above mentioned mobile phone app analyzed data collected from 63,002 participants in the United Kingdom during the delta and omicron variant dominated phases of the pandemic (31). While many symptoms were similar in frequency during the

delta and omicron phases, they also report a significant drop in the frequency of altered smell and more prominently in the frequency of loss of smell, which dropped from 50% during the delta dominated phase to below 20% during the omicron dominated phase, which is very similar to our findings. Interestingly, the frequency of many symptoms such as joint pains, muscle pains, runny nose, sneezing, nausea and diarrhea is higher in this report than in our data, for many of those symptoms by about 10%. A reason for the higher rates might be the daily reporting of symptoms in the mobile phone app, which could be leading to higher reporting rates.

A reduced frequency of loss of smell or taste in the omicron variant dominated phase as we have seen in our survey has also been described in some other reports, interestingly, the frequency in some reports was even lower than in our study, ranging from only 1.2% in a study in Jordan (32) and 2.5% in a study in Korea (33) to 8% in a study in the US (34) and 12% (loss of smell) and 23% (loss of taste) in a study in Norway (35). Loss of smell and taste was reported to be down to 13% in a technical briefing by the United Kingdom Health Security Agency (36); this report was based on the very large number of 182,133 individuals, but as a limitation of this analysis it was indicated that the symptom data was collected early, usually 3 to 4 days, after symptom onset. Very similar frequencies of impaired or lost sense of smell or taste in infected individuals were reported from studies in Italy (37), the Faroe Islands (38), the mobile phone app study mentioned above (31) as well as a community study in the United Kingdom (20). In the latter report from a large community study in the United Kingdom a similar drop in the frequency of loss of smell and taste as we have found in our survey was demonstrated, corroborating our observation. Interestingly, the authors also report a somewhat higher rate of loss of smell and loss of taste as well as of coughing in vaccinated compared to unvaccinated individuals, suggesting that this finding may not be due to an unknown bias but warrants further scrutiny. Of course, the higher frequency in vaccinated individuals found in our study might also be due to a reporting bias, since symptomatic vaccinated individuals, especially those with more pronounced symptoms, might be more motivated to participate in the survey.

The SARS-CoV-2 variants have been fully characterized on the genomic level, and some of the characteristic mutations readily lend themselves for an explanation of the observed changes in symptoms of infected individuals that occurred with the spread of the variants. As described above, a hallmark of the first globally spread SARS-CoV-2 was the mutation D614G, which was retained in later variants (see (13) for a detailed review of SARS-CoV-2 variants with a focus on the spike protein). This variation has been shown to reduce spike S1 subunit shedding (39–44), to stabilize the spike pre-fusion conformation (45–48), and to thereby increase infectivity and transmission due to high ACE2 expression levels in the upper respiratory tract (8, 49). Similarly, the additional N501Y mutation present in the alpha and omicron variants has been shown to directly strengthen the interaction of the SARS-CoV-2 RBD with ACE2 (50, 51). Similarly, mutations in the furin cleavage site (alpha and omicron: P681H; delta: P681R) can lead to increased cleavage of the S1 and S2 spike protein subunits, resulting in higher infectivity and transmissibility (52, 53). A high binding affinity to ACE2 and high fusogenicity of the spike proteins explains the efficient infection of sustentacular cells of the olfactory epithelium and of taste bud cells, which are thought to mechanistically underlie the loss of smell and taste in a large proportion of infected individuals (10, 11). Since the first affinity-enhancing mutation D614G was introduced into the orinial Wuhan Hu-1 strain after its spread into other countries, this may well explain why loss of smell and taste was not observed as a symptom during the earliest phase of SARS-CoV-2 spread in China. While it has been suggested in a large meta-analysis that ethnicity also plays a role in susceptibility of infected individuals for loss of smell and taste, and that individuals of Asian origin are less prone to it (7), viral factors appear to have a much larger role than host factors (54). In comparison to alpha and delta, omicron harbors even more mutations in the spike protein including regions of the RBD involved in ACE2 binding and around the furin cleavage site, and has been shown to have high ACE2 binding affinity (55). Interestingly, however, omicron exhibits an inefficient use of the cellular protease TMPRSS2, which is required for proteolytic cleavage of the spike protein at the S2' site to liberate the fusion peptide, and relies more strongly on the endosomal uptake route (56), suggesting that ACE2/TMPRSS2-expressing cells would no longer be as strongly preferred target cells. This would explain the lower frequency of loss of smell and taste with the omicron variant. Sustentacular cells of the olfactory epithelium have been shown to express high levels of both ACE2 and TMPRSS2 (57), making them direct target cells of the previous SARS-CoV-2 variants as demonstrated both in the mouse model (10) and in human tissue (11). It could be shown *in vivo* in the hamster model that in comparison to G614 and delta, the infection of the olfactory epithelium by the omicron variant was markedly reduced and infection of nasal tissue shifted to the respiratory epithelium (58), which is in accordance with the significantly reduced frequency of loss of smell in omicron infected individuals shown in this study as well as by others.

The mechanism underlying the reduced induction of fever by the SARS-CoV-2 variants is less clear, and possibly more complex and involving proteins other than spike. It has been shown previously that SARS-CoV-2 infection induces cytokines such as interleukin 6 and tumor necrosis factor α (59) that are known mediators of fever. It was shown in the mouse model that the spike protein alone could induce production of these cytokines (60), and it was demonstrated *in vitro* that monocytes produced these cytokines when stimulated with the S1 spike subunit alone (61). Unfortunately, no comparative analysis with the different variants has been performed in these studies, since they were performed earlier in the pandemic. An *in vivo* mouse study showed a differential induction of cytokines by the delta and omicron variants in comparison to an early G614 isolate (62); and similarly, an *in vivo* study in hamsters showed reduced levels of a range of cytokines in omicron infected compared to delta infected animals (63), suggesting that the

variants are indeed inducing different cytokine levels *in vivo*. One study has analyzed various SARS-CoV-2 proteins for the presence of cytokine-inducing peptides and found significant contribution of proteins other than spike to the cytokine response, which was found to be slightly reduced for omicron in comparison to Wuhan Hu-1, gamma (B.1.1.28.1) and delta derived proteins (64). Furthermore it has been shown in *in vitro* studies that omicron is more susceptible to inhibition by type I interferons and induces higher levels of type I interferons than delta (65, 66), which may also play a role in the development of fever. While no direct comparison was made, the authors suggested an increased susceptibility of the omicron variant to interferon, which may be associated with mutations in proteins other than spike, namely variations in the interferon antagonizing proteins non-structural protein 3 (nsp3), nsp12, nsp13, nsp14, M, nucleocapsid and ORF3a (65, 66).

Our study shows interesting changes of symptoms of SARS-CoV-2 infection over time, it does however have a number of limitations that need to be mentioned: since the survey was conducted as an online questionnaire, the participants were likely biased towards younger people, even though we know of instances where younger relatives assisted elderly participants. While the frequency of participants of the 50-59 years of age group was higher in the G614 dominated phase than in the alpha, delta and omicron dominated phases, the participants of the survey from the four phases were largely comparable with regard to age and gender distribution. It has to be noted, however, that there was a far larger proportion of female than male participants of the survey in all four phases of the pandemic. Since the main focus of the presented study was the comparison of the symptoms observed during the different variant dominated phases, this bias should be noted but does not hinder the interpretation of the data as presented here. Furthermore, even though we also saw participants who had been hospitalized or had even required intensive care treatment, we were aware from the beginning that an online questionnaire would be more likely to reach those with mild disease course. Still, those people with more symptoms, or with more pronounced symptoms, might have felt more motivated to participate in the survey, so it would be especially difficult to conclude the number of asymptomatic SARS-CoV-2 infected individuals from this survey, and the overall frequency of the symptoms might be overestimated. However, as discussed above, the frequencies of many symptoms reported in our study are largely in line with the findings of other symptom studies, therefore our detailed results can be regarded as a reliable contribution. While we did see a higher proportion of smoking respondents in the SARS-CoV-2 negative group than in the SARS-CoV-2 infected individuals, we did not perform further analysis on this finding since the present data is likely biased to symptomatic participants and is therefore neither amenable for a calculation of the overall frequency of asymptomatic infection nor to address the question if smoking leads to a reduced incidence of symptomatic SARS-CoV-2 infection. Finally, it also has to be mentioned that we had a very detailed questionnaire, which may have been difficult to complete for participants with an impaired concentration span or severe brain fog, a challenge that has been reported to us by a small number of participants.

With the spread of new variants, the symptoms experienced by SARS-CoV-2 infected individuals have changed during the pandemic, and are likely to change again in the coming months or years with the spread of future variants. Our data show that it remains important to track the symptoms of SARS-CoV-2 infection.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethik-Kommission Medizinische Fakultät Universität Duisburg-Essen Robert-Koch-Str. 9-11 45147 Essen Germany. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HS recruited participants, analyzed data and contributed to writing the manuscript. WB conceived of the study, created the online survey, analyzed data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Spike Mutation Profiles Associated With SARS-CoV-2 Breakthrough Infections in Delta Emerging and Predominant Time Periods in British Columbia, Canada

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Background: COVID-19 vaccination is a key public health measure in the pandemic response. The rapid evolution of SARS-CoV-2 variants introduce new groups of spike protein mutations. These new mutations are thought to aid in the evasion of vaccine-induced immunity and render vaccines less effective. However, not all spike mutations contribute equally to vaccine escape. Previous studies associate mutations with vaccine breakthrough infections (BTI), but information at the population level remains scarce. We aimed to identify spike mutations associated with SARS-CoV-2 vaccine BTI in a community setting during the emergence and predominance of the Delta-variant.

Methods: This case-control study used both genomic, and epidemiological data from a provincial COVID-19 surveillance program. Analyses were stratified into two periods approximating the emergence and predominance of the Delta-variant, and restricted to primary SARS-CoV-2 infections from either unvaccinated individuals, or those infected ≥14 days after their second vaccination dose in a community setting. Each sample's spike mutations were concatenated into a unique spike mutation profile (SMP). Penalized logistic regression was used to identify spike mutations and SMPs associated with SARS-CoV-2 vaccine BTI in both time periods.

Results and Discussion: This study reports population level relative risk estimates, between 2 and 4-folds, of spike mutation profiles associated with BTI during the emergence and predominance of the Delta-variant, which comprised 19,624 and 17,331 observations, respectively. The identified mutations cover multiple spike domains including the N-terminal domain (NTD), receptor binding domain (RBD), S1/S2 cleavage region, fusion peptide and heptad regions. Mutations in these different regions imply various mechanisms contribute to vaccine escape. Our profiling method identifies naturally occurring spike mutations associated with BTI, and can be applied to emerging SARS-CoV-2 variants with novel groups of spike mutations.

Keywords: SARS-CoV-2, COVID-19, vaccine escape, vaccine breakthrough, spike, variants, whole genome sequencing, penalized regression

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent for the ongoing COVID-19 pandemic, which is responsible for 300 million infections and 5.5 million deaths worldwide (1). Public health efforts including hand washing, mask wearing, physical distancing and vaccination are associated with reductions in the spread of COVID-19 (2–5). Both Moderna mRNA-1273 and Pfizer–BioNTech BNT162b2 vaccines have shown high effectiveness at preventing severe COVID-19 related illness by around 95% (3, 5). Despite vaccination, breakthrough infections (BTI) are reported after receiving two vaccine doses. Decreased protection has been attributed to vaccine waning since becoming fully vaccinated (6) and the emergence of SARS-CoV-2 variants capable of evading neutralizing antibodies (7).

SARS-CoV-2 has diversified into many variants with sub-lineages. Several of these have been classified as Variants of Concern (VoC), which includes Alpha (B.1.1.7, Q.*), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2, AY.*) and most recently Omicron (B.1.1.529, BA.*) (8). These VoCs are associated with increased transmissibility, infectivity and/or breakthrough potential (9–13). Their increased fitness has been attributed, in part, to several key mutations spanning the spike protein, which is responsible for binding to the Angiotensin-converting enzyme 2 (ACE2) receptor and subsequent fusion into host cells (14). These features make the spike protein an immunodominant target for neutralizing antibodies (15). In response, spike mutations have emerged and contribute to the evasion of neutralizing antibodies and increased host-receptor affinity. For example, the D614G mutation became predominant early in the pandemic and induces a more open conformation for subsequent ACE2 binding (16). The Alpha-variant harbors several mutations including N501Y and P681R, which enhance ACE2 binding and furin cleavage, respectively (17, 18). The Beta-variant harbors these mutations with the addition of the E484K and K417N mutations, which increase the affinity to the ACE2 receptor and aid in immune escape, respectively (17, 19). Among other emerging variants, the Delta-variant has several spike mutations including T19R, G142D, E156G/Δ 157–158, L452R, T478K and D950N. The combination of these mutations support the escape of neutralizing antibodies and increased affinity to ACE2 (7, 20). With the emergence of Omicron-variant, additional novel spike mutations are still being characterized.

The majority of studies characterizing spike mutations use *in-vitro* assays, protein modeling, and convenient sampling. To date, there are limited studies which characterize vaccine escape mutations at the population level. Genome-wide association studies (GWAS) are suited for finding relationships between mutations and given phenotypes in a population. However, this approach may overlook the interaction or additive effect several mutations have on a complex phenotype. This limitation is further exacerbated by the fact that emerging SARS-CoV-2 variants introduce multiple novel mutations at a time, a phenomenon exemplified by the Omicron-variant. Therefore, we stratify isolates by spike mutation profiles (SMP) and are the first to identify spike mutations and SMPs associated with vaccine BTI within a community setting in British Columbia, Canada. Our analyses take place in two adjacent periods during the pandemic, which are the emergence and predominance of the Delta-variant in British Columbia, Canada in the context of a population with varying degrees of vaccination dosage and coverage.

METHODS

Data Sources

We leveraged laboratory both diagnostic data, including quantitative PCR (qPCR) and whole genome sequencing (WGS), and epidemiological data from an ongoing provincial SARS-CoV-2 surveillance program previously described (21). Briefly, publicly funded diagnostic qPCR testing was widely available for symptomatic individuals and those associated with outbreaks. Testing was implemented through a network of hospital laboratories and the British Columbia Center for Disease Control (BCCDC) Public Health Laboratory (PHL), which serves as a reference laboratory. In addition, vaccination data from BC's Provincial Immunization Registry was retrieved for defining cases and controls. Testing, genomic, case and vaccination data were linked using a minimum of three key personal identifiers, including Personal Health Number (PHN), full name and date of birth. The reported prevalence of the Delta-variant in BC was used to stratify observations into Delta-variant emerging and predominant periods. The Delta emerging period (April 15th–August 31st, 2021) was defined as a period were the prevalence of this VOC increased from 0.24 to 99% of all VOCs reported in BC (22). The Delta predominant period (September 1st–November 30th, 2021) was characterized as a period with sustained prevalence around 99% of all VOCs being reported in BC (22).

Centralized Population Level Genomic Surveillance

Throughout the study period, the genomic surveillance strategy was designed to account for the provincial testing guidelines and case load while ensuring the timely capture of emerging and circulating variants, as well as optimization of sequencing capacity. The sequencing strategy and magnitude is presented in **Figure 1**. Briefly, From April 15th to May 29th, 2021, a combined VOC testing strategy using both "screening" [i.e., targeted VOC single-nucleotide polymorphism (SNP) qPCR] and WGS was applied to specimens to detect and monitor VOC prevalence in British Columbia. Approximately 30–47% of all SARS-CoV-2-positive samples underwent WGS during early April, and 67–78% from April 25th to May 29th, 2021. From June 1st to August 31st, 2021, all positive SARS-CoV-2 samples in BC had WGS attempted. During period 2, all positive samples were sequenced on the first week of each month and a representative 10% of all samples were sequenced in the later weeks between September 1st and November 15th. After November 15th, all positive samples underwent WGS until the end of period 2. Samples' lineage was assigned using WGS if both SNP qPCR screening and WGS was applied. The library preparation and Illumina-based sequencing protocol use a modified version of the Freed et al. 1200bp

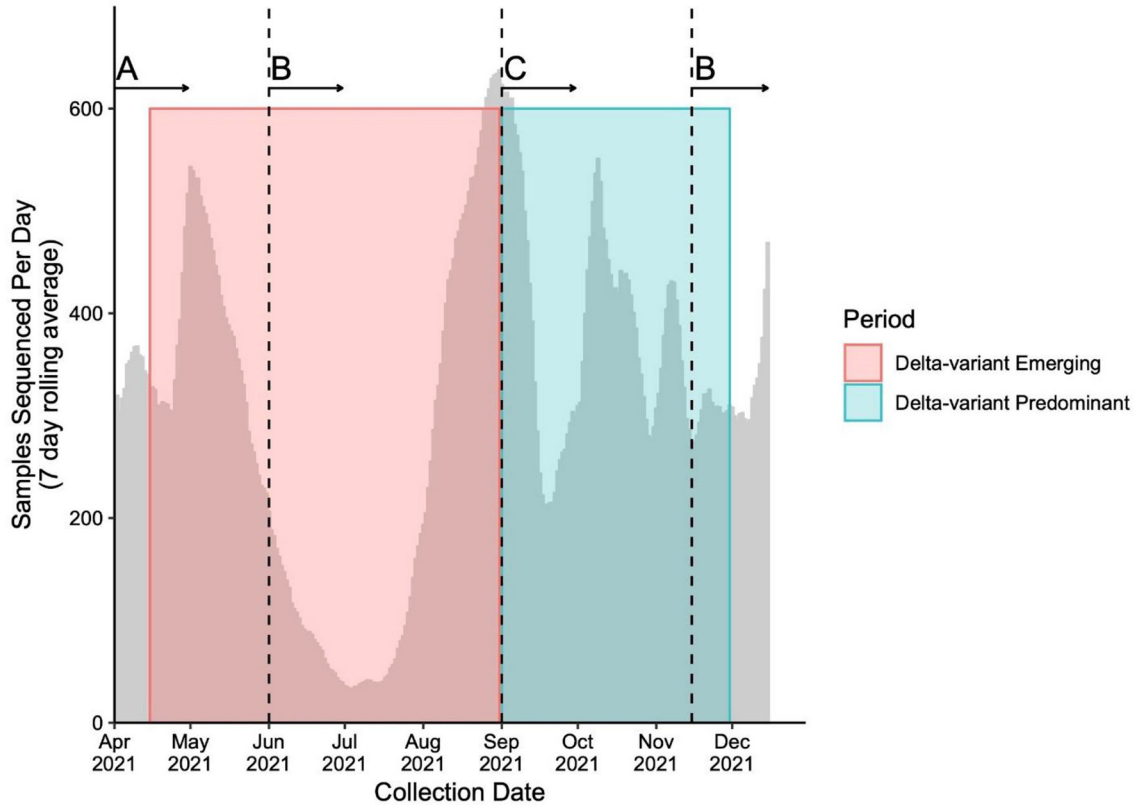

FIGURE 1 | Number of samples sequenced (7 day rolling average) during study period. Sequencing strategies throughout the study periods adapted to changes in testing guidelines and sequencing capacity to capture the evolution of the pandemic. The number of averaged sequenced samples is plotted by time overlapping the Delta-variant emerging (pink box) and predominant (blue box) periods. The vertical dashed lines represent the time when whole genome sequencing (WGS) strategy changed. The sequencing strategies include **(A)** a combination of targeted qPCR-based single-nucleotide polymorphism (SNP) and WGS of between 30 and 78% of all positive SARS-CoV-2 samples, **(B)** WGS of all positive SARS-CoV-2 samples and **(C)** WGS of all positive SARS-CoV-2 samples on the first week of the month, and WGS of a representative subset of 10% of all positive SARS-CoV-2 samples for the second, third and fourth week of the month.

amplicon scheme protocol, which has been previously described (21, 23, 24).

Genomic Sequence Analysis

The BCCDC PHL used a modified ARTIC Network bioinformatics protocol and downstream analysis from the Simpson lab (https://github.com/BCCDC-PHL/ncov2019-artic-nf, https://github.com/BCCDC-PHL/ncov-tools) to process reads, align to the SARS-CoV-2 reference genome, and generate both variant calls and a consensus genome sequence (**Supplementary Material**). All genomic sequence information used in this study has been uploaded to GISAID under the submitter BCCDC PHL. The current study restricted analyses to non-synonymous single nucleotide polymorphisms (SNP) and insertions-deletions variants overlapping the spike region. The filtered spike variants were concatenated to construct unique spike mutation profiles (SMPs) per sample.

Population structure can confound the relationship between mutations and phenotypes of interest. Lack of adjustment can lead to the identification of lineage-defining mutations, which may not be associated with BTI. Therefore, we employed PopPUNK (25), a kmer-based genome clustering method, to generate population structure. Briefly, a PopPUNK database of genome sketches was generated using the trimmed consensus sequences from all samples collected between April 15th and August 31st. The consensus sequences were sketched using the strand-preserved and codon-phased options to account for the structure of the SARS-CoV-2 genome. Next, a lineage-based model using a nearest neighbor approach was used to cluster and assign a distinct lineage to observations by k-mer resolved genetic distance (25). These lineage assignments were used in downstream analyses to control for population structure.

Eligibility Criteria for Defining Study Population

Our study population consisted of those with community-acquired SARS-CoV-2 primary infections with either no vaccination (controls), or those with a BTI occurring $\geq$14 days after receiving a second vaccine dose (cases) (**Figure 2**). Observations were included if collected between April 15th and November 30th, 2021 and their corresponding SARS-CoV-2 genome had <5 ambiguously called nucleotides and a breadth

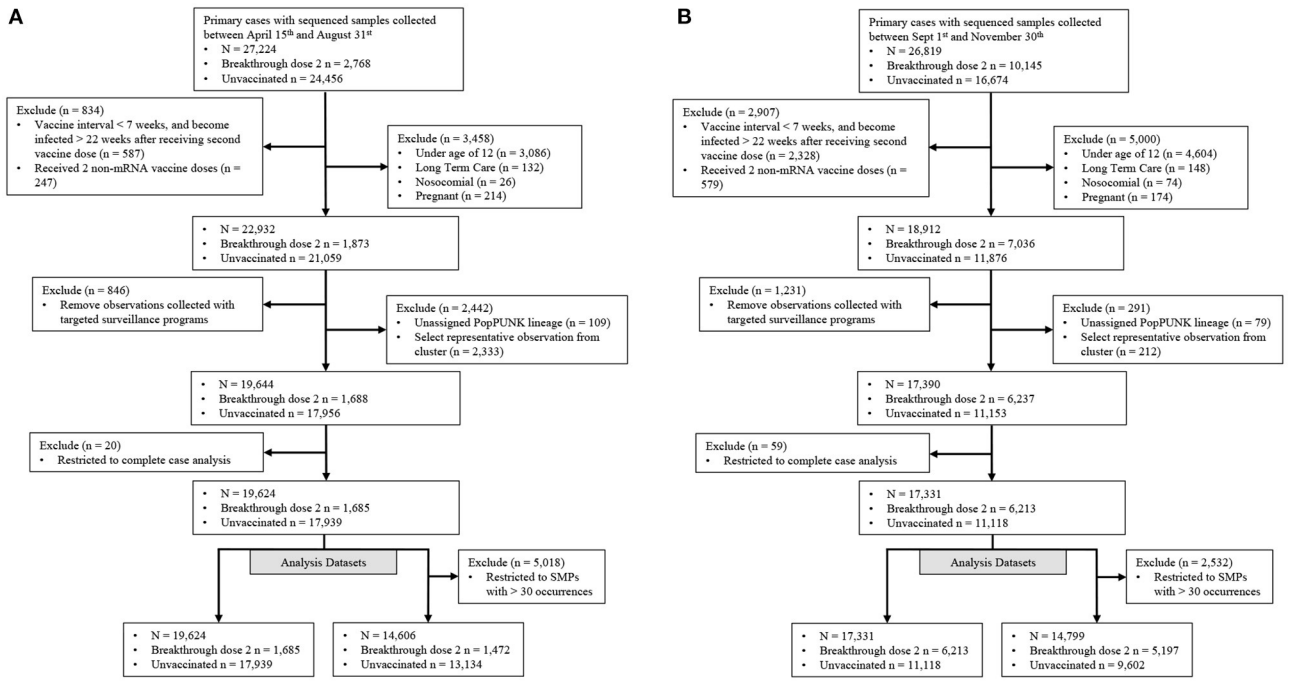

FIGURE 2 | Filtration steps for samples in both Delta emerging and predominance periods. Samples were stratified into two distinct periods representing **(A)** a time when the Delta-variant was emerging and **(B)** when the Delta-variant was the major variant circulating. Targeted surveillance includes individuals regularly tested for work and travel purposes.

of coverage ≥85%. Vaccine effectiveness is associated with vaccine dosage interval, time since vaccination and vaccine type (6, 26, 27). Individuals with a BTI were kept if they met the following criteria: (i) the interval between receiving both vaccine doses was ≥ 7 weeks, (ii) became infected within 22 weeks after receiving the second dose and (iii) received at least 1 mRNA-based vaccine (**Figure 2**). Vaccine inclusion criteria were used to retain vaccinated individuals with sufficient protection against SARS-CoV-2. Individuals were removed if they were not eligible for vaccination in BC (ages below 12), were pregnant or known to be infected in a long-term care facility or hospital. We also removed individuals tested through targeted surveillance programs, which identified foreign temporary workers, individuals requiring regular and repeated testing, and travelers. We selected one observation from known SARS-CoV-2 clusters to limit the artificial inflation of an isolates' ability to cause breakthrough infections due to situational advantages caused by the transmission setting. The selected observation was the earliest infection of the most frequent PopPunk lineage within each cluster to approximate the strain which seeded the cluster. Finally, remaining observations were kept if they had complete sex, age, region (health authority used as proxy), collection date and genomic information recorded (**Figure 2**).

Statistical Analysis

The outcome was defined as either a breakthrough or unvaccinated infection, and covariates included categorical age (12–19, 20–39, 40–59, 60–79 and ≥80), sex (male or female), region (from health authority of residence), isolate collection month, discrete lineage and unique SMP or individual spike mutations.

We employed two strategies for identifying mutations associated with BTI in both time periods. We constructed two datasets, which either consisted of an observation's mutations, or their SMP membership (**Figure 2**). From the mutation dataset, we removed genomic sites if the second highest allele was found in <10 cases or controls. Similarly, we removed observations in SMP with <30 occurrences and at least 3 occurring in vaccinated individuals (**Figure 2**). Next, elastic net penalized logistic regression was used to identify either single spike mutations, or SMPs, predictive of BTI, while adjusting for age, sex, geography, and population structure. Elastic net permits variable selection in the presence of highly correlated variables in high dimensional data. Selection is performed via coefficient shrinkage achieved by minimizing residual sum of squares and a penalty term. This penalty is a mixture of the ridge, and lasso penalties, which is the summation of either squared or absolute regression coefficients, respectively. The mixture of these penalties is controlled by a mixing parameter (α), which ranges between 0 (only applying the ridge penalty) and 1 (only applying the lasso penalty). In addition, the regularization (λ) parameter controls the contribution of the penalty term in model fitting. The caret R package (version 6.0-86) was used to perform an 80–20% train-test split of the data. The glmnet package (version 2.0–16) was used to fit a penalized model with a grid

of α values between 0.3 and 1. λ parameter in each model was chosen using 10-fold cross validation to maximize the weighted area under the curve (AUC). The elastic net model was run with class weights defined as:

$$class\ weight = 1 - \left(\frac{\#\ in\ outcome\ class}{\#\ of\ total\ members}\right)$$

The predictive performance of each elastic net model on the test dataset was evaluated using AUC. The best performing model's non-zero penalized mutations or SMP coefficients were collected.

Lastly, we ran logistic regressions to quantify the association between identified features and breakthrough status, adjusting for age, sex, geography, and collection month. Single identified mutations were separately quantified, and their corresponding coefficient's *p*-value was corrected for multiple comparisons using the false discovery rate (FDR) adjustment. All analyses and visualizations were conducted in R, version 3.5.2 (28).

Ethics

This study's protocol was approved by The University of British Columbia's institutional review board (REB H21-01206).

RESULTS

Study Characteristics

The Delta-variant emerging and predominance period consisted of 19,624 and 17,331 individuals, respectively (**Table 1**). Most COVID-19 isolates were from people under 60 years old, with an even distribution between sexes (**Table 1**). The majority of infections in both periods occurred in unvaccinated individuals, but the frequency of BTI increased over time with the majority being associated with the Delta-variant (**Table 1**). The most common Delta sub-lineages for period 1 belong to AY.25 and AY.27, which constituted 69.3 and 21.2% of all Delta cases in this period, respectively. For period 2, the AY.25 and AY.27 were responsible for 72.0 and 19.8% of Delta cases.

Breakthrough Infections in the Delta Emerging Period

There were 729 unique positions across the spike gene harboring non-synonymous mutations. Frequency based filtration of sites resulted in the retention of 29 sites which were supplied to the penalized regression models. An elastic net model with $\alpha = 0.4$ and $\lambda = 0.0076$ maximized predictive performance (AUC = 0.87). This model recognized 12 spike mutations to be most predictive of BTI (**Supplementary Results**). The relationship between these mutations and BTI were quantified with subsequent regression models, adjusting for age, sex, region, and collection time. All spike mutations which remained positively associated with BTI include: T19R, G142D, E156G/Δ 157-158, L452R, T478K, P681R, A846S, D950N and P1162L (**Figure 3A**). These identified mutations span multiple regions of the spike protein including the N-terminal domain (NTD), receptor binding domain (RBD), S1/S2 cleavage region and heptad regions (**Figure 3A**). Adjusted odds ratio estimates for these mutations range between 2.00 and 4.56, and are reported in **Supplementary Table S1**.

No isolate harbored all identified mutations positively associated with breakthrough. In response, we sought to identify naturally occurring SMPs positively associated with BTI. All SARS-CoV-2 isolates from this period were clustered into 1,218 unique SMPs. We restricted our population to individuals infected with isolates in SMP groups with frequencies ≥ 30 to justify multivariate analyses (total $n = 14{,}606$, unvaccinated $n = 13{,}134$, dose 2 breakthrough $n = 1{,}472$). This filtering resulted in further analysis of 17 SMPs for which an elastic net model with $\alpha = 0.5$ and $\lambda = 0.0085$ provided the best predictive performance (AUC = 0.86). A Delta-variant SMP, containing T19R, G142D, E156G/Δ 157-158, L452R, T478K, D614G, P681R, and D950N, was used as a reference group to quantify identified SMPs association with BTI. Two SMPs were positively associated with BTI, and shared the Delta-variant mutations, with the addition of either A846S ($OR_{Adj} = 2.37$, 95% CI 1.26–4.29, *P*-value = $5.4e^{-3}$), or P1162L ($OR_{Adj} = 3.78$, 95% CI 1.79–8.00, *P*-value = $4.5e^{-4}$) amino acid mutations (**Figure 3B**). The frequency of these identified SMPs, stratified by periods, is reported in **Supplementary Table S2**.

Breakthrough Infections in the Delta Predominance Period

For this period, the study samples contributed 732 unique positions across the spike gene, of which 50 remained after frequency pre-screening. An elastic net model, parametrized with $\alpha = 0.7$ and $\lambda = 0.0034$, maximized performance (AUC = 0.67). This model identified 25 spike mutations to be predictive of BTI (**Supplementary Results**). The S45F, A647S, Q675H, P812S, A845V and G1124V mutations remained positively associated with BTI, and span both inter and intra functional spike regions (**Figure 3C**). The adjusted odds ratio for these identified mutations range between 2.04 and 18, and are reported in **Supplementary Table S1**. No isolate contained all identified mutations, so we employed the SMP analysis.

This time period contained 1,090 unique SMPs. Frequency filtration resulted in 36 SMPs (total $n = 14{,}799$, unvaccinated $n = 9{,}602$, dose 2 breakthrough $n = 5{,}197$). An elastic net model, parametrized with $\alpha = 0.3$ and $\lambda = 0.017$, achieved optimal performance (AUC = 0.67), and subsequent multivariate analysis identified 6 SMPs to be positively associated with BTI, relative to a Delta-variant SMP (**Figure 3D**). The SMPs shared the Delta-variant mutations, with the addition of either N74I ($OR_{Adj} = 2.49$, 95% CI 1.24–5.10, *P*-value = $1.0e^{-2}$), T95I ($OR_{Adj} = 1.68$, 95% CI 1.22–2.31, *P*-value = $1.4e^{-3}$), A647S ($OR_{Adj} = 2.65$, 95% CI 1.38–5.26, *P*-value = $4.1e^{-3}$), A684V ($OR_{Adj} = 2.25$, 95% CI 1.22–4.19, *P*-value = $9.0e^{-3}$), P812S ($OR_{Adj} = 2.11$, 95% CI 1.30–3.53, *P*-value = $3.2e^{-3}$) or A845V ($OR_{Adj} = 3.73$, 95% CI 2.12–6.80, *P*-value = $8.9e^{-6}$) amino acid mutations (**Figure 3D**). The frequency of each identified SMP in both time periods is presented in **Supplementary Table S2**. The two methods showed discordant results where the individual method identified the Q675H and G1124V mutations, while the SMP approach selected the N74I, T95I, and A684V mutations.

TABLE 1 | Characteristics of study population stratified by period and vaccination status.

	Delta-variant emerging period (April 15th–Aug 31st)			Delta-variant predominance period (Sept 1st–Nov 30th)		
	Unvaccinated	**Breakthrough dose 2**	**Overall**	**Unvaccinated**	**Breakthrough dose 2**	**Overall**
	(*N* = 17,939)	(*N* = 1,685)	(*N* = 19,624)	(*N* = 11,118)	(*N* = 6,213)	(*N* = 17,331)
Sample type						
Gargle	8,226 (45.9%)	791 (46.9%)	9,017 (45.9%)	5,652 (50.8%)	2,894 (46.6%)	8,546 (49.3%)
LRT[a]	5 (0.0%)	0 (0%)	5 (0.0%)	0 (0%)	1 (0.0%)	1 (0.0%)
NP[b]	9,684 (54.0%)	892 (52.9%)	10576 (53.9%)	5,444 (49.0%)	3,309 (53.3%)	8,753 (50.5%)
Other[c]	2 (0.0%)	0 (0%)	2 (0.0%)	11 (0.1%)	3 (0.0%)	14 (0.1%)
Missing	22 (0.1%)	2 (0.1%)	24 (0.1%)	11 (0.1%)	6 (0.1%)	17 (0.1%)
Health authority						
1	7,696 (42.9%)	465 (27.6%)	8161 (41.6%)	3,804 (34.2%)	2,420 (39.0%)	6,224 (35.9%)
2	5,668 (31.6%)	571 (33.9%)	6239 (31.8%)	3,148 (28.3%)	1,283 (20.7%)	4,431 (25.6%)
3	745 (4.2%)	63 (3.7%)	808 (4.1%)	2,161 (19.4%)	773 (12.4%)	2934 (16.9%)
4	2,861 (15.9%)	468 (27.8%)	3,329 (17.0%)	1,002 (9.0%)	1,147 (18.5%)	2,149 (12.4%)
5	969 (5.4%)	118 (7.0%)	1,087 (5.5%)	1003 (9.0%)	590 (9.5%)	1,593 (9.2%)
Sex						
Male	9,762 (54.4%)	788 (46.8%)	10,550 (53.8%)	5,945 (53.5%)	2,827 (45.5%)	8,772 (50.6%)
Female	8,177 (45.6%)	897 (53.2%)	9,074 (46.2%)	5,173 (46.5%)	3,386 (54.5%)	8,559 (49.4%)
Collection month						
2021–04	3,439 (19.2%)	0 (0%)	3,439 (17.5%)	–	–	–
2021–05	5,128 (28.6%)	1 (0.1%)	5,129 (26.1%)	–	–	–
2021–06	988 (5.5%)	5 (0.3%)	993 (5.1%)	–	–	–
2021–07	1,010 (5.6%)	64 (3.8%)	1,074 (5.5%)	–	–	–
2021–08	7,374 (41.1%)	1,615 (95.8%)	8,989 (45.8%)	–	–	–
2021–09	–	–	–	4,020 (36.2%)	1,443 (23.2%)	5,463 (31.5%)
2021–10	–	–	–	4,248 (38.2%)	2,558 (41.2%)	6,806 (39.3%)
2021–11	–	–	–	2,850 (25.6%)	2,212 (35.6%)	5,062 (29.2%)
Age group						
12–19	2,042 (11.4%)	46 (2.7%)	2,088 (10.6%)	1,550 (13.9%)	232 (3.7%)	1,782 (10.3%)
20–39	9,891 (55.1%)	677 (40.2%)	10,568 (53.9%)	4,470 (40.2%)	2,174 (35.0%)	6,644 (38.3%)
40–59	4,515 (25.2%)	549 (32.6%)	5,064 (25.8%)	3,392 (30.5%)	2,220 (35.7%)	5,612 (32.4%)
60–79	1,363 (7.6%)	347 (20.6%)	1,710 (8.7%)	1,504 (13.5%)	1,285 (20.7%)	2,789 (16.1%)
80+	128 (0.7%)	66 (3.9%)	194 (1.0%)	202 (1.8%)	302 (4.9%)	504 (2.9%)
CT value						
Mean (SD)	22.1 (4.80)	23.0 (4.92)	22.1 (4.82)	22.8 (4.64)	23.2 (4.80)	23.0 (4.70)
Median (Min, Max)	22.0 (10.0, 37.4)	22.9 (10.7, 36.4)	22.1 (10.0, 37.4)	22.9 (10.4, 36.5)	23.1 (10.4, 37.6)	23.0 (10.4, 37.6)
Missing	4,267 (23.8%)	255 (15.1%)	4,522 (23.0%)	3,312 (29.8%)	1,663 (26.8%)	4,975 (28.7%)
Vaccine type						
No vaccination	17,939 (100%)	0 (0%)	17,939 (91.4%)	11,118 (100%)	0 (0%)	11118 (64.2%)
Mix[d]	0 (0%)	127 (7.5%)	127 (0.6%)	0 (0%)	444 (7.1%)	444 (2.6%)
mRNA[e]	0 (0%)	1,558 (92.5%)	1,558 (7.9%)	0 (0%)	5,769 (92.9%)	5769 (33.3%)
Lineage						
Alpha	4,878 (27.2%)	4 (0.2%)	4,882 (24.9%)	3 (0.0%)	0 (0%)	3 (0.0%)
Beta	12 (0.1%)	0 (0%)	12 (0.1%)	0 (0%)	0 (0%)	0 (0%)
Delta	8,477 (47.3%)	1,665 (98.8%)	10,142 (51.7%)	11,111 (99.9%)	6,210 (100.0%)	17,321 (99.9%)
Gamma	3,780 (21.1%)	14 (0.8%)	3,794 (19.3%)	3 (0.0%)	1 (0.0%)	4 (0.0%)
Omicron	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0.0%)	1 (0.0%)
VOI[f]	288 (1.6%)	0 (0%)	288 (1.5%)	0 (0%)	0 (0%)	0 (0%)
VUM[g]	32 (0.2%)	0 (0%)	32 (0.2%)	0 (0%)	0 (0%)	0 (0%)
Other	472 (2.6%)	2 (0.1%)	474 (2.4%)	1 (0.0%)	1 (0.0%)	2 (0.0%)
Unique PopPUNK lineages	155	29	155	135	127	144

[a]Lower respiratory tract sampling method. [b]Nasopharyngeal sampling method. [c]Other includes upper respiratory tract, nares, and other mis-specified sampling method. [d]Receiving a combination of a mRNA vaccine (Moderna mRNA-1273 and Pfizer–BioNTech BNT162b2) and a viral-vector-based vaccine (AstraZeneca). [e]Receiving a combination of the Moderna mRNA-1273 and Pfizer–BioNTech BNT162b2 vaccines. [f]Variant of interest, including the Epsilon, Eta and Mu variants. [g]Variants under monitoring, including Iota and Kappa variants.

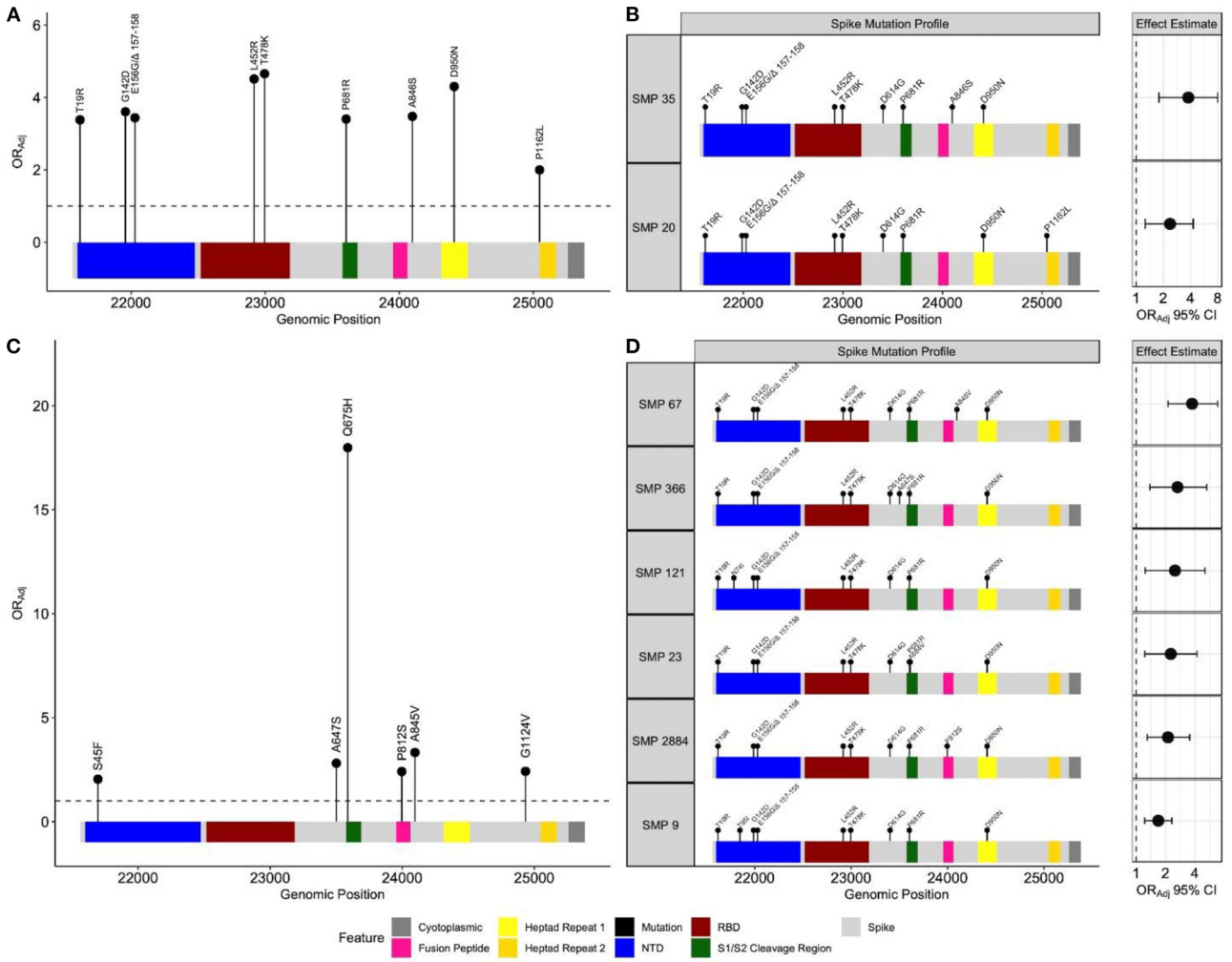

FIGURE 3 | Spike mutation variants and profiles associated with breakthrough infections during the emergence and predominance of the Delta-variant. Separate elastic net models were fit to identify either individual spike variants, or spike mutation profiles (SMP) associated with breakthrough infections. **(A)** Depicts individual mutations associated with breakthrough during the emergence ($n = 19{,}624$) and **(C)** predominance of the Delta-variant ($n = 17{,}331$). The black circles represent the odds ratio for each mutation, adjusting for age, sex, health authority, and month of collection. The profiles for each SMP identified during the **(B)** emergence ($n = 14{,}606$) and **(D)** predominance periods ($n = 14{,}799$) are plotted with their corresponding odds ratio and 95% confidence intervals, adjusting for age, sex, health authority, and month of collection. The reference group SMP consisted of the most common Delta spike variants (T19R, G142D, E156G Δ 157–158, L452R, T478K, D614G, P681R and D950N). The N-terminal domain and receptor binding domain are abbreviated with NTD and RBD, respectively.

DISCUSSION

Recent studies have relied on protein modeling, *in-vitro* experiments, and frequency-based trends to examine the relationship between BTI and SARS-CoV-2 spike mutations. In this work, we extend the current knowledge by examining both spike mutations, and SMPs associated with vaccine BTI in a community setting during the emergence and predominance of Delta, a SARS-CoV-2 variant of concern. Our findings corroborate Delta-defining spike mutations, along with A846S and P1162L to be associated with BTI during the emergence of the Delta-variant. We also find A647S, P812S, and A845V mutations confer additional vaccine escape potential.

The COVID-19 pandemic has seen several SARS-CoV-2 variants emerge. We found T19R, G142D, E156G/Δ 157-158, L452R, T478K, D614G, P681R, D950, A846S, and P1162L mutations to be positively associated with BTI during the emergence of the Delta-variant. These mutations cover multiple spike domains including the NTD, RBD, S1/S2 cleavage region, fusion peptide and heptad regions. This suggests various mechanisms may contribute to SARS-CoV-2 vaccine BTIs. A previous study showed full-length Delta spike protein constructs have an increased rate of membrane fusion, relative to other lineages (29). The previous study also shows T19R, G142D, and E156G/Δ 157–158 mutations decreased affinity of NTD-targeted antibodies, while L452R and T478K do not aid in the evasion of several neutralizing antibodies (29). Others suggest L452R

and T478K mutations increase the stability of the ACE2-RBD complex (20). In addition to effective binding, the identified P681R mutation has been associated with increased viral fusion (18). We also identified A846S, D950N, and P1162L to be positively associated with BTIs. The A846S mutation is adjacent to the fusion peptide and both D950N and P1162L are within or proximal to heptad regions, which are important for membrane fusion (30). Both A846 and P1162 sites are associated with decreased spike protein stability (31), but their proximity to key spike domains may provide increased fitness. Interestingly, our identified SMPs harbor the exact same set of mutations. All Delta-defining spike mutations were found in these SMPs, with the addition of either the A846S, or P1162L mutations. In addition to agreeing with the individual spike analysis, the proposed SMPs analysis allows further evaluation of these novel mutations. Both methods indicate that Delta-defining spike mutations likely contributed in overcoming the previous co-dominance of the Alpha and Gamma variants in BC between April and August, 2021 (22).

In the Delta-predominant period, we identified S45F, N74I, T95I, A647S, Q675H, P812S, A845V, and G1124V to be positively associated BTIs. There is sparse information about the S45F and T95I mutations, but their position in the NTD may contribute to evasion of neutralizing antibodies. The frequency of T95I has increased overtime (32), and is present in other SARS CoV-2 variants including Omicron. The N74I has not been previously associated with breakthrough infections. This position is glycosylated and adjacent to a NTD "super site" recognized by neutralizing antibodies (33). The loss of the glycan would decrease glycan shielding, which is sparse relative to other densely glycosylated viruses (34). However, the proximity to this "super site" may be associated with increased evasion of the immune system. We also identified A647S and A845V, which are positioned between functional domains of the spike protein. These mutations increase the spike protein's stability (31, 35). Other *in-silico* studies show Q675H is associated with decrease protein stability (31), but provides increased furin affinity (36). Lastly, we identified P812S to be positively associated with BTIs, but an *in-silico* study suggests this mutation decreases spike-TMPRSS2 binding (37). This difference is likely related to reporting the isolated effect P812S has on protein interactions compared to the effect a group of mutations has on a complex phenotype. Finally, we note G1124V is positively associated with BTIs at the population level, which agrees with a previous study showing epitopes with this mutation decrease the affinity to several HLA alleles (38). This finding further highlights alternative mechanisms for immune evasion. The SMP approach reached similar conclusions in this period. However, the SMP method did not identify Q675H and G1124V, as these mutations were at low frequencies, but instead identified the T95I, N74I, and A684V mutations. The A684V is located in the S1/S2 cleavage region and could interact with the proximal P681R mutation to aid in furin binding. Interestingly, positively associated mutations did not remain associated with BTIs in both periods, except for the Delta defining mutations found with the SMP approach. This may be explained by the transient nature of mutations circulating in the population, which has shown rapid fluctuations in several spike mutations including S477N, A222V, H49Y, and V1176F (32). Furthermore, several identified mutations are characterized as destabilizing. However, protein stability has not been previously quantified in the presence of additional spike mutations, which could interact to become neutral or beneficial.

The current study has several strengths. First, we utilize both population-based epidemiological, and WGS data from prospectively collected COVID-19 samples across BC. This information allowed us to stringently define community-acquired infections, avoid misclassification bias in our outcome group, and increased external validity. Second, the sequencing strategy ensured adequate and accurate representation of circulating variants. Despite our strengths, the study has a limitation in that the majority of cases analyzed are symptomatic. This limitation may underestimate some non-synonymous spike mutations. In conclusion, we identify novel BTI mutations and propose the use of SMPs, which concur with traditional methods, prioritizes naturally occurring isolates and highlights the affect coupled mutations have on an outcome. These results extend our knowledge of SARS-CoV-2 vaccine breakthrough mutations to the population level, and provide a robust method for analyzing variants emerging with novel groups of spike mutations.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.gisaid.org/, Submitter: BCCDC PH.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of British Columbia's institutional review board (REB H21-01206). 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

HS, AJ, and NP conceptualized the study. CDF conducted the literature review, was responsible for the analyses, figure generation, and writing the manuscript. NJ, CF, and HS were responsible for acquiring demographic and epidemiological data for the population of interest. NP and JT oversaw the collection, processing, and reporting of genomic data for study subjects. CDF, YJ, and HS were responsible for data linkage, cleaning, and implementing inclusion criteria for the study. HS and YJ have reviewed the analyses. HS, AJ, NP, JT, and CC aided in the interpretation of the data. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.915363/full#supplementary-material

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Conflict of Interest: NJ participates in Abbvie Advisory Board meeting.

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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REVIEW
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SARS-CoV-2 and the Missing Link of Intermediate Hosts in Viral Emergence - What We Can Learn From Other Betacoronaviruses

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The emergence of SARS-CoV-2 in 2019 has resulted in a global pandemic with devastating human health and economic consequences. The development of multiple vaccines, antivirals and supportive care modalities have aided in our efforts to gain control of the pandemic. However, the emergence of multiple variants of concern and spillover into numerous nonhuman animal species could protract the pandemic. Further, these events also increase the difficulty in simultaneously monitoring viral evolution across multiple species and predicting future spillback potential into the human population. Here, we provide historic context regarding the roles of reservoir and intermediate hosts in coronavirus circulation and discuss current knowledge of these for SARS-CoV-2. Increased understanding of SARS-CoV-2 zoonoses are fundamental for efforts to control the global health and economic impacts of COVID-19.

Keywords: SARS-CoV-2, intermediate hosts, emergence, coronavirus, spillover, variants, zoonosis, reverse zoonosis

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INTRODUCTION

History of *Betacoronavirus* Emergence

Coronaviruses were first identified in 1937 with the identification of avian infectious bronchitis virus (1). The first human coronaviruses (HCoV) were discovered in 1967, and found to be predominantly associated with mild, self-limiting, cold-like illnesses: HCoV-OC43, a *Betacoronavirus* of subgenus *embecovirus* (also known as *Betacoronavirus 1*), and HCoV-229E, an *Alphacoronavirus* of subgenus *Duvinacovirus* (2, 3). For more than three decades, HCoVs were not regarded as emerging global health threats (4). However, the emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002 rapidly changed this view; however, no cases have been identified since 2004 (5). In 2004, two additional HCoVs were identified - HCoV-NL63, an *Alphacoronavirus* of subgenus *Setrecovirus*, was identified and found to primarily infect children, the elderly and immunocompromised patients, followed by the identification of HCoV-HKU1, a *Betacoronavirus* of subgenus *Embecovirus*, in a patient admitted to hospital in early 2004 (6, 7). Concerns regarding the global health threat of HCoVs were again fueled by the identification of Middle East respiratory syndrome coronavirus (MERS-CoV) infection in humans in 2012; more than 2500 confirmed infections have subsequently been recorded with a case fatality rate of ~35% (8,

9). At the end of 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel *Betacoronavirus*, was identified from a cluster of patients in Wuhan, Hubei Province, China, and has subsequently resulted in a pandemic with devastating economic and public health impacts (10–13). With three HCoVs that have had profound effects on global health having been identified in the last two decades, this demonstrates the pressing need to better understand the zoonotic origins and circulation patterns of these viruses (14).

The emergence of coronaviruses within the human population are believed to have occurred through spillover events from animal reservoirs, such as bats and rodents, to humans (15–18). Reservoir hosts, wherein a pathogen can be maintained through persistent infection, may experience asymptomatic or mild infections while carrying the virus, the mechanism of which is still not well understood, but appears to be due to immune tolerance by the reservoir host (19).

4Transmission of viruses from a reservoir host to a susceptible host can occur directly or indirectly. Direct transmission occurs through direct contact with the reservoir host carrying the pathogen (20); this was observed during the SARS outbreak of 2003 when butchers at live animal markets in Guangdong province, China, had a higher infection rate than the general population (21). Indirect transmission occurs when the virus is shed by the reservoir host to the environment (e.g. airborne or vehicle) or to a vector (e.g. intermediate host) with the potential to be transmitted to humans (20). For example, during the first MERS outbreak in 2012, it is thought that bats infected with MERS-CoV shed the virus in their feces contaminating nearby water and food of domesticated camels (22). The camels are then thought to have transmitted MERS-CoV to the humans they came in contact with (15, 23). The mechanisms by which these cross-species transmission events, or spillovers, occur varies depending on a number of factors such as intermediate host species, geographical locations, climate, and seasonal patterns (24). Many human activities, including urbanization, can increase contact between humans and reservoir/intermediary species and thus increase the likelihood of future spillover events (25). The coronavirus disease 2019 (COVID-19) pandemic caused by SARS-CoV-2 demonstrated the public health and economic tolls following the emergence of SARS-like-coronaviruses (SL-CoVs). In order to minimize the impact of future *Betacoronavirus* spillover events, we need to better understand the interactions between virus and reservoir, and subsequent interactions between animal reservoirs and humans (26). This review aims to compile and discuss available data on betacoronaviruses and their reservoir and intermediary hosts to highlight knowledge gaps and the importance of ongoing virus surveillance efforts.

SARS-CoV-2, COVID-19 and the Origins of Coronaviruses

In late December 2019, the first cluster of COVID-19 cases in Wuhan, China, was reported by the World Health Organization (WHO) (27–29). While initially described as an atypical pneumonia and SARS-like illness, viral genome sequencing quickly identified the causative agent as a SARS-like coronavirus, initially termed nCoV-2019 but subsequently named SARS-CoV-2 (10, 27, 30). A unique feature that contributed to the rapid global spread of SARS-CoV-2 is the ability for the virus to spread prior to outward signs or symptoms of COVID-19 (31). Within four months of the initial outbreak, SARS-CoV-2 spread across the globe while governments rapidly instituted mitigation measures, including lockdowns and border closures, in an effort to reduce viral spread (32). Though the speed at which SARS-CoV-2 spread took the world by surprise, viral spillover events have been increasing in frequency in recent decades (33). Early analysis identified similarity of SARS-CoV-2 to bat coronaviruses, suggesting that SARS-CoV-2 may have originated as the result of a natural spillover event from bats to humans in late 2019 (34–36). Genomic analyses were conducted on samples from nine patients exhibiting COVID-19 symptoms in Wuhan China (37). Eight of the nine tested patients had either visited or worked at a wet market in the region, which suggested that infections could have been acquired from contact with animals or food products sold at the market (38). There were two initial lineages of SARS-CoV-2 circulating in the early days of wet market circulation termed lineages A and B (29). Lineage B became the dominant lineage linked to early cases from the Hunan market, environmental samples at the time of identifying the outbreak and eventually spread globally (29). While lineage A was associated with cases at other markets in Hunan Province and other areas of China (29 However, the identification of cases from early December not linked to the Huanan seafood market suggest that initial spillover occurred prior to subsequent cases that had contact with the market in mid-to-late December (29). The potential emergence of SARS-CoV-2 across multiple markets mirrors what occurred with the emergence of SARS-CoV with high levels of genetic diversity observed in both cities where SARS-CoV emerged (39–41). This type of emergence pattern suggests SARS-CoV-2 emergence involved multiple contacts with infected animals/traders resulting in multiple spillover events (42). There is evidence to support this with potential infected or susceptible animals transported to/between Wuhan animal markets through supply chains (42). Based on recent models, the first SARS-CoV-2 case in Hubei Province likely occurred between mid-October to mid-November (43). Worobey and colleagues have recently provided supportive evidence that the Huanan market was the geographical epicentre for the pandemic based on both geospatial analysis of the earliest COVID-19 cases in humans as well as spatial analysis of SARS-CoV-2 positive environmental samples with vendors selling live animals (44). There are ongoing investigations into potential intermediate hosts of SARS-CoV-2 which will be discussed in detail in this review (36). The COVID-19 pandemic highlights the continual risks of cross-species transmission and spillover events that can rapidly lead to large-scale outbreaks due to the variability of host-pathogen dynamics and the unpredictability of emerging pathogens.

Virology of Betacoronaviruses

Phylogeny and Genome

Coronaviruses possess the largest genomes of all RNA viruses at 26 – 32 kilobases (kb) in length (45). These are enveloped, single-stranded, positive-sense RNA viruses whose genomes contain at least 6 open reading frames (ORFs) that encode 16 non-structural proteins (NSP), 4 structural proteins, a 5' cap structure and a 3' poly (A) tail (46, 47). The family *Coronaviridae* belongs to the order

Nidovirales (48). Within the family *Coronaviridae* is the subfamily *Orthocoronavirinae* which is subdivided into four genera, each containing a type species: *Alphacoronavirus (alphacoronavirus 1), Betacoronavirus (murine coronavirus), Gammacoronavirus (avian coronavirus)*, and *Deltacoronavirus (bulbul coronavirus HKU11)* (46). This review will be focused on the Betacoronavirus genera which is divided into five subgenera: *Embecovirus, Sarbecovirus, Merbecovirus, Nobecovirus* and *Hibecovirus* with *Embecovirus, Sarbecovirus* and *Merbecovirus* relevant to humans, as seen in **Figure 1** (10, 26, 49–51).

Structure and Replication

Betacoronaviruses have a helical nucleocapsid contained within spherical envelopes which are coated with characteristic S glycoproteins protruding from the surface of the envelope (47). When observed *via* cryo-transmission electron microscopy, the S proteins of the virus have the appearance of a crown, which is where the name coronavirus originates (48). Each spike on the envelope of betacoronaviruses is a homotrimer of the glycosylated S protein. Each monomer S protein contains an S1 binding domain and an S2 fusion domain catalyzing anchoring to the membrane (52). The S protein, a class-1 fusion protein, is processed through the Golgi apparatus where it is heavily glycosylated *via* an encoded N-terminal signal tag. The M protein, the most abundant protein in the envelope, consists of three transmembrane domains, all of which give the virion its spherical form as well as binding to the nucleocapsid, serving as the scaffold for the virion (53). The E protein consists of 2 domains functioning to assist in the assembly and release of the viral particle from the host cell. Lastly, the N protein consists of two domains allowing binding to the genomic RNA and formation of the nucleocapsid (54). Both the N-terminal and C-terminal domains have RNA binding capacity, however the mechanism by which binding occurs varies. Due to large amounts of observed phosphorylation of the C-terminal domain of the N protein, it is hypothesized that phosphorylation catalyzes a conformational change, increasing the binding affinity between the N protein and viral RNA (55). Phosphorylation of the N protein also acts to tether the replicase-transcriptase complex (RTC) to the viral genome during virion assembly (48). The structural proteins play critical roles in virulence and establishment of infections within reservoirs and other hosts by facilitating viral attachment and entry into host cells.

Betacoronavirus genomes are positive sense and therefore in the same orientation as host mRNA. This allows for direct translation by the host to produce the two viral polyproteins (48) (pp1a and pp1ab) which are then cleaved into the 16 NSPs (**Figure 2**). Some non-structural proteins exhibit host suppression, for example nsp3 and nsp16 seem to block the interferon-mediated immune response through inhibition of interferon (IFN) pathways (56). Through degradation of host mRNA and suppressing translation of host proteins, nsp1 contributes to the regulation of host cells and the immune response, while also promoting viral production (57). The use of 5' caps on the viral genome is involved in immune evasion, by disguising as host mRNA which does not activate pattern recognition receptor pathways that would normally lead to the destruction of viral RNA (58, 59). The S protein is critical in viral infection as this is the protein responsible for binding and gaining entry into host cells (60).

Betacoronavirus Host Range

The host specificity of betacoronaviruses is determined by their S protein, which binds host receptors that include aminopeptidase N, angiotensin converting enzyme 2 (ACE2) and dipeptidyl peptidase 4 (DPP4) in order to gain entry to host cells (61). Receptor-recognition of the S protein is facilitated by the S1 domain, which is composed of the N-terminal domain (NTD) and C-terminal domain (CTD) (62). The NTD is involved in receptor recognition whereas the CTD contains the receptor-binding domain (RBD) that binds the host cell receptor and determines specificity (63). In the case of SL-CoVs, there is a strong binding affinity between the viral S protein and the host cell receptor ACE2 (52), whereas MERS-like coronaviruses have a strong binding affinity to the DPP4 receptor in the host (64). Reservoir species, such as bats, have a protein homologous to the human ACE2 receptor which may enable transmission of SL-CoVs from bats to humans (65). As different

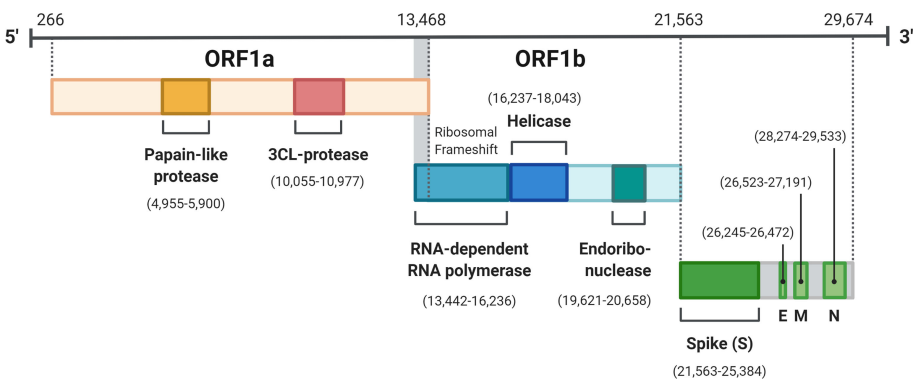

FIGURE 1 | SARS-CoV-2 genome organization. ORF 1a and 1b translated to pp1a and pp1ab are the two polypepdies which are processed into the 16 nonstructual proteins. The four structural proteins are S, spike; E, envelope; M, membrane; N, nucleocapsid. Created with BioRender.com.

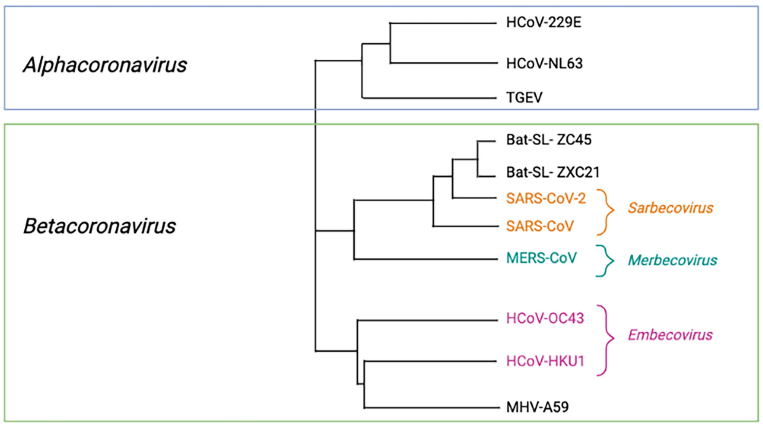

FIGURE 2 | Phylogeneic tree of relevant Orthocoronaviruses. Phylogenetic tree with representative species CoVs. Virus names: HKU, coronavirus identified at Hong Kong University; HCoV, human coronavirus; MERS, Middle Eastern respiratory syndrome; MHV, murine hepatitis virus; SARS, severe acute respiratory syndrome; SL, SARS-like; TGEV, transmissible gastroenteritis virus. Created with BioRender.com.

SL-CoVs have varying binding affinity to ACE2 receptors, intermediate host infections can facilitate viral mutation of S protein allowing recognition and binding of ACE2 receptor in humans (66). Following binding of the S protein to its host cell receptor and endosomal uptake of the virus into the cell, the viral genome is subsequently released into the cytoplasm for transcription and replication (67).

ACE2 is the receptor used for cell entry by many *Sarbecoviruses*, including SARS-CoV and SARS-CoV-2 (65). ACE2 is a type-I transmembrane protein found in epithelial cells of the lung, vascular endothelial cells, and renal tubular epithelium (68, 69). This receptor plays an important role in cardiac pathophysiology acting as a negative regulator of the Renin-angiotensin pathway in the lung, which regulates blood pressure and electrolyte levels (70). Numerous studies have been done to investigate the role of ACE2 in SARS-CoV infection. For example, viral loads in *ACE2* knockout mice were far lower than those in the wild-type control mice, suggesting the virus was unable to enter host cells without ACE2 (71). The ability of the S protein to bind ACE2 is largely dependent on the affinity of the viral RBD - which may vary due to mutations in this area of the genome (39, 66, 72). Researchers were able to show that minor variations within the S protein in the RBD of *Sarbecoviruses* can lead to binding of ACE2 receptors in other animals (intermediate hosts) and humans (73). Investigating the S protein variability within different *Sarbecoviruses* and SL-CoVs has allowed for greater understanding of how cross-species transmission occurs.

MECHANISMS OF *BETACORONAVIRUS* EMERGENCE

Betacoronavirus Spillover Events, Sylvatic Cycles and Synanthropy

There are many examples of coronavirus spillover from intermediate hosts from recent history (23, 26, 47, 74). Many betacoronaviruses are known to originate from bats, including SARS-CoV, BtCoV-WIV1, MERS-CoV, BtCoV-HKU4, BtCoV-HKU5, and Ro-BtCoV-HKU9 (74–77). Since >70% of emerging infectious diseases are zoonotic in origin (78), human-animal interfaces, such as transitional zones bordering wild habitats, are an important factor that should be considered when analyzing viral emergence (79). Li and colleagues assessed bat coronavirus spillover potential in rural districts of Southern China and found serological evidence of SL-CoV antibodies despite the low probability of community exposure to SARS-CoV (79). It was determined that any antibodies detected were likely the result of SL-CoV exposure by cross-species transmission from bats, which are known hosts for these viruses (79). In this example, it had been reported that bats were living within the community which would increase the opportunity for spillover. This is one of many communities that are found within transitional zones, a number that is rapidly increasing due to the encroachment of humans on shrinking wild habitats.

Human incursion into wild habitats is facilitated through activities such as farming, wild animal hunting and rapid transportation (80–82). These activities have a direct effect on the circulation of zoonotic pathogens between their reservoir hosts, intermediate hosts and humans; this is referred to as the sylvatic cycle (83–85). Sylvatic cycles are also affected by climate change as global warming can broaden habitat ranges, allowing species to migrate into geographical locations they previously did not inhabit (25). Broadening of these habitat ranges can lead to increased interactions with humans which increases the probability of a spillover event (86). Human-wildlife interactions will likely continue to increase and therefore the rate of zoonotic pathogen emergence will also increase if these factors are not controlled.

Asymptomatic Nature of Infections in Bats and Implications for Spillover Events

The evidence of bats harbouring and propagating virus while exhibiting little to no signs of disease when considered with the

diversity of bat CoVs and close relationship to HCoVs make a case for bats to be considered the reservoir for CoVs (36, 87, 88). Horseshoe bats (*Rhinolophus*) are the most relevant natural CoV host demonstrated through the diversity of SL-CoVs discovered in several species in Africa, Asia and Europe (89–91). It is not well understood how or why this occurs and therefore, the current research focuses on elucidating the underlying molecular mechanisms through the use of bat cells (15). The work so far suggests that there is early cellular recognition and response to viral replication coupled with moderate suppression of the immune system to tolerate low level infection by these viruses (92). Adaptation of immune system functioning, including variations in the expression levels of type I IFNs, has been demonstrated in different bat species. This may be due to co-evolution resulting from the long-term presence of these viruses among bat species (93). Major histocompatibility complex class one molecules (MHC I) have been found to differ among bat species in both the presentation and structure providing a partial explanation for the different levels of pathogenesis observed in bats (94). Among the differences observed, variations in the MHC I peptide binding groove that recognize distinct peptide epitopes are linked to alterations of bat immune responses (94). Immune suppression by the host in addition to viral evasion of the host's immune system allows for viral replication to continue uninterrupted, which in turn leads to increased viral shedding (95). These processes are integral to spillover events and thus understanding the complex relationship between the host and the virus is a key part of the transmission dynamic of which our understanding is severely lacking. Further research, specifically *in vivo* studies, are needed to further our understanding of the molecular mechanisms behind this suppression of symptomatic viral infections observed in numerous bat species.

EVIDENCE FOR ORIGINS OF BETACORONAVIRUSES

Embecoviruses

Human *Embecoviruses* (previously lineage A *Betacoronaviruses*) consist of HCoV-OC43 and HCoV-HKU1. Both HCoVs are globally endemic and most often present clinically as the common cold through upper respiratory tract infections (16). Rarely these viruses can cause more severe illnesses such as pneumonia, especially in immunocompromised individuals (96) and detection of HCoV-OC43 in patients with encephalitis hints that this virus has limited neuroinvasive capacity (97–99). Studies suggest both HCoV-OC43 and HCoV-HKU1 originated from rodents (42). This notion has gained support through the discovery of *Embecoviruses* in rats from Norway and south western China (16, 100–102) in addition to the high degree of sequence homology of HCoV-HKU1 and mouse hepatitis virus (MHV) (16). Further evidence came with the identification of China Rattus CoV HKU24, an *Embecovirus* found in Norwegian rats in 2015 and it is believed to represent a lineage of CoVs that were present before HCoV-OC43 spilled over into humans in the late 1800s (100). This occupies an early branch of the *Embecovirus* subgenera and provides more support to suggest that rodents may be an important and understudied reservoir for *Embecoviruses*.

Sarbecoviruses

SARS-CoV

The *Sarbecoviruses* consist of SARS-CoV and SARS-CoV-2, two of the most pathogenic coronaviruses identified to date (103). SARS-CoV was originally believed to have emerged from *Paguma larvata* (masked palm civets) after a case of SARS transmission from masked palm civets to humans (83, 104). However, this changed in 2005 when SL-CoVs were discovered in *Chiroptera* spp. bats, suggesting that bats may be the true reservoir for SARS-CoV (105) and that civets may instead be an intermediary host. This is not entirely a surprise as there are many coronaviruses which have been identified in various bat species, and SL-CoVs have been found in bat species such as *Rhinolophus* spp., *Hipposideros* spp. and *Chaerophon* spp. (5, 89, 104, 106). More specifically, SL-CoVs that bind ACE2 to mediate cell entry have been found in *Rhinolophus sinicus* (Chinese horseshoe bats) (65, 107). Further, in 2017 there were 11 new SL-CoVs identified in *Rhinolophus sinicus* bats from a cave in Yunnan province, China that shared 92-99% sequence homology to SARS-CoV (91). Today, *Rhinolophus sinicus* is considered the main reservoir of SL-CoVs and should likely be considered the origin point of SARS-CoV as well (108).

SARS-CoV-2

While SARS-CoV-2 has not been identified in bats to date bats are known to be an original source of alpha- and betacoronaviruses, with other Sarbecoviruses similar to SARS-CoV-2 known to be harboured in *Rhinolophus* spp. bats (109, 110). However there remain only speculations about the origins of SARS-CoV-2 with no direct evidence for the original source, leaving room unfortunately for wild hypotheses around the origins of this virus. The existence of a major virology laboratory (Wuhan Institute of Virology) with a program studying coronaviruses in the city where SARS-CoV-2 was first identified proved to be too large a coincidence for some, subsequently birthing several lab origin hypotheses (14). The first claim is of the virus being of manmade origin involves the observation of human immunodeficiency virus (HIV) sequences in the SARS-CoV-2 genome in a now retracted article by Pradhan et al. and again in another article (111). These findings were quickly refuted through bioinformatic analyses demonstrating the four short sequences occurred at different times and independent of each other and are insufficient evidence for a common ancestor (112, 113). Similarly there have been hypotheses around creation of a virus through gain of function experiments with both recombination and engineered mutations through serial passaging in animal models to obtain these changes to a SL-CoV suggested as possible routes of engineering this recombinant virus (114, 115). However, many of these proposed mutations may be present in other coronaviruses such as the furin cleavage site observed in the RBD of SARS-CoV-2 or a N501Y mutation that would have occurred for efficient replication in animal models (which was not observed in the early stages of the pandemic) and there remains no evidence of engineering within the RBD with the

only explanation for their presence being viral evolution (14, 34, 116–121). There has been concern that the emergence of the virus into the human population may have resulted from a laboratory release. The two main hypotheses are, SARS-CoV-2 was created through gain of function experiments on related viruses *via* serial passaging though this has been refuted (34, 122, 123). The second hypothesis has posited that an accidental laboratory release of SARS-CoV-2 precipitated movement of the virus from the laboratory to the community though this has also been refuted (29, 122). Recent investigation of SARS-CoV-2 genomic diversity by Pekar and colleagues has provided evidence to support the emergence of SARS-CoV-2 through multiple zoonotic events (124). The analysis supports that SARS-CoV-2 lineages A and B resulted from at least two separate spillover events into humans in late 2019.

However, despite these hypotheses there are historical patterns of zoonotic emergence and circulation for coronaviruses as well as the increasing identification of SARS-CoV-2 in numerous nonhuman animal species (14, 125). This is supported by the similarity in the route to human exposure through animal markets as this follows previous coronavirus outbreaks (39). There remains much to be understood about SARS-CoV-2 spillover into humans, and much of our current understanding has relied on epidemiological modeling. Molecular clock modeling of the genome for SARS-CoV-2 and the subsequent mutations suggest a recent emergence, some suggest however, this may not be true due to the highly mutated state of the genome and the effect this may have had on the linearity of the analysis (126–128). As a recent phylogenetic analysis suggests that the lineage of SL-CoVs that SARS-CoV-2 originates from diverged from ancestral bat CoVs sometime between 1948-1982 leaving the door open to the possibility of circulation under the radar (103). It is also unknown if a bat was the only animal involved in the evolution and emergence of SARS-CoV-2. Ongoing and future studies will continue to provide context and nuance for these questions.

In 2020, a group of researchers collected and sequenced samples from *Rhinolophus* spp. bats in Yunnan province, China, to better understand the zoonotic origins of SARS-CoV-2. Of interest, two bat CoVs had high nucleotide sequence homology to the full-length genome of SARS-CoV-2: RaTG13 (96.1%) and RmYN02 (93.3%) (129, 130). These viruses were collected from *Rhinolophus affinis* and *Rhinolophus malayanus* bats, respectively. While RaTG13 maintained high nucleotide sequence homology to the S gene (92.9%) and the RBD (85.3%), RmYN02 showed far lower sequence homology for the S gene (71.9%) and RBD (61.3%). In contrast, they also compared Pangolin viruses GD/2019 and GX/P5L/2017 to SARS-CoV-2 and found lower levels of nucleotide homology for the whole genome (GX/P5L/2017 = 85.2%) but high levels of amino acid sequence homology in the S gene (GX/P5L/2017 = 92.4% and GD/2019 = 90.7%) and RBD (GD/2019 = 97.4% and GX/P5L/2017 = 86.8%) (130, 131). It seems that while pangolins may have been involved in a recombination event affecting the RBD, the high level of homology between the genome sequences of SARS-CoV-2 and bat CoVs suggest that the virus originated from bats rather than pangolins (132, 133).

To further investigate the bat origin hypothesis, investigations have assessed the selective pressures driving viral adaptation and evolution. MacLean and colleagues identified weak purifying selection among SARS-CoV-2 strains from the first 11 months of the pandemic (36). For the spike protein, diversifying selection occurred deeper in the phylogenetic branches of the *Sarbecovirus* clade, leading to a very generalist SARS-CoV-2 virus and is supported by the wide host range (36, 134). Others have suggested that the closest ancestral divergence of this virus is likely to be approximately four or five decades ago, based on similarities to bat CoVs RmYN02 and RaTG13, respectively (135, 136). Additionally, investigations have looked at the CG suppression within the viral genome (cytosine followed by a guanine in the 5' to 3' direction) due to their link to antiviral mechanisms in the host (137). Many vertebrate RNA viruses demonstrate similar patterns of 5'-CG-3' dinucleotide suppression, where there is a lower number of CG dinucleotides than expected, as found within vertebrate genomes (137). Further, Takata and colleagues suggested that this suppression may highlight an adaptation with RNA viruses to evade host immunity through reduced discrimination of self- and non-self RNA (137). Analysis of the *Sarbecovirus* clades identified a phylogenetic shift towards CG suppression followed by an elevated substitution rate (36). This suggests an increase in selective pressure in the surrounding environment at the time (138, 139). These evolutionary factors taken together suggest that the virus evolved prior to the spillover event into humans, rather than through human-to-human infection during the pandemic (103). Thus, it is likely that SARS-CoV-2 was highly capable of infecting humans prior to the spillover event which led to the first COVID-19 case (140). Adding to this, the fact that SARS-CoV-2 can transmit readily to other animals (mink, cats, dogs, etc) - and in some cases transmit back to humans (mink) - supports the possibility of a generalist virus, where the virus already contained generalist ACE2 binding properties that could aid in efficient host switch across multiple intermediate animal species (139, 141).

Merbecoviruses

The *Merbecoviruses* (formerly lineage C betacoronaviruses) consist of MERS-CoV, the only known *Merbecovirus* to infect humans, as well as several bat CoVs including HKU4 and HKU5 (61). The Egyptian tomb bat, *Taphozous perfortus* is believed to be the reservoir species for MERS-CoV (142). Fecal samples collected from a trapped Egyptian tomb bat tested positive for MERS-CoV in the same region that the MERS-CoV index patient was found (143). These findings have been supported by studies demonstrating that the bat receptor dipeptidyl-peptidase 4 (DPP4) potentially co-evolved with MERS-CoV, supporting Egyptian tomb bats as an appropriate reservoir for MERS-CoV (102). HK4U and HK5U share over 65% amino acid sequence identity to MERS-CoV (61, 144); HKU4 was first identified in lesser bamboo bats, while HKU5 has been found to circulate in Japanese pipistrelle bats (7). Using molecular clock analysis, studies have shown MERS-CoV, HKU4, and HKU5 have a common ancestor as recently as several centuries ago

(100). Due to this phylogenetic ancestry, researchers believe the possibility of a species jump is high enough to warrant further surveillance (61, 145).

INTERMEDIATE HOSTS OF BETACORONAVIRUSES AND THEIR ROLE IN SPILLOVER EVENTS

It is generally accepted that HCoV spillover to humans is often facilitated through an intermediate host (4, 102). Intermediate hosts are animals that are biologically similar to the natural reservoir host and more frequently come in contact with humans; therefore, these hosts allow for opportunity to mutate to a form that is more easily transmissible to humans (146). There is some consensus on the intermediate hosts of three of the five human betacoronaviruses: SARS-CoV (masked palm civets), MERS-CoV (camelids) and HCoV-OC43 (bovine) (21, 74, 147). The same cannot be said for the remaining two HCoVs, HCoV-HKU1 and SARS-CoV-2, where intermediate hosts are the subject of continuing investigation. It is thought that each of these viruses have individually spilled over into intermediate hosts facilitating zoonotic transfer to humans (130, 132). The known and proposed intermediate hosts of human betacoronaviruses are presented in the following sections. Nonhuman animal species that have been reported to be susceptible to *Betacoronavirus* infection are presented in **Table 1**.

Embecovirus Intermediate Hosts

Both known human embecoviruses (HCoV-OC43 and HCoV-HKU1) are suspected to use an intermediate host in their emergence. While it is generally accepted that HCoV-HKU1 likely originated from rodents as it is related to MHV (16, 100, 101), there is no evidence that points to an intermediate host for this virus. One hypothesis is that HKU1 was transmitted directly from rodents to humans, either through contact with rodent excrement, exposure to their blood or other biological products, or through consumption of rodents (150). HCoV-OC43 is also believed to have originated in rodents however, this virus has 96.6% sequence identity to Bovine Coronavirus (BCoV) (16, 168) and is therefore thought to have used cattle as an intermediate host between rodents and humans (102, 148). Estimations based on evolutionary rates of betacoronaviruses place HCoV-OC43 spillover from cattle into humans around 1890 (16, 148, 169). Serological studies have shown that HCoV-OC43 antibodies are also present in other animals including llamas, alpacas, guanaco, and Bactrian camels (149, 170). These animals may have been exposed to HCoV-OC43 or BCoV if they had previous close contact with cattle, demonstrating the wide intermediary host range embecoviruses and other betacoronaviruses can have (171).

Sarbecovirus Intermediate Hosts – SARS-CoV

In 2002, the first recorded emergence of a *Betacoronavirus* that was highly pathogenic in humans occurred (80). The emergence of SARS-CoV and subsequent epidemic ignited interest in the origin of betacoronaviruses as prior to these, HCoVs were not considered global health threats (4). Zoonotic transmission of SARS-CoV was considered early on due to the fact that many of the early SARS cases appeared to have a common connection to an animal market in Shenzhen, China (80, 102). An investigation into animals sold at the market identified both SARS-CoV and another SARS-related coronavirus in palm civets (*Paguma larvata*) and racoon dogs (*Nyctereutes procynoides)*, and SARS-CoV antibodies in Chinese ferret badgers (*Melogale moschata*) (80, 151, 172). Evidence of palm civet-to-human transmission arose during a small SARS outbreak in Guangdong Province in 2003-2004 where four individuals tested positive for SARS-CoV and three of these patients had either direct or indirect contact with masked palm civets (21). While this provided evidence that palm civets may have been a source for the 2003-2004 SARS outbreak, it does not indicate that civets were the original source for the jump of SARS-CoV to humans. It was found that SARS-CoV isolates from palm civets at the Shenzhen market had 99.6% sequence identity to SARS-CoV samples collected from infected patients (21); however, there was a 1000-fold difference in their affinity for the human ACE2 receptor (173, 174). Additionally, while ~80% of the animals tested in Guangdong had SARS-CoV antibodies, infectious virus was not recovered from additional samples collected from wild and farmed palm civets (40). These data suggest that SARS-CoV does not naturally circulate in palm civet populations and it was likely introduced in the markets through storage of animals in close quarters (21, 80, 171). The high degree of sequence homology and lack of mutations suggests that SARS-CoV may have recently spilled over into masked palm civets not long before spillover to humans (175).

Additional animal species susceptible to SARS-CoV have been identified including house cats, ferrets, Chinese ferret badgers and racoon dogs; however, limited study has occurred in these species (152, 171). The route and timing of SARS-CoV transmission to raccoon dogs in the Shenzhen market remains unknown (21). Investigation of raccoon dogs at a market in Guangzhou, China, did identify SARS-CoV antibodies (21). To date, available evidence supports the hypothesis that masked palm civets acted as an intermediate host for SARS-CoV.

Sarbecovirus Intermediate Hosts – SARS-CoV-2

Investigations on SARS-CoV-2 origins and potential intermediate hosts have focused on the linkages of patients to the Huanan market in Wuhan (176). Approximately two-thirds of patients from the initial cluster of COVID-19 cases in 2019 had visited this market prior to contracting the virus while others were in contact with people involved in live animal trade (133). There were also similarities to the emergence of SARS-CoV in Foshan and Guangzhou, Guangdong, China in 2002 (177). Yunnan province has been hypothesized as the originating region for SARS-CoV and SARS-CoV-2 since the discovery of animal traders with prevalence of SL-CoVs and high IgG levels there in 2003 (177). Sampling of various animals pointed to bats as one of the early candidates for zoonotic transmission due to the presence of SL-CoVs with high levels of homology to SARS-

TABLE 1 | Nonhuman animal species identified to be susceptible to *Betacoronavirus* infection. Source of evidence, confirmation of infection and suggested role in viral transmission are provided.

Viral Genus	Viral species	Host Species	Latin name	Infection Evidence	Suggested Role	Evidence	References
Embecovirus	HCoV-OC43	Rodents	*Rodentia*	Natural	Reservoir host	Hypothesized	102, 148
	HCoV-OC43	Cattle	*Bos taurus*	Natural	Intermediate host	Hypothesized	102, 148
	HCoV-OC43	Llama	*Llama glama*	Natural	Intermediate host	Serology	149
	HCoV-OC43	Alpacas	*Llama pacos*	Natural	Intermediate host	Serology	149
	HCoV-OC43	Guanaco	*Llama guanicoe*	Natural	Intermediate host	Serology	149
	HCoV-OC43	Bactrian camels	*Camelus bactrianus*	Natural	Intermediate host	Serology	149
	HCoV-HKU1	Rodents	*Rodentia*	Natural	Reservoir host	Hypothesized	150
Sarbecovirus	SARS-CoV	Horseshoe bats	*Rhinolophus*	Natural (hypothesized)	Reservoir host	Hypothesized	5, 106
	SARS-CoV	Palm civets	*Paradoxurus hermaphroditus*	Natural	Intermediate host	Isolates closely matched patients	21
	SARS-CoV	Racoon dogs	*Nyctereutes procyonoides*	Natural	Intermediate host	Evidence for infection	151
	SARS-CoV	Ferrets	*Mustela furo*	Experimetal	Intermediate host	Experimental infection	152
	SARS-CoV	Chinese ferret badgers	*Melogale moschata*	Natural	Intermediate host	Serology	151
	SARS-CoV	Domestic cats	*Felis catus*	Experimental	Dead end host	Experimental infection	152
	SARS-CoV-2	Horseshoe bats	*Rhinolophus*	Natural	Reservoir host	Hypothesized	132, 133
	SARS-CoV-2	Malayan pangolins	*Manis javanica*	Natural	Intermediate host	Hypothesized	153
	SARS-CoV-2	White tailed deer	*Odocoileus virginianus*	Experimental	Intermediate host	Experimental infection/	154
	SARS-CoV-2	Domestic cats	*Felis catus*	Both	Intermediate host	Serology	155; 156
	SARS-CoV-2	Rabbits	*Oryctolagus cuniculus*	Experimental	Intermediate host	Experimental infection	157
	SARS-CoV-2	Bank voles	*Myodes glareolus*	Experimental	Intermediate host	Experimental infection	158
	SARS-CoV-2	Syrian gold hamsters	*Mesocricetus auratus*	Experimental	Intermediate host	Experimental infection	159
	SARS-CoV-2	Deer mice	*Peromyscus maniculatus*	Experimental	Intermediate host	Experimental infection	160
	SARS-CoV-2	Bushy tailed wood rats	*Neotoma cinerea*	Experimental	Intermediate host	Experimental infection	160
	SARS-CoV-2	Skunks	*Mephitis*	Experimental	Intermediate host	Experimental infection	160
	SARS-CoV-2	Mice	*Mus musculus*	Experimental	Intermediate host	Experimental infection	161
	SARS-CoV-2	Racoon dogs	*Nyctereutes procyonoides*	Experimental	Intermediate host	Experimental infection	162
	SARS-CoV-2	Rhesus macaque	*Macaca mulatta*	Experimental	Dead end host	Experimental infection	163
	SARS-CoV-2	Cattle	*Bos taurus*	Experimental	Dead end host	Experimental infection	164
	SARS-CoV-2	Tigers	*Panthera tigris*	Natural	Dead end host	Serology	165
	SARS-CoV-2	Dogs	*Canis familiaris*	Both	Dead end host	Serology	166; 156
	SARS-CoV-2	Snow leopards	*Panthera uncia*	Natural	Dead end host	Serology	165
	SARS-CoV-2	Gorillas	*Gorilla gorilla*	Natural	Dead end host	Serology	165
	SARS-CoV-2	Lions	*Panthera leo*	Natural	Dead end host	Serology	165

(Continued)

TABLE 1 | Continued

Viral Genus	Viral species	Host Species	Latin name	Infection Evidence	Suggested Role	Evidence	References
	SARS-CoV-2	Cougar	*Puma concolor*	Natural	Dead end host	Serology	165
Merbecovirus	MERS-CoV	Egyptian tomb bat	*Taphozous perforatus*	Natural	Reservoir host	Isolates match index patient	142, 143
	MERS-CoV	Dromedary camels	*Camelus dromedarius*	Natural	Intermediate host	Hypothesized	4
	MERS-CoV	Alpacas	*Llama pacos*	Both	Dead end host	Serology	167
	HKU4	Lesser bamboo bats	*Tylonycteris pachypus*	Natural	Reservoir host	First Identified	7
	HKU5	Japanese pipistrelle bats	*Pipistrellus abramus*	Natural	Reservoir host	Found to circulate	7

CoV-2 like viruses (RaTG13 and RmYN02) (178). In-depth phylogenetic analysis suggests that SARS-CoV-2 is a generalist virus which has been circulating in bats for some time, and that there was little mutation and adaptation required to be capable of infecting humans (36). This is highlighted by the fact that many animals can host productive SARS-CoV-2 infections and some animals, such as mink, are capable of transmitting the virus back to humans (141). In the following sections, we will examine the possible intermediate hosts of SARS-CoV-2 and what they may have contributed to the evolution of this virus.

Potential Intermediate Hosts and Animals With Role in Viral Dissemination

We summarize the growing evidence of SARS-CoV-2 circulation and transmission patterns across various animal species in **Figure 3**. Ferrets (*Mustela putorius furo*) are instrumental models for studying respiratory pathogenicity and transmission of viruses (181). Experimental studies show that ferrets are susceptible to SARS-CoV-2 infection of the upper respiratory tract early in the disease course (166). However, clinical signs of illness appear to be uncommon with transient fever, and mild respiratory symptoms reported (166, 182–185). There is still substantial viral shedding observed in the respiratory tract during infection making ferrets a useful model for studying transmission. During infection ferrets have been shown to infect healthy ferrets in close contact through the high degree of viral shedding in their feces, nasal secretions, urine and saliva (12). Direct and indirect transmission of SARS-CoV-2 has been demonstrated in ferrets and healthy ferrets become symptomatic following direct contact with infected ferrets, though separation of the animals with maintenance of shared airspace did result in some viral positivity in the absence of symptoms (139). The lack of clinical signs of illness in conjunction with the high amount of viral shedding suggest mostly asymptomatic infection and evidence that mustelids such as ferrets may have played a role as an intermediate host in SARS-CoV-2 emergence. While infection of ferret badgers has not been documented, they have a high degree of similarity to other animals which are permissive to SARS-CoV-2 infection and therefore are still an animal of interest.

Mink (*Neovison vison*) are also a potential SARS-CoV-2 intermediate host given the SARS-CoV-2 detection and onward transmission in mink from two farms in the Netherlands (186). The animals showed respiratory and gastrointestinal symptoms (187), and approximately 1.2-2.4% of animals succumbed to infection, the majority of which were pregnant females (188). Necropsies found signs of interstitial pneumonia and lung lesions (186). There is supportive evidence that SARS-CoV-2 was introduced to mink by farm workers with subsequent transmission between the animals (186). This was supported by sequencing of viral samples from both mink and humans which revealed significant homology between the viruses present in each sample (187). This became a cause for concern as it was also observed that variants of the virus had been transmitted from mink to humans (189), suggesting an intermediate host which could support viral recombination and rapid transmission (190). Transmission between humans and mink was observed in ten countries: Canada, Denmark, France, Greece, Italy, Lithuania, the Netherlands, Spain, Sweden and the USA (190, 191). It was determined early on that the mode of transmission was not direct as mink are housed separately. Viral RNA was also detected in early collection of inhalable dust samples indicating a potential route of exposure (186).

Raccoon dogs (*Nyctereutes procyonoides)* initially gained attention as possible intermediate hosts of SARS-CoV (21, 80, 151) for two main reasons: i) susceptibility to SARS-CoV infection (192); and ii) high ACE2 sequence similarity between raccoon dogs and humans (193, 194). Raccoon dogs can be productively infected with SARS-CoV-2 through experimental inoculation and transmit the virus to other healthy animals (162). However, clinical signs of illness such as increased body temperature or weight loss were not observed and virus isolated from infected animals had 100% sequence homology to the viral inoculum.

The susceptibility of white tailed deer (*Odocoileus virginianus*) to experimental infection was assessed in early 2021 (154). Intranasal inoculation of deer resulted in virus shedding through nasal secretions and transmission to naïve deer who later seroconverted. Indirect transmission was also observed as virus was identified in nasal swabs and transiently in fecal samples from naïve fawns housed in separate pens from infected animals, and which included plexiglass barriers (154). Additionally, RNA was detectable in infected animal tissues for up to 21 days post-infection. Other woodland animals such as bushy-tailed wood rats and skunks are capable of shedding virus in respiratory secretions (160). As these animals are shedding the virus and, in some cases, appear capable of transmitting to their

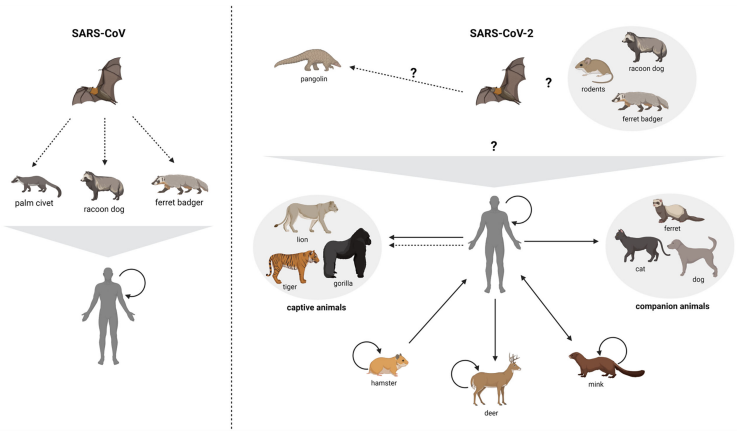

FIGURE 3 | Zoonotic circulation of SARS-CoV and SARS-CoV-2. Suspected and confirmed zoonotic circulation of both SARS-CoV and SARS-CoV-2 are presented. Suspected routes of transmission are presented by dashed arrows. Question marks designate routes that have yet to be demonstrated by direct or indirect methods (though are theorized or probable). Note that there is epidemiological and genetic evidence for some human-to-captive animal transmission events (179, 180) while others are suspected/probable. Solid lines represent confirmed transmission events. Created with BioRender.com.

surrounding environments, their role as intermediate hosts and potential future sources of spillback of novel SARS-CoV-2 variants to humans needs to be investigated.

Rodents are believed to have played a significant role in the emergence of human *Embecoviruses* (HCoVs OC43 and HKU1) and have therefore been hypothesized as potential intermediate hosts of SARS-CoV-2. Early studies suggested that mice were unlikely to be an intermediate host candidate for SARS-CoV-2 (130, 195, 196). However, subsequent work by Griffin and colleagues has demonstrated that deer mice are susceptible to infection resulting in asymptomatic or mild disease (197). Infected deer mice could also transmit virus to co-housed naïve mice. More recently, Stone and colleagues demonstrated that the SARS-CoV-2 Alpha and Beta variants could result in productive infection of wild-type C57BL/6 mice *via* intranasal inoculation (198). Alternatively, Syrian hamsters are susceptible to infection with SARS-CoV-2 presenting with clinical disease that resembles respiratory infection in humans as well as weight loss (159, 199–201). One study demonstrated that Syrian hamsters that had previous infection to SARS-CoV-2 had protection from re-infection with reduced replication in the upper respiratory tract and no observed transmission to naïve contact animals (202). Thus, hamsters have become widely used for investigations of SARS-CoV-2 infection (7, 161, 200). A recent preprint provides evidence for transmission of SARS-CoV-2 to humans from naturally infected hamsters, though the implications of this on the initiation of new human-to-human transmission chains remains to be determined (203).

Susceptibility of Additional Species to SARS-CoV-2

ACE2's ubiquitous presence within the animal kingdom and its high degree of similarity amongst mammalian species is a major contributor to the spread of betacoronaviruses around the world, most recently with SARS-CoV-2 (204). *In silico* modelling predicted the following species may exhibit binding affinity for the SARS-CoV-2 S protein: cats, cattle, monkeys, dogs, pigs, horses, sheep, and rabbits (in decreasing order) (205). Many other animals have been infected with SARS-CoV-2 experimentally or in nature and are able to host a productive infection. These cases will be discussed in the following paragraphs.

Initial attention was given to domestic animals such as dogs and cats as they would be high risk to transmit to humans if they could host a productive infection. Reports showed domestic cats had tested positive for SARS-CoV-2 in Europe, Asia, and North America (163, 205). Cats have also been shown to spread SARS-CoV-2 *via* respiratory droplets (166) though viral RNA has also been detected in nasal, oropharyngeal and rectal swabs (155, 206). This was concerning due to the proximity of cats to humans however, one study showed that cats exposed to the virus did not exhibit any symptoms (88, 207). There is conflicting data as to whether or not cats are asymptomatic throughout the infection (208), but if cats are in fact asymptomatic they may not be effective intermediate hosts. As for dogs, studies show that SARS-CoV-2 replicates poorly in these animals and that healthy dogs who come into contact with SARS-CoV-2-positive dogs remain seronegative (166). Overall, infected dogs showed no obvious signs of infection and most often did not have detectable levels of RNA present in biological samples (88, 166, 206). In an attempt to determine why this occurs, scientists analyzed the ACE2 receptor in dogs and found 5 amino acid substitutions but none within the RBD; therefore, this is believed to have minimal impact on binding of the S protein (196, 209). Based on the studies conducted so far it is unlikely cats or dogs played a role in the emergence of SARS-CoV-2.

Investigations into agriculturally significant animals such as cattle were an important consideration as there are previous bovine links to coronaviruses in addition to an identified bovine coronavirus (210). For the most part, cattle did not seem to be able to host a productive SARS-CoV-2 infection with little to no viral replication or immune response detected in these animals.

They also did not exhibit clinical signs of infection or transmit to nearby animals (164). Cattle and other farm animals are important to consider as they are commonly in contact with humans. One study in particular looked at susceptibility to SARS-CoV-2 in poultry and determined that chickens along with turkeys, geese, quail and ducks are not susceptible to infection (166, 211). Similarly, many studies have investigated SARS-CoV-2 infection in pigs, and it seems that they are resistant to infection as there are no signs of infection, no pathology and no viral RNA detected (212–214).

SARS-CoV-2 infections in a variety of zoo animals have been widely reported, including tigers, snow leopards, lions, gorillas and pumas. Animal testing followed signs of respiratory symptoms of disease and while transmission between animals in the same enclosures was reported, widespread transmission was observed in any of these cases (215). It is thought that the animals contracted the virus from asymptomatic workers.

Aside from these examples of natural infection, much of what we know of SARS-CoV-2 infection in animals has come from experimental infections. This has led to the discovery that rhesus monkeys can be experimentally infected (163). Two Rhesus macaque species (*M. fascularis and M. mallata*) and one common marmoset species (*C. jacchus*) were able to be infected with SARS-CoV-2 *via* the intratracheal and intranasal routes and demonstrated clinical signs of infection (216, 217). It has been reported old world monkeys are susceptible to infection and new world monkeys have much lower susceptibility when compared to human ACE2 there are 4 amino acid differences (218). Nonhuman primates have similar clinical presentation of COVID-19 to humans including viral replication in the upper and lower respiratory tracts, inflammation and focal edema among other less frequent signs and symptoms (13). Due to these clinical presentations, the phylogenetic relatedness of nonhuman primates to humans, and previous reports of HCoV-OC43 infections in nonhuman primates, concerns have been raised regarding the potential impacts of SARS-CoV-2 on endangered species, including great apes (88, 219).

Bank voles have been experimentally infected and had viral RNA present in nasal tissue for up to 21 days post infection, though no transmission to animals in direct contact was observed (220). Rabbits have been reported to be susceptible to infection with SARS-CoV-2 (164) however, there is conflicting data stating that in fact white cotton tail rabbits are resistant to infection (160). Additional studies have determined that squirrels (211), raccoons and black-tailed prairie dogs (160) are resistant to SARS-CoV-2 infection [Recommended further readings on the topic of experimental and natural infections of animals. (221–223)].

Evidence Regarding the Role of Pangolins

Malayan pangolins (*Manis Javanica*) and Chinese pangolins (*Manis pentadactyla*) are considered vulnerable or critically endangered as they are the most trafficked mammals in the world (224) due to the use of their meat and scales in traditional African and Chinese medicine (225). Pangolins are solitary, nocturnal mammals that dwell in remote sandy forests (226) away from humans (227), and thus, poaching provides the only real opportunity for human contact with wild populations (37). Pangolins were proposed as an intermediate host in the emergence of SARS-CoV-2 in the early days of the pandemic due to a high degree of sequence identity to pangolin-CoVs, their interaction with bat populations and their presence at the Hunan wet market during the time when the first documented cases of SARS-CoV-2 were identified (153).

Sequence similarity was identified by two independent studies that identified SL-CoV in Malayan pangolins confiscated from illegal wildlife traders (77, 228). The identified SL-CoVs had 85.5-92.4% sequence identity to SARS-CoV-2; however, these pangolin-CoVs had 97.4% sequence identity to the RBD of the SARS-CoV-2 S protein (specifically pangolin CoVs GD/P1L and GD/P2S) (77, 131, 132, 228). Specifically, the six amino acid residues in the RBD of the S1 protein identified as critical for binding the host ACE2 receptor are conserved between pangolin-CoVs and SARS-CoV-2 (34, 131, 229). This high degree of sequence identity in the RBDs of SARS-CoV-2 and pangolin-CoVs suggests either a recombination event occurred or these viruses have highly similar RBDs due to convergent evolution (77, 228). For the recombination hypothesis it was posited to have occurred between pangolin-CoVs from Malayan pangolins and RaTG13 from *R. affinis* bats due to the high degree of similarity in the RBD of pangolin-CoVs to SARS-CoV-2, while SL-CoV RaTG13 is the closest relative to SARS-CoV-2 albeit with a distinct RBD site of the S protein (77, 131, 132, 228). However, when analyzed through alignment there is low nucleotide similarity compared to the high amino acid similarity and many misalignments within the sequence, suggesting recombination is unlikely and merely convergently evolved features of these distinct SARS-CoV-2 and pangolin CoV viruses (77, 103, 172, 229, 230). Although recombination has been observed in CoVs there is no evidence in the S protein of the SARS-CoV-2 lineage and instead appears to be an artifact of the metagenomic analysis that detected recombination initially (103, 123). Supporting the idea of convergent evolution, a recent analysis of SL-CoV RaTG13, pangolin-CoVs and SARS-CoV-2 finding that pangolin-CoVs diverged from the SL-CoVs approximately 150-180 years ago (103). If recombination occurred it would be expected for these viruses to have a higher degree of similarity in specific regions, which has not been observed (29, 132, 174). For recombination to occur it also implies the viruses, in this case SL-CoV RaTG13 and pangolin-CoV GD/P1L or GD/P2S, would co-infect the same cell; however, *R. affinis* bats (Species SL-CoV RaTG13 was found in) do occupy the same natural range as *M. javanica* pangolins (122, 231, 232).

To date, pangolin-CoVs have not been found in Chinese pangolins, which share the same habitat range as *R. affinis* bats (228). There is limited evidence for cohabitation of bats with pangolins of any species and does not appear very common, with one study finding bats and pangolins in Gabon within the same burrows (233). Follow up studies to detect pangolin-CoVs in Malayan pangolins have been unsuccessful with a study of 334 confiscated pangolins finding no sarbecoviruses raising doubts

around the role of pangolins in the emergence of SARS-CoV-2 (123, 228, 234). Further to this point, a study of wild Malayan pangolins in Malaysia determined that there is no observed circulation for betacoronaviruses, filoviruses and flaviviruses, suggesting that any detected sarbecoviruses are most likely acquired through contact within the smuggling trade of these animals (234). The animals that pangolin-CoVs have been isolated were found to be either severely ill or already dead (82, 228). Animals becoming severely ill is not what is expected for intermediate hosts as this would greatly limit viral amplification through host immune response and ultimately death, as well as interactions with other species limiting ability to spread the virus to other species (110). Some have presented the possibility for contributing factors to the severe illness and death of the tested animals, including due to the stressful environment they find themselves in in close contact with other animals and humans as well as other viruses that are commonly found in pangolins including Sendai virus (235, 236). While some studies have shown the presence of coronaviruses in captivity (82), there remains no evidence of SARS-CoV-2 or SL-CoVs in wild pangolins. More to that point, Yuan *et al.* claim that pangolins were not present at the Huanan Wildlife Market during the initial identification of SARS-CoV-2 in 2019 (237). In addition to the lack of circulating coronaviruses, the solitary lifestyle of pangolins makes it difficult for pangolin populations to amplify pangolin-CoVs, which suggests they would be a poor intermediate or reservoir host candidate from an epidemiological standpoint (103). Considering the studies conducted to date on the role of Malayan pangolins in the emergence of SARS-CoV-2 as an intermediate host does not line up with the hypothesis and the initial phylogenetic reports have been refuted.

No Intermediate Host

There is continuing evaluation of the susceptibility and onward transmission potential of various animal species to SARS-CoV-2. However, it has also been hypothesized that SARS-CoV-2 emergence may have occurred in the absence of an intermediate host (36). Here, the authors suggest that diversifying positive selection was limited to the early phase of the pandemic and that SARS-CoV-2 has much weaker purifying selection as compared to related sarbecoviruses. The authors further suggest that the transmission of SARS-CoV-2 to additional nonhuman animal species supports the generation of a generalist virus in a bat reservoir.

Ongoing research continues to actively investigate the hypotheses for the emergence and transmission of SARS-CoV-2 demonstrating the collective effort of the scientific community to determine the origins of this pandemic, there is still much to be known and continued collaboration will be essential.

Merbecoviruses Intermediate Hosts

Dromedary camels were identified as the intermediate host of MERS-CoV after camels tested positive for virus with 100% sequence identity to viral isolates from humans that were infected through close contact with the animals (238). MERS-CoV can be transmitted from dromedary camels to humans *via* respiratory droplets as well as the fecal/oral route (4). It is postulated that MERS-CoV can spread between camelids when kept in close contact (167) but more studies are needed to confirm this (239). MERS-CoV outbreaks usually occur when an infected camel transmits to a human, who can then transmit to their close contacts (240). During the 2012 outbreak there were multiple lineages of MERS-CoV circulating, indicating that multiple zoonotic transmission events may have contributed to this outbreak (241). Dudas et al. estimate that hundreds of spillover events between camels and humans have resulted in the cases of MERS that we know of today (242). This is supported by the fact that neutralizing MERS-CoV antibodies have been found in dromedary camels in Africa, Asia and the Middle East (102, 243, 244). Experimental and serological studies have also shown that alpacas can be infected with MERS-CoV and may potentially serve as intermediary hosts however, this appears to be restricted to regions where MERS-CoV is endemic in dromedary camels (149, 167, 239, 245). Since MERS-CoV is believed to have spilled over into dromedary camels from bats more than 20 years ago (106, 244), it is thought that the camel coronavirus adapted to the dromedary camel hosts and therefore caused minimal health effects (242, 245, 246). For this reason, dromedary camels have been reclassified as the reservoir hosts of MERS-CoV (242, 245, 246).

DISCUSSION

Human Interactions With Zoonotic Coronavirus Hosts

Understanding how viruses emerge and the role that humans play in these emergence events are of central importance to early detection and prevention of large-scale outbreaks (204). As the human population grows, a greater area of land is being converted into farmland and housing to meet the demand. This naturally means that humans, and the domesticated animals that accompany them, will be overlapping more with wild animals like bats (15). Sharing habitats like this facilitates cross-species transmission of viruses and emergence of infectious diseases (4). However, eliminating all human contact with possible *Betacoronavirus* hosts is not feasible due to urbanization and in many cases the cultural and economic importance of these animals (247). A recent example of this is the role of dromedary camels in MERS-CoV circulation and spillover. Camels are a central part of the livelihoods of many families, providing a source of transportation, food, and commodity trade (248). Culling dromedary camels to prevent the spread of MERS-CoV would negatively impact the well-being of the people in these communities and the local economy (248). Preventative strategies for MERS instead relies on recognizing illness in camels, rapid testing, national surveillance, international communication, and the development of vaccines for dromedary camels to decrease MERS-CoV transmission to humans (249).

Surveillance and Monitoring for Future Zoonotic Outbreaks

The increase in globalization and urbanization over the last half-century have led to a dramatic change in both the mode and the

frequency in which humans and animals come in contact. As we continue to piece together the roles of intermediate hosts in zoonoses, including for SARS-CoV-2, we must continue to examine the magnitude of their role in viral emergence and subsequent public health emergencies. Public health responses to outbreaks have primarily been reactionary in nature (i.e. quarantines, travel restrictions, vaccine and therapeutic development) as opposed to preventative including global surveillance, pandemic prediction, early warnings and control (250, 251). Precautionary rather than reactive responses would seem far more logical given the global health and economic toll of the COVID-19 pandemic. One Health approaches to emerging virus surveillance and preparedness are critical in this regard given that ~ 60% of emerging infectious disease outbreaks are of zoonotic origin (domestic or wildlife), with almost 75% of zoonotic emergence events originating with wildlife (78). However, it is imperative to consider the potential for bidirectional transmission between humans and animals for emerging viruses, such as has been identified for SARS-CoV-2, when considering outbreak response and containment plans (252). Indeed, observations from mink and white-tailed deer during the COVID-19 pandemic highlight the importance of consideration for the complexities of routes of transmission and reservoir-host interactions (141, 253). However, there is ongoing development of emerging infectious disease surveillance systems which utilize wildlife screening techniques to sample and test for various pathogens in healthy animals, and monitor morbidity and mortality rates of regional animal species (254). This information can then be collated and relayed to additional research groups conducting similar surveillance programs across the globe (255). Understanding that the most effective way to combat future outbreaks is with a preventative/precautionary approach as opposed to a responsive/reactive approach, researchers continue to lobby for more holistic approaches to monitor animal-human interfaces (256). There are independently-funded holistic programs such as the One Health Project which takes into consideration environmental, animal, and human factors to understand and monitor disease spread from animals-to-humans as well as from human-to-animal (256). Surveillance work is currently focused on regions that have high potential for inter-species viral transmission. Some of these environmental factors include regions experiencing extreme effects of climate change, or regions with tropical rainforests, high population density, and high numbers of mammalian species (251). Based on these criteria, Sub-Saharan Africa, Southeast-Asia, and Latin America are the focus of current research into surveillance and are considered high risk regions for emerging infectious disease (EID) events to occur (257, 258). It will be of paramount importance to invest into monitoring and surveillance of animals in these regions, ensuring that any future outbreaks are detected early and minimized.

A One Health approach to limiting the exposure and spread of emerging disease is a better model for outbreak/pandemic response because despite the availability of vaccines for SARS-CoV-2 these alone are not enough with the likelihood of endemicity due to demonstrated instances of zooanthroponosis (259). As new variants of SARS-CoV-2 continue to emerge the protective coverage that vaccines have provided will continue to wane with diverging spike proteins and have demonstrated a comprehensive One Health approach is needed to bring the current and future pandemics under control (260). An approach of this kind would encompass public health and human vaccination campaigns already being implemented globally as well as animal vaccination campaigns and wildlife surveillance (259). The development and integration of animal vaccines for zoonotic viruses could have great impacts on zoonoses and zooanthroponoses as well as impacting reservoir establishments. Animal vaccination against SARS-CoV-2 have already been approved and demonstrated to be safe and effective with administration of the Zoetis vaccine being utilized at zoos, on mink farms as well as domestically (261). Early detection and prevention measures should be implemented within a One Health model beyond humans alone as humans are only a part of the story of outbreaks and pandemics (223, 260). Global collaboration and cooperation are necessary in tracing the source and will be necessary for mitigation of outbreaks and pandemics in the future (125).

Conclusions

Many facets of coronaviruses are yet to be uncovered. Here we provide a collection of evidence for the complexities of coronavirus transmission patterns across species. There are some important clarifications that have been identified for sarbecoviruses. For example, embecoviruses likely emerged from rodents with cattle acting as an intermediate host in HCoV-OC43 whereas HCoV-HKU1 is suspected to have used an intermediate host that has yet to be identified. The transmission of SARS-CoV to humans utilized an intermediate host believed to be palm civets or raccoon dogs and MERS-CoV utilizes dromedary camels as a reservoir and intermediate host following an original spillover event from bats to camels. Other human coronaviruses pose a greater challenge. While there is evidence that SARS-CoV-2 originated from bats, and there was likely the involvement of an intermediate host, the specific details of these events have yet to be conclusively determined.

It is critical to appreciate that while spillover events of viruses that rapidly become public health threats occur unpredictably, this should not preclude global investments in robust surveillance and prediction systems, in particular within regions that are 'hot spots' for emergence events. For decades infectious disease experts have studied how increased contact with wild animals – whether it be through deforestation, climate change, or other factors – leads to new diseases spilling over to humans. In our fast-growing world, expansion is not slowing (80–82). We should not expect the spillover rate of infectious diseases to humans to slow either. Thus, several strategies need to be utilized to limit the economic, health and social impacts of these events.

- Reduction of transmission risk through preventative hygiene measures and public health education campaigns in place early on following identification of a spillover of a new virus.

- Have a framework in place, infrastructure and trade agreements in place to allow for accelerated development and deployment of therapeutics and vaccines to all countries to shorten the duration of a pandemic through reduced risk of variants.
- Global education campaigns for risks of contact with certain wild and domestic hunted/farmed species as well as the sale and consumption of species. In addition to the appropriate aid work to find safe sustainable alternatives for impacted communities
- The identification of reservoir and intermediate hosts of known infectious diseases is important for the prevention of future viral outbreaks/pandemics through understanding the viral ecology of animal populations and the circulating pathogens within these animal communities.
- Proactively reduce the risk of spillover events through implementing ecosystem stewardship measures in conjunction with prioritizing climate change reduction measures and biodiversity conservation measures. While also ensuring access to proper sanitation, safe and sustainable food sources and clean water sources.

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

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Mutations in SARS-CoV-2 nucleocapsid in variants of concern impair the sensitivity of SARS-CoV-2 detection by rapid antigen tests

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Rapid antigen tests (RATs) are used as convenient SARS-CoV-2 tests to minimize infection risks in the private and public domain (e.g., access to shops, concerts, sports, and other social events). RATs are: however, less sensitive than quantitative reverse transcription Polymerase chain reaction (RT-qPCR) assays; hence, samples with low viral loads may be misdiagnosed. Reports on the ability of RATs to detect SARS-CoV-2 variants of concern (VOCs) Delta and Omicron are often only qualitative. We, therefore, examined the analytical sensitivities of four different RATs for the detection of both full virus and recombinant proteins of relevant VOCs. Since most RATs are based on the detection of the SARS-CoV-2 nucleocapsid protein (N-protein), we constructed multiple N-protein mutants (mirroring specific amino acid exchanges of VOC N-proteins) using prokaryotic expression plasmids and site-directed PCR mutagenesis. Testing of recombinant proteins by four RATs revealed amino acid substitutions R203K and R203M, are critical for the sensitivity of some RATs. Interestingly, R203M mutation completely abrogated antigen detection even at high protein concentrations in the Delta variant. As a proof-of-concept study, we show that one or two specific amino acid changes in the N-protein can negatively impact the analytical sensitivity of RATs. Hence, antibodies used in such lateral flow assays should be optimized and target preferentially more conserved regions of N-protein.

KEYWORDS

SARS-CoV-2, rapid antigen test, nucleocapsid mutation, variants of concern, sensitivity loss

Introduction

SARS-CoV-2 is an RNA virus constantly mutating and evolving, with new variants emerging over time. Only a few of those variants are of public health concern in humans because of their high transmission rates, severe pathogenicity, or ability to evade acquired immune responses (1). The five SARS-CoV-2 lineages B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.617.2 (Delta), and B.1.529 (Omicron) constitute variant groups of concern (VOCs) and hence have received vigilant monitoring for their potential impact on SARS-CoV-2 diagnostics, therapeutics, and vaccines (2). In late 2020, the Alpha was first detected in the UK, and was the most predominant cause of new cases worldwide in early 2021 (3). Also, Beta, initially found in South Africa in 2021, is 6-fold less susceptible to vaccine-derived neutralizing antibodies than the SARS-CoV-2 wild-type virus (4). Indeed, as SARS-CoV-2 variants become more contagious or influential on countermeasures, their potential to exacerbate the trajectory of the pandemic *via* triggering new waves increases. We have seen this with Delta, that has driven the deadly second wave of infections in summer 2021 in India (5), and also the more recent surge of Omicron. Currently, Omicron, with its sublineages, has been identified in more than 150 countries, outcompeting other variants and causing increasing numbers of infections, raising alarms for the need for immediate proactive measures (6). One of these measures is establishing novel platforms for validating the currently available diagnostic tools against the circulating variants.

Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) is the gold standard for accurate and reliable COVID-19 diagnostics. However, the time needed for sample collection, transport to the laboratory, the assays' performance, and patient notification are limitations of RT-qPCR. Besides, the need for specific chemicals, laboratory capacity, and trained staff to accurately execute RT-qPCR assays remains an additional immense challenge. Hence, the development of rapid antigen tests (RAT) for easy detection of SARS-CoV-2 by trained personnel and by ordinary persons was a big step forward to the expeditious testing of a broader scale of the human population. Unfortunately, compared to the RT-qPCRs, the RATs show a lower sensitivity (7), and therefore the confirmation of a negative result in a clinical sample may require confirmation by validated RT-qPCRs in peculiar cases (8).

Recently, several hundred RATs have become available in Germany, and more than 200 different RATs have been licensed for professional use, including 43 tests for self-application. A recent study using the Wuhan SARS-CoV-2 strain, evaluated these tests regarding their diagnostic specificity and sensitivity as listed by the Federal Institute for Drugs and Medical Devices (BfArM) (9, 10). Most of the evaluated RATs have values higher than 95% for both diagnostic criteria, and are therefore suitable for detecting high viral loads in the range of effective viral transmission. Anti-SARS-CoV-2 N-protein antibodies in RATs seem to be the most appropriate, as the nucleoproteins are abundant in infectious virus particles (bound to viral genomic RNA in the nucleocapsid complex) (11). Furthermore, most of the single nucleotide polymorphisms (SNPs) defining specific variants of SARS-CoV-2 are in the spike protein (S) of the viral particle (12, 13) and not in the nucleoprotein. Therefore, detecting a different protein with fewer SNPs should ensure, at least in theory, that RATs also recognize all variants.

In the first part of this study, we used a RAT from BioNote (Nowcheck) to analyze its sensitivity for the detection of non-VOC and VOCs. This particular RAT has been used in previous studies and has been found to detect the non-VOC Strain reliably. In a subsequent study, it was tested whether RATs from other companies yield the same results or whether they produce deviating results, especially for VOCs. For this purpose, RATs were selected from three vendors listed in the BfArM list with good results. Finally, the influence of individual amino acid exchanges typical of certain VOCs on the sensitivity of the RATs used was tested. Taking all parts together, we show that SNPs affect the functionality of RATS and are present not only in S but also in N genes of VOCs.

Materials and methods

Viruses and cells

Infections of Vero E6 (African green monkey (Chlorocebus spec.) kidney cells, Collection of Cell Lines in Veterinary Medicine CCLV, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany) were performed using the following viruses (Non-VOC hCoV-19/Germany/BY-ChVir-929/2020|EPI_ISL_406862|2020-01-28, Alpha hCoV-19/Germany/NW-RKI-I-0026/2020|EPI_ISL_751799|2020-12-07, Beta hCoV-19/Germany/NW-RKI-I-0029/2020|EPI_ISL_803957|2020-12-28, Delta hCoV-19/USA/PHC658/2021, and Omicron hCoV-19/Czech_Republic/KNL_2021-110119140/2021|EPI_ISL_6862005| 2021-11-26) as previously described (14). Cells were maintained in Eagle´s minimal essential medium (Lonza, Germany) with 8% foetal bovine serum (PAA) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Sigma, Germany) and incubated at 37°C under 5% CO_2 in locked boxes as previously published (15). Virus stocks were preserved at -80°C, and titers were calculated as $TCID_{50}$/mL in a biosafety-level 3 (BSL-3) laboratory at Friedrich-Loeffler-Institut, Germany, before using viruses for downstream analysis. Due to biosecurity reasons, all experiments using virus have been performed with heat-inactivated material. Heat inactivation of viruses was performed at 95°C for 20 minutes.

SARS-CoV-2 rapid antigen assays

For this study, four rapid antigen tests evaluated by the Paul-Ehrlich-Institut have been used; The BioNote NowCheck

COVID-19 Ag Test (Sensitivity 89,2%; 95% CI: 81,7% – 93,9%, Specificity 97.6%; 95% CI: 95.1% - 98.8%), the Panbio ™ Covid-19 Ag Rapid Test Device (Nasal) from Abbott (Sensitivity 98.1%; 95% CI: 93.2% - 99.8%; Specificity 99.8%; 95% CI: 98.6% - 100%), the SARS-COV-2 ANTIGEN SCHNELLTEST from Xiamen Boson Biotech Co., Ltd (Sensitivity 96.17%; 95% CI: 94.04% - 98.29%, Specificity 99.16%; 95% CI: 98.49% - 99.83%) and theSARS-CoV-2-Antigen-Schnelltest-Kit from Triplex International Biosciences (China) Co., LTD (Sensitivity 96.17%; 95% CI: 94.04% - 98.29%, Specificity 99.16%; 95% CI: 98.49% - 99.83%). All these assays optimally test human naso/oropharyngeal swabs and are based on SARS-CoV-2 N-protein by using two anti-N-antibodies; a dye-labeled antibody and immobilized fixed antibody. We tested sensitivities of these RATs for the detection of heat-inactivated viruses by following the previously published protocol (16). For the investigations of recombinant proteins, we diluted the proteins in PBS to 8.333 ng/µl (corresponding 1000 ng/120µl). From this amount, we further diluted proteins to 100, 50, 25, 10 and 1 ng/120µl (8.33 pg/µl) in LFD extraction buffer and inoculated these amounts on RATs. Depending on the type of swab used, up to 1450 ng/ml of nucleocapsid were extracted in elution buffer, if 5 x 10^5 virus particles were loaded experimentally (17). Wolfel et al. showed that 6.76 × 10^5 genome copies per swab were present until day 5 after symptom onset, and it was concluded that at least 10^6 viruses per ml were needed for successful cultivation (18). Assuming that 10^6 viral particles are indeed collected with a swab when sampling a patient with symptoms, this means an estimated amount of 1.5 ng nucleocapsid in approx. 300 µl elution buffer for one RAT, correlating to a concentration of 5 pg nucleocapsid/µl buffer. All concentrations used were above this value. All experiments were carried out in duplicates at least.

Detection of SARS-CoV-2 RNA by real-time RT-PCR

Viruses from diluted cell culture supernatants were subjected to RNA extraction using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following their standard protocol. Viral RNA was analyzed by CFX real-time PCR systems (Bio-Rad, Germany) using real-time RT-PCR kits (SuperScript III Reverse Transcriptase, Invitrogen, Germany) and the *envelope* E gene primers and probes set (19). Dilutions of RNA isolates from standardized samples (provided by INSTAND e.V., Germany) were used to generate standard curves.

Scoring and densitometric analysis of RATs

Immediately after 15 min incubation, RATs were visually assessed (at least four-eyes-principle) as follows: - no test line visible; +: test line weakly visible; ++: test line less intensive than control line; +++: test line as intensive as control line or more intensive. For densitometric analysis, RATs were digitalized using either a Canon flatbed scanner or the BioRad Chemidoc Imaging system. Digitalized images were composed with Adobe Photoshop CS5 software (version 12.0 x64) and densitometrically analyzed using ImageLab 6.0.1 software. Adjusted volumes of control lines and test lines (internal units) were used to normalize the ratio of the test line to the control line.

Cloning of N gene

Sequences of the N-protein open reading frame (ORF) were cloned into the pET19b prokaryotic expression plasmid (Novagen; Cat. No.69677-3) by the use of specific primers (Table 1). The forward primer contains a *NdeI* recognition site, and the reverse primer harbors a *BamHI* recognition site for the directed insertion into the expression plasmid. Amplified PCR products were first cloned into the pCR2.1 vector by TA cloning, and an internal *NdeI* site was silently mutated by site-directed PCR mutagenesis where necessary. Using the *NdeI* and *BamHI* (New England Biolabs, MA; USA), the N protein-coding region was inserted with an 8x histidine peptide (His-Tag) into the vector pET19b. Sequence identity and correct in-frame insertion were verified by Sanger sequencing (Eurofins Genomics, Germany) with standard sequencing primer T7 and T7term. Geneious Prime® 2021.0.1 was used to analyze the constructs and sequences.

TABLE 1 Primer for cloning of the N-proteins (*NdeI* and *BamHI* sites are underlined).

Primer	Sequence 5'➔ 3'	Binding region regarding reference sequence GISAID accession number EPI_ISL_402124
N-28220-F	TACCATATGATGTCTGATAATGGACCCCAAAATCA	28,265 – 28,299
N-29479-R	AATATGGATCCTTATGATTAGGCCTGAGTTGAGTCAGCA	29,550 – 29,512

➔ defines direction (5' to 3').

PCR mutagenesis of N-protein expression plasmids

Complementary mutagenesis primers (Table 2) were designed and used to induce in-frame single nucleotide exchanges in VOCs N genes. These nucleotide exchanges result in substituting one specific amino acid or two neighboring amino acids (Supplementary Figure 3). We used the described primers in Table 2 to amplify the pET19b-N-VOC template by 20 PCR rounds Subsequently, plasmids were digested with DpnI to remove methylated target DNA. Plasmids were further transformed into E. coli strain XL1 and plated onto LA-Amp agar plates. One single colony from a plate was amplified in LB-Broth on a Mini-prep scale. The identity of isolated plasmids and nucleotide exchanges were further proved by Sanger sequencing using the standard T7 and T7term primers.

Production of recombinant SARS-CoV-2 N-proteins

E. coli bacteria of the BL21(DE3) strain were transformed with the non-VOC-N-protein and N-protein-mut expression plasmids. Protein expression was induced by adding IPTG to the LB-Broth growing medium at an OD600nm of 0.6. After 4 hours of expression, bacteria were sedimented, and His-tagged protein was isolated using Ni-NTA (nickel-nitrilotriacetic acid) technology (Thermo ScientificTM, Germany) according to the manufacturer's instructions and under denaturing conditions described elsewhere. Expression of specific proteins has been confirmed by Coomassie SDS-PAGE and Western blot using His-Tag specific antibody (Supplementary Figure 1). The concentration of the proteins was measured in a microtiter plate using Nanoquant (Carl Roth, Germany), a modified Bradford method (20).

Results

SARS-CoV-2 variants are detected with reduced sensitivity by rapid antigen assays

To test the analytical sensitivity of RATs against SARS-CoV-2 VOCs, equal amounts of heat-inactivated viruses (non-VOC, Alpha, Beta, Delta, and Omicron) were loaded onto the BioNote/NowCheck RATs. These amounts correlated to Ct values (of 24, 27, 30, and 33) known to be in or close to the detectable range of RATs limit of detection (LoD) (16). To confirm the similar amounts of viral antigen in the dilution, Western blot analysis using 20 µl of the Ct 24 dilution has been performed (Supplementary Figure 4). Fifteen minutes post-application, the images of RATs were recorded, and test line intensities were quantified by densitometric analysis. The highest concentrations, corresponding, to Ct of 24 for all strains (non-VOCs and VOCs) were detected by the BioNote RAT (Figures 1A–E). However, cell culture supernatants correlating to Ct values of 27 and 30, showed only positive results in the case of non-VOC (Figure 1A). Visual examination of RATs with Alpha and Beta revealed a negative result, indicating a lower sensitivity of this RAT toward these variants (2 logs, Figures 1B–F).

Interestingly, Delta and Omicron showed a faint test line in the sample corresponding to Ct 27 (Figures 1D, E), albeit it was also detected with a lower sensitivity by this RAT as shown in Figures 1D–F (1 log lower than the non-VOC strain). Dilutions corresponding to Ct value 33 were not detectable for any viral strains, as they are likely to be beyond the LoD of this particular RAT (16). Together, these data confirm the lowered sensitivity of this RAT to detect Alpha, Beta, Delta, and Omicron (Table 3).

TABLE 2 Primer used for PCR mutagenesis.

Mutation	Backbone	Primer	Sequence (5' ➔ 3')
Internal *NdeI* deletion		N-Ndemut-F	GTGCTAACAAAGACGGCATTATCTGGGTTGCAACTGAGGG
		N-Ndemut-R	CCCTCAGTTGCAACCCAGATAATGCCGTCTTTGTTAGCAC
K203R L204G	VOC Beta	N-Mut1-Fw	CAACTCCAGGCAGCAGTAGGGGAACTTCTCCTGCTAGAAT
		N-Mut1-Rev	ATTCTAGCAGGAGAAGTTCCCCTACTGCTGCCTGGAGTTG
R203K	Non-VOC	N-Mut2-Fw	CAACTCCAGGCAGCAGTAAAGGAACTTCTCCTGCTAGAAT
L204G	VOC Beta	N-Mut2-Rev	ATTCTAGCAGGAGAAGTTCCTTTACTGCTGCCTGGAGTTG
G204L	Non-VOC	N-Mut3-Fw	CAACTCCAGGCAGCAGTAGGCTAACTTCTCCTGCTAGAAT
K203R	VOC Beta	N-Mut3-Rev	ATTCTAGCAGGAGAAGTTAGCCTACTGCTGCCTGGAGTTG
R203M	Non-VOC	N-Mut4-Fw	CAACTCCAGGCAGCAGTATGGGAACTTCTCCTGCTAGAAT
		N-Mut4-Rev	ATTCTAGCAGGAGAAGTTCCCATACTGCTGCCTGGAGTTG

➔ defines direction (5' to 3').

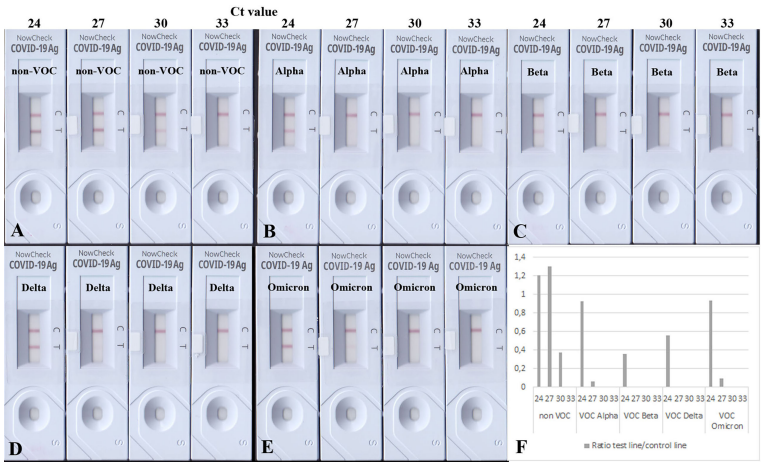

FIGURE 1
BioNote RATs loaded with samples of heat-inactivated cell culture supernatants. **(A)** non-VOC, **(B)** Alpha, **(C)** Beta, **(D)** Delta, **(E)** Omicron. **(F)** Densitometric analysis of VOCs shown on **(A–E)**. Supernatants were adjusted to comparable viral loads represented by Ct values. All viruses are detected in samples with the highest viral loads but VOCs with less sensitivity (1-2 logs). Densitometric analysis shows comparable detection of VOCs Alpha and Omicron. At Ct = 30, only the non-VOC strain is detected.

Amino acids sequence analysis of N-proteins from different SARS-CoV-2 variants revealed N-gene mutations

In order to focus the investigations on the target proteins and to avoid side effects, the further investigations on the reasons for the loss of analytical sensitivity of the RATs against VOCs were carried out with recombinant N-protein. Therefore, we amplified N-protein sequences of the non-VOC and VOC viruses and cloned them into prokaryotic expression vectors. Further, we sequenced the constructs to ensure the correct insertion of the coding region into the vector backbone (Supplementary Figure 1). Interestingly, our alignment analysis revealed that the N proteins derived from the non-VOC strain and Alpha and Beta have 14 differences on nucleic acid level, resulting in seven amino acid changes, respectively (Supplementary Figure 2). Moreover, Delta has differences at seven amino acid positions compared to the non-VOC sequences, 13 to Alpha and Beta, and 12 to Omicron (Supplementary Figure 2). VOC Omicron has a deletion of three amino acids and changes at five positions, including R203K, compared to the non-VOC strain. All other nucleotide changes were silent. We observed that the exchange of amino acids 203 and 204 alone or in combination (grey in Table 4) occurs in different VOCs. Primarily at position 203, three different amino acids (R, K, M) are found, making this region interesting for mutagenesis and possible alteration of efficient antibody binding. The amino acid exchanges, including the most VOC defining SNPs (underlined), are listed in Table 4. It should be noted that the detected amino acid exchanges are not necessarily characteristic for the particular VOC in the public SARS-CoV-2 genomic repositories. However, they were found in the isolated viruses, most likely in the context of further viral evolution *in-vivo*. Mutations due to serial virus passages in cell culture are also possible. To minimize this risk, we used only the third passages in the experiments.

TABLE 3 Detection of SARS-CoV-2 variants by BioNote RAT: VOCs are detected with less sensitivity compared to non-VOC

	Ct-value			
	24	27	30	33
non-VOC	+++	+++	++	+
Alpha	++	+	–	–
Beta	++	–	–	–
Delta	+++	+	–	–
Omicron	+++	+	–	–

Test line intensities are depicted as follows, +++, very intense; ++, intense; +, faint; -, negative.

TABLE 4 Amino acid exchanges in recombinantly expressed N-proteins in comparison to the non-VOC strain (VOC defining SNPs are underlined and investigated amino acid substitutions are shaded).

Type of VOC	Amino acid exchange						
VOC Alpha	D3L	R203K	G204P	S235F	S255P	G295V	M411K
VOC Beta	R203K	G204L	S235F	K249R	V270I	G295V	M317T
VOC Delta	D63G	R203M	Y268C	I337T	D358G	D377Y	K405E
VOC Omicron	P13L	Δ31-33	D63G	G99S	R203K	G204R	

Beta and Delta are the most poorly recognized VOCs by some RATs

Next, we aimed to test the performance of the RATs by using 100 ng purified recombinant N-protein variants per RAT. All analyzed RATs (BioNote, Abbott, Boson, TIB) detected the N-protein of the non-VOC SARS-CoV-2 at this amount (100 ng). Nonetheless, the Beta and Delta recombinant N-proteins were only detected by Boson and TIB RATs and faintly detected by BioNote and Abbott (Figures 2, 3 and Table 5). Densitometric analysis of the BioNote RAT loaded with VOCs showed that the intensity of the test line produced by the N protein of Beta is less than 20% compared to the control line (Figure 2B). The N protein of Delta produced a test line with higher intensity, which was also under 40% of the intensity of the control line (Figure 2C). In contrast, Omicron was easily detected, and the test line was even more intense than the control line. Except for Alpha, these findings are consistent with the results of applying cell culture supernatants to the BioNote RAT (Figure 1). Unsurprisingly, though, although VOCs are primarily defined by differences of the amino acid sequence in the spike (S) protein, changes in other viral proteins also contribute to the definition of the PANGOLIN lineages. Therefore, it is very likely, that amino acid exchanges in N-protein might impair the binding of a monoclonal antibody to its epitope. For this, we tested the function of the RAT by adding purified recombinant N-protein to the extraction buffer at defined amounts.

The reduced detection of VOC Beta and Delta is due to the amino acid exchange at position 203 of the N-protein

We tried to understand the reason for such lowered sensitivities for VOCs in some RATs. Therefore, we decided to introduce specific mutations in the N-protein using PCR-based mutagenesis (Table 6). This allowed us to analyze whether the band intensity reduction results from the described amino acid exchange/s. Interestingly, we found that applying the mutated N proteins R203K and G204L onto BioNote lateral flows did not decrease the detection capability of this particular RAT. In contrast, an R203M mutation (a defining SNP of VOC Delta) completely impaired the detection (Figure 4A). This evidence is a clear proof of concept that even one amino acid exchange can affect protein epitopes or stability, allowing abrogated detection by RATs. Interestingly, the R203K mutation on VOC Omicron does not alter the detection of this variant by RATs.

Since we observed a significant reduction in BioNote RAT analytical sensitivity for detecting Beta, we also introduced single aa changes reflecting the Beta in an attempt to recover the band intensity of this protein variant, as these amino acids exist in the non-VOC protein. An exchange of amino acid 203 alone in the VOC Beta from Lysin to Arginin did not alter the detection, but a Leucine to Glycine exchange at position 204 enhanced the detection (Figures 4B, C). The latter was also true when both amino acids (203, 204) were exchanged (Figure 4B).

The mutated N proteins were also used in other RATs. Boson and TIB RATs that detected all non-VOC and VOCs (Figure 2) were not affected by the introduced mutations

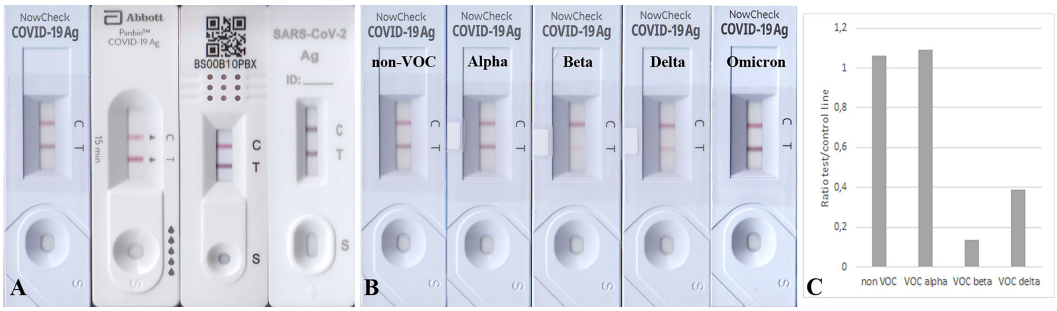

FIGURE 2
(A) RATs (BioNote, Abbott, Boson, and TIB) loaded with 100 ng recombinant N-protein of the non-VOC strain. All used RATs detected this amount of protein. **(B)** The exact amount of protein produced a comparable test line in the case of Alpha and Omicron, but the test lines using protein from Beta and Delta appeared much weaker. **(C)** Densitometric analysis of RATs shown in **(B)**. The N protein from all variants was detected, but for Beta and Delta, the sensitivity seems much lower.

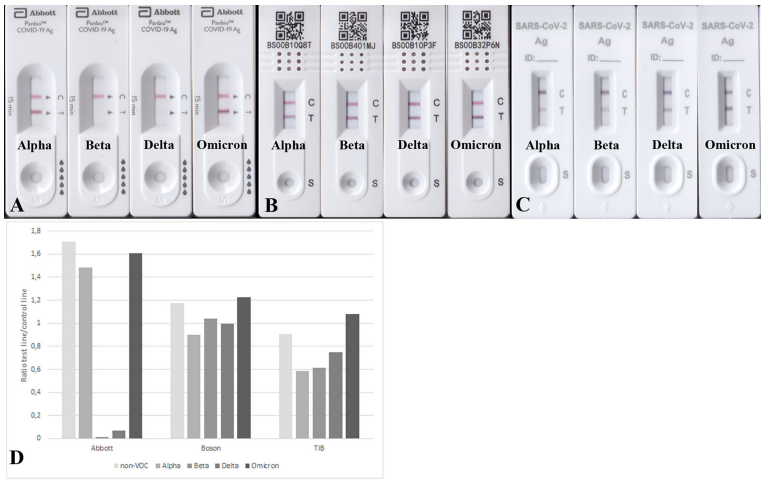

FIGURE 3
RATs detecting 100 ng N protein of Alpha, Beta, Delta, and Omicron. RATs were from different companies: Abbott **(A)**, Boson **(B)**, and TIB **(C)**. **(D)** Densitometric analysis of RATs shown in **(A-C)**. Corresponding RATs loaded with non-VOC strain are shown in Figure 2A.

(Figures 3B–D). However, RATs produced by Abbott did not detect the N protein mutants on the Beta background, or the R203M mutation (Figure 5). We could not explain why the K203R/L204G mutations retrieved the band intensity in one RAT (BioNote) and not in the other (Abbott), but both tests are presumable to use monoclonal antibodies whose target site is not *bona fide* identical. Hence, we concluded that the mutation at position 203 (R203K or R203M) on non-VOC background seems to be responsible for a decreased intensity of the test line.

Limits of detection (LOD) for VOC delta N protein and non-VOC R203M

As the R203M mutation was one of the decisive SNPs for Delta, we further aimed to determine the limits of detection of different RATs for N proteins of the non-VOC strain, Delta, and the non-VOC protein mutant with R203M exchange. Therefore, 50, 25, 10, and 1 ng of recombinant proteins were applied to the RATs from BioNote, Boson, and TIB. RATs from BioNote and

TABLE 5 Detection of 100 ng recombinant N-protein of non-VOC and VOCs by RATs.

	non-VOC	Alpha	Beta	Delta	Omicron
BioNote	+++	+++	+	++	+++
Abbott	+++	+++	(+)	+	+++
Boson	+++	+++	+++	+++	+++
TIB	+++	++	++	++	+++

Test line intensities are depicted as follows: +++: very intense, ++: intense, +: faint, (+); very faint, almost not visible, -: negative.

TABLE 6 Overview of the induced amino acid exchanges (PCR-based mutagenesis) in SARS-CoV-2 recombinant N-protein.

N-protein backbone	Amino acid substitutions	Emulating*
Non-VOC	R203K	VOC Alpha, Beta, Omicron
	G204L	VOC Beta
	R203M	VOC Delta
Beta	K203R	Non-VOC**
	L204G	
	K203R, L204G	

* Other mutations in the sequence have not been done, so the protein's primary structure is identical to that of the original protein, as has been shown by sequencing of the expression plasmid (supplementary figure 2). ** This can be considered as a revertant mutant.

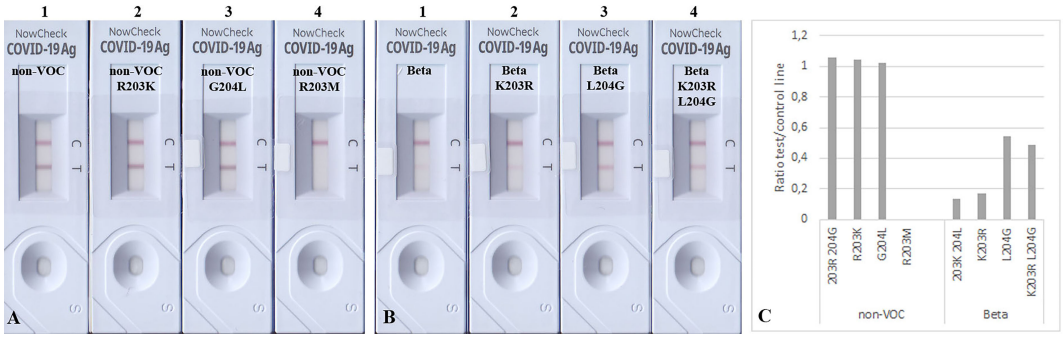

FIGURE 4
Detection of 100 ng mutated N proteins by RATs from BioNote. Numbers label specific RATs in **(A)** and **(B)** for easier orientation. Changes at positions 203 (A2) and 204 (A3) of the non-VOC N protein to the amino acids found in VOC Beta at these positions do not alter the detection of the N protein by the BioNote RAT as seen by densitometric analysis **(C)**. In contrast, R203M mutation (A4) impairs the detection ultimately. Whereas the K203R mutation (B2) in the Beta N protein does not alter the test line intensity, the L204G (B3, alone and B4 in combination with K203R) mutation enhanced the detection of Beta compared to Beta (B1).

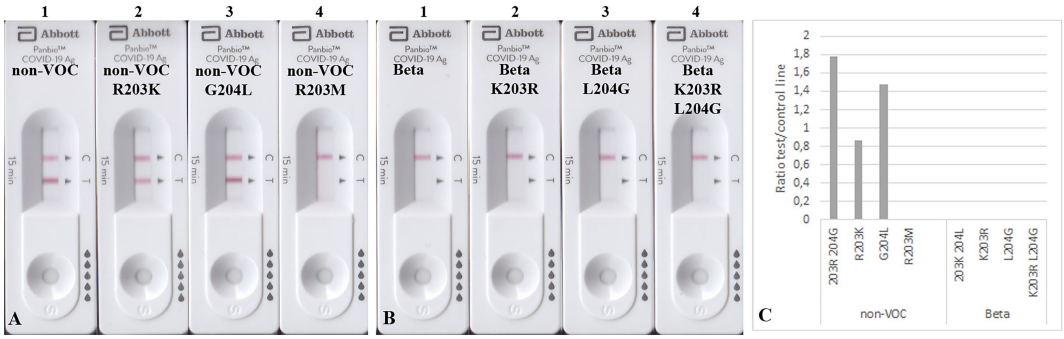

FIGURE 5
Detection of 100 ng mutated N proteins by RATs from Abbott. Numbers label specific RATs in **(A)** and **(B)** for easier orientation Amino acid change at position 203 **(A2)** decreases the test line intensity, whereas the change at position 204 **(A3)** of the non-VOC N protein does not have an effect. R203M mutation **(A4)** also impairs the detection by these RATs. **(B)** All N proteins on the Beta background were not detected. **(C)** Densitometric analysis of RATs shown in **(A, B)**.

Boson detected 1 ng of the non-VOC N protein but the same amount is not detected by TIB RAT. BioNote RAT detected the Delta protein until 50 ng, whereas the R203M protein variant was not detected at all by this RAT (Table 7 and Figure 6). This can be explained by the fact that Delta harbors additional mutations in the N-protein and not only the R203M that exist in the mutated protein (Table 7 and Supplementary Figure 2). These mutations might retain protein stability and/or epitopes.

TABLE 7 Summary of RAT sensitivity for N-protein from non-VOC, Delta, and non-VOC carrying the R203M amino acid exchange.

	N-Protein											
	non-VOC				Delta				R203M			
	50	25	10	1	50	25	10	1	50	25	10	1
BioNote	+++	+++	++	+	+	–	–	–	–	–	–	–
TIB	++	+	+	–	+	+	+	–	++	+	+	–
Boson	+++	+++	++	+	+++	+++	++	+	+++	+++	++	+

Whereas proteins with 203M are less or even not detected by BioNote RAT, Boson RATs show reliable test lines even with loads of 1 ng. Test line intensities are depicted as follows: +++: very intense, ++: intense, +: faint, -: negative.

We found that TIB RATs detected 10 ng of Delta and non-VOC R203M but not the lowest amount of 1 ng, raising questions about the LoD of this particular RAT. Strikingly, Boson RATs showed the best performance in this study since all proteins (VOC and non-VOC) were recognized even at 1 ng, and the test to control lines had the best ratios in all experiments (Figure 6).

Discussion

Negative RAT results can be used to minimize SARS-CoV-2 exposure risks, especially when combined with the user's immune status (vaccinated, recovered, versus non-immune) (21). Therefore, RATs must work for most, if not all, circulating VOCs at an

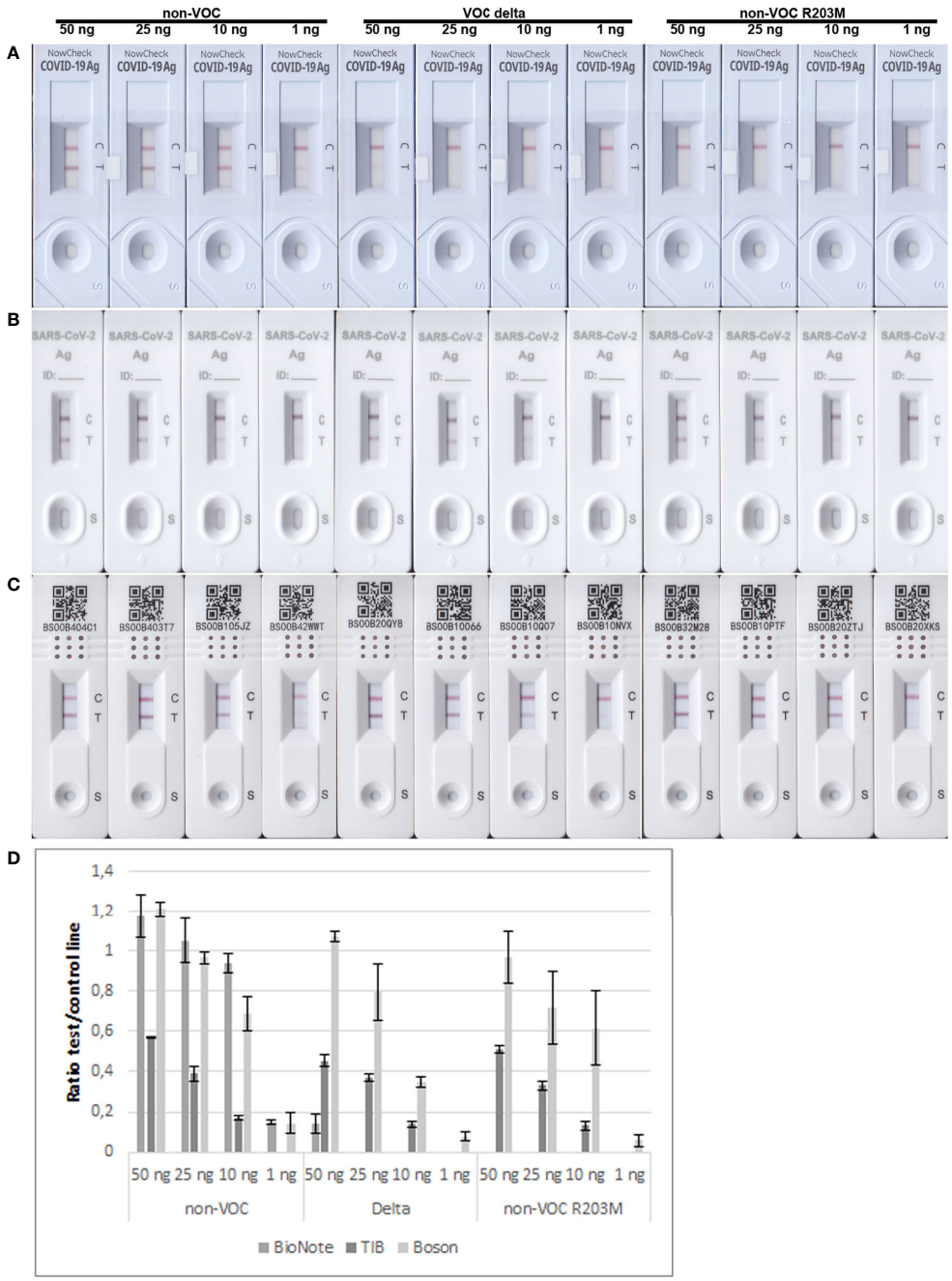

FIGURE 6
Limits of detection of BioNote, TIB, and Boson RATs for detection of non-VOC, Delta, and non-VOC R203M recombinant proteins. **(A)** BioNote, **(B)** TIB, **(C)** Boson, using amounts of 50 ng, 25 ng, 10 ng, 1 ng respectively. **(D)** Means of densitometric analysis of RATs is shown in **(A–C)** (n=2).

analytical sensitivity equaling one infectious dose for humans (conservatively estimated: ~10^6 to ~10^7 copies/mL) (16, 18, 22).

Previously, we have shown that the limit of detection for the non-VOC strain was up to the level of Ct value 32,25 and gene copy number of 10^3-10^4 copies/mL using randomly selected RATs (16). In the present study, we in assessed the ability of RATs to detect formerly and currently circulating VOCs by using heat-inactivated cell culture supernatant and recombinant proteins. Differences between the data of this study and previous studies regarding the sensitivity of RATs against the different VOCs presented are most probably due to different study designs. Standardized procedures, such as those used in large-scale series before (23, 24), would make the results of different studies more comparable. However, this was not the primary goal of the study, but rather to show how much the analytical sensitivity of the RATs can vary depending on amino acid variations in the target regions of the VOCs.

Alpha and Beta VOCs were not well-detected by one particular RAT (Abbott) as already published (16). Here, we also included the most recently circulating VOCs, Delta, and Omicron. Initially, Ct values of RNA isolations from native (Ct 14.35) and heat-inactivated (Ct 20.4) cell culture supernatants have been compared to quantify the loss of sensitivity due to inactivation. Data illustrate that heat-inactivation of SARS-CoV-2 results in a loss of sensitivity of up to 2 logs in RT-qPCR, which is in good accordance with published data (25). To increase the comparability of the assays, recombinant proteins were used in the present study in addition to heat-inactivated cell culture supernatants. This excludes the possibility that the lower sensitivity of the RATs is solely due to the denaturation of the antigen.

This reduced sensitivity to VOCs is due, at least in part, to a mutation at position 203 (R203K, R203M) in the N protein of SARS-CoV-2 variants. Since many RATs are based on the less-variable N-protein, they are thought to be able to detect all the variants. However, this study clearly shows that the performance of RATs depends heavily on the anti-N-antibodies used for detection. RATs rely on two anti-N-antibodies; one in the sample pad and another immobilized capture antibody at the test line (16). Both antibodies are not necessarily the same, but they may be. If one of the antibodies cannot bind N-protein (e.g., due to an amino acid exchange), the test will not or only partially detect the SARS-CoV-2 antigens in the sample.

In both VOCs Beta and Delta, amino acid 203 (Arginine) of the N-protein has been changed to Lysine in Beta and Methionine in Delta, respectively. Using PCR-mediated mutagenesis, it was possible to mimic this amino acid exchange in the non-VOC N-gene *in vitro*, and use it to evaluate different RATs. While the R203K mutation only reduced the intensity at the test line, an R203M mutation (as in the Delta) completely impaired the antigen detection. Therefore, antibodies binding to a more conserved part of the N-protein or polyclonal antibodies should be used preferentially.

Interestingly, the R203K mutation seems not to influence the RAT sensitivity in Omicron. Reasons for this could be the replacement of other single amino acids in other domains, especially the three amino acid deletions in the N-terminal domain of the protein. The possible interaction of different secondary structures due to the changes in the primary structure may expose or mask epitopes in the tertiary structure of the proteins, which may result in different binding properties of the antibodies. This has to be further evaluated in future studies using correspondingly mutated N-protein.

Several studies have been conducted using RATs for special cohort screening (26–29), and several assays have been recommended to be useful for the early detection of infection with SARS-CoV-2. This study is not intended to warn users about specific products. The selected sample size of RATs from only 4 manufacturers is too small and not representative for this purpose, but the aim of this article is to raise awareness in terms of a VOC specific evaluation of RATs.

Data availability statement

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

Author contributions

ITH, MG, MK conceived and designed the experiments. ITH, MK performed experiments and analyzed data. ITH, SW, KP, AB-B, MK contributed reagents/materials/analysis tools. MHG attained funding. ITH, MHG, MK wrote the paper. All authors reviewed, edited, and approved the final version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fviro.2022.971862/full#supplementary-material

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Assessing the impact of the four COVID-19 variants and the vaccine coverage on mortality in Malta over 2 years: An observational case study

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Background: Mortality may quantify a population's disease burden. Malta, like other European countries, experienced COVID-19 surges in cases and mortality across the pandemic. This study assesses COVID-19's mortality impact, while exploring the effects of the four dominant COVID-19 variants and that of the vaccination coverage on the Maltese population.

Methods: COVID-19 data (cases, mortality, positivity, and vaccination rates) was obtained from the websites of the European Center for Disease Prevention and Control and the Malta Ministry of Health. Data was categorized into the four periods according to reported dominant COVID-19 variant. Years of life lost (YLL) and Case-Fatality-Ratio (CFR) for each period were estimated. CFR was also estimated for the pre-vaccine and post-vaccine periods.

Results: The original COVID-19 period (36 weeks) had the highest YLL (4,484), followed by the Omicron variant period (12 weeks; 1,398). The Alpha variant period (7 weeks) had the highest CFR (1.89%) followed by the Original COVID-19 (1.35%). The pre-vaccine (1.59%) period had higher CFR than the post-vaccine period (0.67%).

Conclusion: Various factors contributed to mortality, but the variant's infectivity, transmissibility, and the effectiveness of the vaccine against the variant play an important role. Reducing mortality by embracing mass vaccination that targets current variants along with other non-pharmaceutical interventions remains paramount.

KEYWORDS

COVID-19, mortality, vaccination, disease burden, mutation, Malta

Introduction

The novel coronavirus SARS-CoV2 was first reported in Wuhan, China at the end of 2019 and within weeks spread globally, resulting in the COVID-19 pandemic (1). During the pandemic various mutations occurred to the original viral strain resulting in the emergence of several variants of interest and variants of concern (2). The small Mediterranean islands of Malta with a total population of 514,564, like the rest of

Europe, were affected by COVID-19. The first COVID-19 case reported in Malta was in March 2020 and for a whole year the original SARS-CoV2 virus dominated the island's population (3). The Beta variant (B.1.351) was first detected in Malta in February 2021 although only a couple of cases were identified before the Alpha variant (B 1.1.7) took over the scene from March 2021 (4, 5). This led to a spike in cases bringing about Malta's second lockdown (6). At the time, COVID-19 vaccination rollout was well underway with a substantial proportion of the Maltese elderly population fully vaccinated, while the younger age groups were progressively being inoculated (7). June 2021 saw the first case of the Delta variant (B.1.617.2) in Malta, which became the dominant variant across the islands within weeks (8). The new surge in cases and mortality led to the initiation of the booster dose vaccination rollout targeting the elderly in September 2021 (9). The first Omicron variant (B.1.1.529) cases were reported during the end of December 2021 and in days became the new dominant variant (10, 11). By the end of February 2022, total reported COVID-19 cases since the onset of the pandemic in Malta were 13,308 cases per 100,000 population and 126 per 100,000 population deaths (12).

Mortality is an important index for quantifying the burden of a disease among the population and also constitutes a fundamental pillar for public health decision making (13, 14). In this study, we set to assess the impact of COVID-19 in terms of population mortality, while exploring the effect of the four dominant variant phases and the vaccination coverage on the Maltese population. The small population size of Malta provides a unique opportunity to evaluate the burden of COVID-19 at a population level, and the evidence generated by this exercise is of importance to both local and international public health authorities and policymakers in their role in the prevention and control of the ongoing pandemic.

Methods

This observational study was based on freely available epidemiological data and public health announcements reported in local newspapers from the onset of COVID-19 till the end of February 2022. The European Center for Disease Prevention and Control (ECDC) database (https://www.ecdc.europa.eu/en/covid-19/data) was utilized to obtain Malta's COVID-19 data for weekly cases, positivity rate and vaccination rate stratified by age and gender. The Ministry of Health official repository (https://github.com/COVID19-Malta/) was used to obtain the daily mortality data stratified by age and gender. Excess mortality data was obtained from the Eurostat website (15).

Weekly cases, positivity, mortality, and vaccination data were categorized according to these four phases: (i) Original COVID-19* from week 30 of 2020 to week 14 of 2021; (ii) Alpha variant from week 15 of 2021 to week 22 of 2021; (iii) Delta variant from week 23 of 2021 to week 48 of 2021; (iv) Omicron variant from week 49 of 2021 to date (end of February 2022). For this study's analyses, Original COVID-19 phase* was considered to start from week 30, i.e., with the onset of the second wave since during the first wave, the COVID situation in Malta was well-controlled with low positive cases and deaths (3). It needs to be noted that only the dominating variant in a particular phase was considered for the purpose of the study analyses, but this does not preclude that a small proportion of cases and deaths were due to different variant/s.

Data analyses

Year of life lost

The Years of Life Lost (YLL) is a metric used in population health to measure the number of years lost due to premature death from a particular cause. The YLL calculation provides a good comparative insight into the impact of death on the population as it recognizes deaths occurring at a younger age group as having a greater impact on population health as opposed to deaths occurring at an advanced age group (16). Following the Global Burden of Disease (GBD) Study methodology, the years of life lost (YLL) was estimated by combining the death counts by five-year age-groups and sex (17). The estimates were calculated by multiplying the number of deaths in each age-group by the age-conditional remaining life expectancy from the GBD Study 2019 reference life table, where the same values are assigned to both males and females (18). The YLL for each of the four COVID-19 variant phases was estimated using the described calculation. In view that the YLL metric considers the mortality impact over a period of a year, but the different variants were dominant for weeks, the YLL established was divided by 52 (number of weeks in a year) and then multiplied by the total number of weeks each variant dominated (Original 36 weeks; Alpha 7 weeks; Delta 26 weeks; Omicron 12 weeks). The YLL per week was also calculated i.e., YLL/52. This calculation is expected to provide an indication of the impact of a variant on premature mortality over the duration of its dominance.

Case-Fatality-Ratio

The Case-Fatality-Ratio (CFR in %) for ongoing epidemic was calculated using the formula below (19). The CFR for each of the four COVID-19 variant phases was estimated. The CFR was re-calculated to consider the impact of COVID-19 vaccine roll-out on mortality. Therefore, the pre-vaccine CFR (week 30/2020 till week 52/2020) and the post-vaccine CFR (week 1/2021 till week 9/2022) were also calculated. The post-vaccine period covered from the start of the first dose up till the booster

dose among the study population.

$$\text{Case fatality ratio (\%)} = \frac{\text{Number of COVID} - 19 \text{ deaths}}{\text{Number of COVID} - 19 \text{ deaths} + \text{Number of recovered}} \times 100$$

Vaccination

Data on cumulative full dose vaccination, cumulative booster dose and mortality were stratified by age groups (25–49; 50–59; 60–69; 70–79; 80+ years). For trend analysis the Jonckheere-Terpstra test was used for both the cumulative full dose vaccination and cumulative booster dose against the mortality by age groups at 50% vaccination uptake at a population level. Full vaccination was estimated to have reached 50% uptake at week 21/2021 for the 25–49 years, week 23/2021 for 50–59 years; week 20/2021 for 60–69 years; week 14/2021 for 70–79 years and week 8/2021 for 80+ years. While booster dose was estimated to have reached 50% uptake at week 52/2021 for 25–49 years; week 50/2021 for 50–59 years; week 47/2021 for 60–69 years; week 42/2021 for 70–79 years and week 40/2021 for 80+ years.

Results

Across the 2 years of the pandemic, Malta reported three dominating COVID-19 variants apart from the original SARS-CoV2, leading to several surges in infection and mortality cases as shown in Figures 1A,B. Over 12 weeks, the Omicron variant appeared to have had the worse infectivity spread with an average positivity rate of 6.7 when compared to the rest of the variants (Original: dominated 36 week, average 6.7 positivity rate; Delta variant: dominated 26 weeks, average 2.10 positivity rate; Alpha variant: dominated 7 weeks average 0.84 positivity rate).

In the initial pandemic phase, the original variant had low mortality (Figure 1B), but the second wave led to a surge of deaths. Weekly deaths continued to be reported until mid-May 2021, when the Alpha variant surge subsided. During the original and delta COVID-19 phases, the surges in deaths could be observed on average 5 weeks following the spike in positive cases. The mortality rate during the Delta variant phase was much lower than that of the original COVID-19. Conversely, this was not the case following the Alpha variant surge, where mortality declined (Figure 1B). The aftermath of the Omicron variant peak led to the highest death rate over a period of a week from the onset of the pandemic (Figure 1B).

When comparing the four COVID-19 variant phases, the original COVID-19 phase contributed to the highest adjusted YLL (4,484 years), followed by the Omicron phase (1,398 years–Table 1). YLL contributed by the original COVID-19 was for a period of 36 weeks as opposed to the 12 weeks period of Omicron. A similar YLL distribution could be observed when comparing the YLL per week across the different variants, as shown in Table 1. On comparing the case-fatality ratio (CFR) of the four COVID-19 phases, the Alpha variant was observed to contribute to the highest ratio, followed by the original COVID-19 (Table 1). The CFR for the pre-vaccine period (1.59% over 22 weeks) was higher than that of the post-vaccine period (0.67% over 61 weeks). Excess mortality per month was reported throughout the pandemic and across all the four phases as shown in Supplementary Table 1.

Impact of vaccination coverage on mortality

The COVID-19 vaccine rollout started on the 27th December 2020 in Malta and by summer 2021 almost the entire eligible population had been fully vaccinated. Evaluation of the effect of vaccination coverage on the mortality rate (per 100,000 population) across the different age groups, shows that the mortality rate declined until the Omicron outbreak (Figure 2). A borderline significance was established for these trends ($p = 0.05$). The elderly were invited to take the booster dose as the Delta variant predominated at the end of summer 2021. As shown in Figure 3, when the Omicron variant was detected in Malta a large proportion of the elderly (60+ years) were already inoculated by the booster dose. Despite this, an increase in the mortality rate can be observed across all age groups, with borderline significance ($p = 0.05$).

Discussion

Mortality is a useful measure to assess the magnitude of the pandemic as well as act as a tracking tool of the pandemic's impact on the population (20). Since the onset of the pandemic, the mortality rate has been on the incline, with certain COVID-19 phases experiencing a higher rate than others, as was observed in this study. It needs to be acknowledged that although this was out of the scope of this study, the excess mortality noted since the onset of the pandemic is not only a direct effect of the COVID-19 infection but also due to secondary indirect causes such as economic turmoil, lockdowns and pandemic related anxiety leading to higher suicide deaths among other factors (14, 21, 22).

The only preventive measures available during the first year of the pandemic were non-pharmaceutical interventions (NPI). Their success in pandemic control was dependent on timely measures instituted by the country's authorities and the population's compliance. During the first COVID-19 wave, Malta was praised for its effective pandemic management resulting in low infectivity and mortality rates (3), as supported by this study. Yet, abrupt lifting of the measures and mass

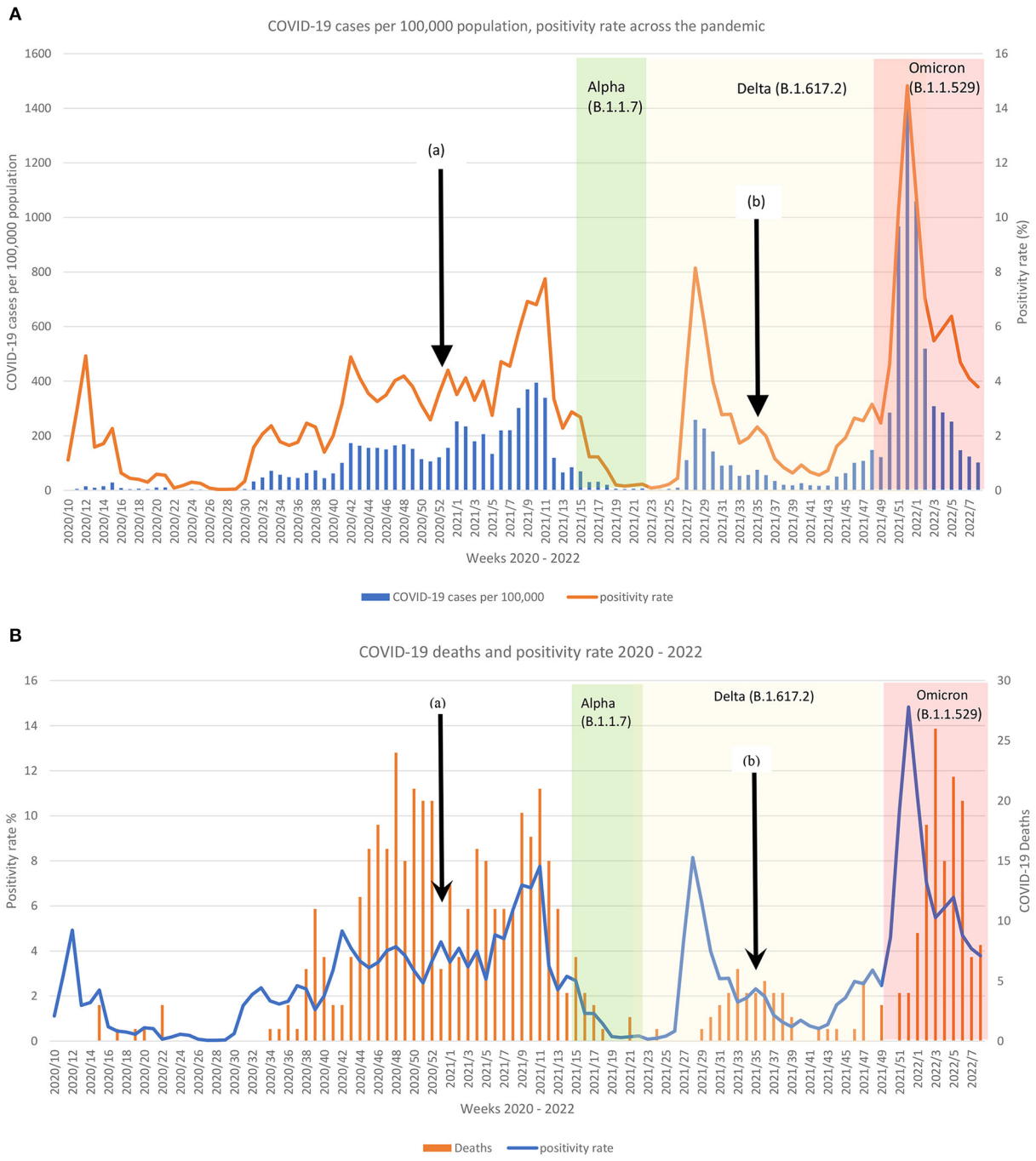

FIGURE 1
Comparison of the reported **(A)** COVID-19 cases per 100,000 and positivity rate per week and **(B)** COVID-19 deaths and positivity rate per week according to the dominating variant across 2 years in Malta. Black arrow (a) indicates start of COVID-19 vaccination and (b) start of booster dose. White background = original SARS-CoV2; Green background = Alpha variant; Yellow background = Delta variant; Red background = Omicron variant.

gatherings brought about the second wave (23). At the time the dominant original COVID-19 variant resulted in a sharp rise in mortality, as noted in this study. Indeed, this period contributed to the highest years of life lost (YLL) out of the four variant phases, yet not the highest CFR. COVID-19 has affected individuals across all age groups with premature deaths occurring even among the young generation (24). Hence, the YLL metric provides a good indication of the COVID-19 impact

TABLE 1 Comparison of the four COVID-19 phases epidemiological outcomes.

	Original (36 weeks)	Alpha (7 weeks)	Delta (26 weeks)	Omicron (12 weeks)
Total cases (*N*)	29,166	901	9,280	29,055
Total mortality (*N*)	393	17	49	181
Case-Fatality Ratio (CFR–%)	1.35	1.89	0.53	0.62
Years of life lost (YLL—years)	4,484	46	444	1,398
YLL/week	125	7	17	52

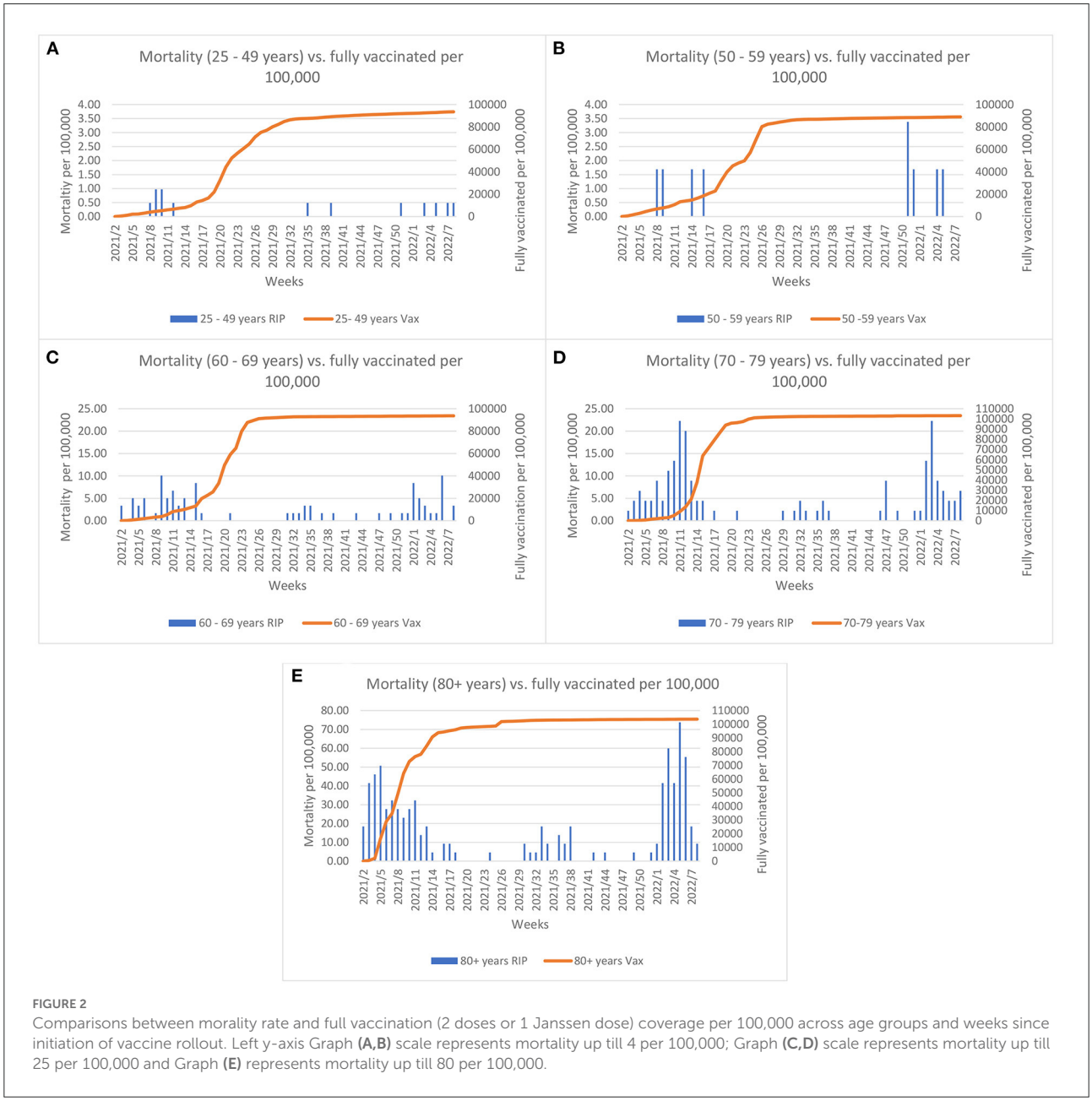

FIGURE 2
Comparisons between morality rate and full vaccination (2 doses or 1 Janssen dose) coverage per 100,000 across age groups and weeks since initiation of vaccine rollout. Left y-axis Graph **(A,B)** scale represents mortality up till 4 per 100,000; Graph **(C,D)** scale represents mortality up till 25 per 100,000 and Graph **(E)** represents mortality up till 80 per 100,000.

on the population in terms of premature mortality. Much of the original COVID-19 disease phase relied on just NPI's for prevention, as the COVID-19 vaccine became available when the original COVID-19 began to phase out and new variants took over. This may explain the high YLL attributed to this phase as mortality occurred among young adults apart from more

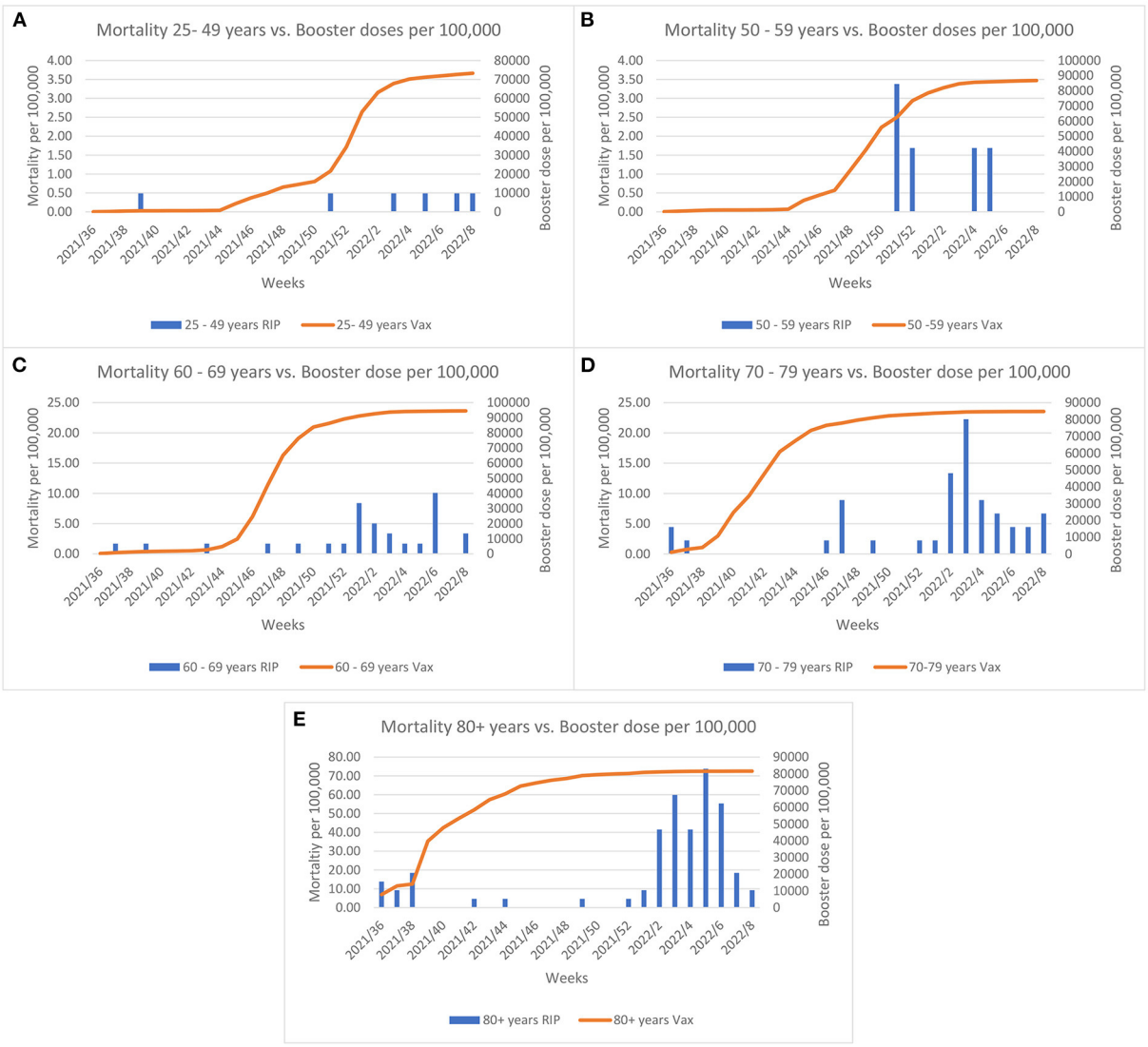

FIGURE 3
Comparisons between morality rate and the booster dose coverage per 100,000 across age groups and weeks since initiation of the booster rollout. Left y-axis Graph **(A,B)** scale represents mortality up till 4 per 100,000; Graph **(C,D)** scale represents mortality up till 25 per 100,000 and Graph **(E)** represents mortality up till 80 per 100,000.

advanced adults. Additionally, this phase lasted for a longer duration than the other variants. Yet, despite the presence of the vaccine and the booster dose, the Omicron variant phase was observed to have the highest positivity rate and mortality occurrence at a population level as well as having the second highest YLL in this study. This may imply that mortality among the younger generation occurred even if the percentage vaccinated adults was high, although other confounding factors might also have been present. It has been reported that two doses vaccination does not provide adequate protection against the Omicron variant, while the addition of a booster dose only adds a low protective effect, with a decline in its effectiveness after some 4 months post vaccination (25, 26). This relationship could be clearly observed in this study, where despite high vaccination uptake, the mortality rate did not decline. Another feature exhibited by the Omicron variant is its ability to evade the immune system, with those having the booster still susceptible to infection (27). These Omicron features might have played a role in the rise in the mortality rate observed in this study. The elderly were inoculated with the booster between September and October 2021, with wanning immunity when the Omicron variant dominated the scene in Malta. Of note, the Omicron variant phase considered in this study was of a period of 12 weeks, as opposed to the original COVID phase (36 weeks). Therefore, with caution one might project that if the Omicron variant continuous to dominate the landscape

with the same virulence level, it will lead to a higher level of premature mortality.

From the start of the pandemic, Malta, had followed a high swab testing policy and progressively increased swabbing hubs across the islands to make testing accessible to all of the population (3, 28). Therefore, calculating the case-fatality ratio can be considered as reliable measure of severity and a valuable piece of policymaking (29). When evaluating the impact of mortality in terms of CFR, the Alpha variant phase contributed to the highest CFR proportion, even though this had the shortest phase. This points to the highly transmissible feature of this variant over a short period of time along with its associated high mortality (30, 31). Despite this, a mortality decline was observed across this Alpha variant phase which corresponds to the high uptake of COVID-19 vaccination among the population. Indeed, it has been reported that the COVID-19 vaccine is highly effective in decreasing transmission and mortality vs. the Alpha variant (32). The pre-vaccine period was noted to hold a higher CFR proportion than the post-vaccine period. With caution this may indicate that vaccines had a positive effected on the overall mortality incidence even if the dominating variants during the vaccination phase resulted in a substantial high positivity and mortality rate. Although the vaccine efficacy could not be measured for this study population, other studies have reported a relationship between vaccine efficacy and a decrease in the all-cause mortality and hospitalisations irrespective of the dominating variant (33–35). This is an important public health finding as it provides evidence how the impact of future COVID-19 waves can be reduced by enhancing mass population vaccination while safeguarding the healthcare systems. However, when interpreting this study's findings, the duration of both periods need to be considered, i.e., the pre-vaccine period was shorter than the post-vaccine period, which might have had an effect to this finding, apart from other potential confounding factors. Further research is therefore recommended to investigate the effect of vaccination on mortality outcome.

Several strengths and limitations need to be acknowledged. The study was based in a small country making it easier to explore the COVID-19 impact at a population level. From the onset of the pandemic, Malta had a high swab testing capacity including testing every individual that is admitted to hospital and post-mortem (3), so the detection of COVID-19 can be considered as being representative of the population. This study was an observational study based on epidemiological data freely available through ECDC and the Maltese government repository. The authors did not have direct access to the genotyping or medical history of the infected population nor to those that died, which might have impacted on the study's outcome including the inability to perform regression analyses and other complex analyses pertaining to the different variants. Furthermore, individualized vaccination data was not available to estimate the vaccine efficacy. Other underlying confounding factors, apart from vaccination, might have influenced the mortality outcome across the four variant phases. Assumptions had to be made that once the authorities reported that a variant is dominant within the population, the recorded cases, and deaths from that point in time were affected by that same dominant COVID-19 variant. However, this may have overestimated the effect of the dominant variant as other variants might have been present. Delayed mortality reporting might have occurred possibly leading to under reporting or overreporting of deaths during a particular COVID-19 variant phase. The authorities report daily mortality but do not differentiate between individuals dying due to COVID-19 or dying while also having COVID-19. Therefore, in this study we were unable to take this in consideration.

Conclusion

Morality data provides an indication of the burden of COVID-19 within a population. Various factors contribute to mortality, yet the variant's infectivity, transmissibility, and the effectiveness of the vaccine against the variant play an important role. The pandemic is far from over and reducing mortality should remain high up on the agenda by embracing mass vaccination that targets the current variant as well as the institution of timely preventive measures across countries.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found at: https://www.ecdc.europa.eu/en/covid-19/data, https://www.facebook.com/sahhagovmt.

Author contributions

SC was responsible for the design of the study, data collection, data analyses, and writing of the draft article. SG and VG were contributed to the study design and critically reviewing the article. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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Supplementary material

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Commentary: MSH3 homology and potential recombination link to SARS-CoV-2 furin cleavage site

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SARS-CoV-2, FCS, homology, Markov, evolution

A commentary on

MSH3 Homology and potential recombination link to SARS-CoV-2 furin cleavage site.

By Ambati BK, Varshney A, Lundstrom K, Palú G, Uhal BD, Uversky VN and Brufsky AM (2022) *Front. Virol.* 2:834808. doi: 10.3389/fviro.2022.834808

Introduction

Coronaviruses are characterized by the spike glycoprotein, which consists of two domains: S1, which binds to angiotensin-converting enzyme 2 (ACE2) receptors on the host cell, and S2, which drives membrane fusion. A 12-nucleotide insertion (i.e., 5′-CCTCGGCGGCGA-3′) that codes a furin cleavage site (FCS) between S1 and S2 has been discovered in SARS-CoV-2 (1). FCS insertion at the S1–S2 junction is unique among known sarbecoviruses (SARS-CoV-2 subgenus) and offers a functional advantage (2). FCS presence is surprising, and its origin is debated. Ambati et al. (3) reported a sequence homology between SARS-CoV-2 FCS and the negative strand of a patented sequence, with a coincidence probability of 3.21×10^{-11}. Therefore, the authors suggested that the SARS-CoV-2 FCS could originate from a copy choice recombination in human

cells in the context of viral research. This scenario is molecularly possible, but the computed coincidence probability may be erroneous.

Irrelevant probability and *a posteriori* information for *a priori* computation

The authors computed the probability of randomly finding the FCS pattern in a database of patented sequences and in a viral genome, such as SARS-CoV-2. This probability is irrelevant, as the authors decided to search for this pattern because it appeared in SARS-CoV-2. Hence, to assess if their finding could be due to chance, they should have computed the probability of finding the FCS pattern in only the database they queried, given that the appearance of the FCS in SARS-CoV-2 was the starting point.

In addition, instead of computing the probability of finding the 12-nucleotide pattern coding for the FCS identified *a priori* (before the BLAST research), the authors computed the probability of finding the 19-nucleotide 5′-CTCCTCGGCGGGCACGTAG-3′ pattern. This latter pattern is the original pattern extended by two nucleotides before and five nucleotides after the FCS. It corresponds to the extended homology that they found between SARS-CoV-2 and the patented sequence, which is, therefore, *a posteriori* information (after the BLAST research). To correctly consider the expandability of their homology, they should have computed the probability of finding one of the eight possible 19-nucleotide-long extensions without presuming its form: 5′-TAATTCTCCTCGGCGGGCA-3′, 5′-AATTCTCCTCGGCGGGCAC-3′, 5′-ATTCTCCTCGGCGGGCACG-3′, 5′-TTCTCCTCGGCGGGCACGT-3′, 5′-TCTCCTCGGCGGGCACGTA-3′, CTCCTCGGCGGGCACGTAG-3′, 5′-TCCTCGGCGGGCACGTAGT-3′, and 5′-CCTCGGCGGGCACGTAGTG-3′.

Finally, the reported match is on the negative strand of the patented sequence identified. This indicates that matches with the pattern's reverse complement are also considered a discovery. Hence, they should have computed the probability of finding one of the eight possible extensions or one of their reverse complements (i.e., 16 patterns) in the queried database.

Probability to find patterns of length *m* in a sequence of length *n*

The authors computed the probability of finding a pattern (e.g., 5′-CTCCTCGGCGGGCACGTAG-3′) of length m in a sequence of length n as: $(n - m + 1)(\frac{1}{4})^m$.

This formula is inexact; as an illustration, let us compute the probability of finding the CG pattern ($m = 2$) among the 256 sequences of length $n = 4$. Using the above-mentioned formula, 48 occurrences of the CG pattern are counted (16 occurrences for each sequence structure XXCG, XCGX, and CGXX). However, the number of sequences containing CG is not 48. Indeed, the sequence CGCG is counted twice: in both XXCG and CGXX structures. Therefore, the formula used by Ambati et al. leads to a probability of 48/256 = 18.75%, whereas the correct probability is 47/256 = 18.36%. If we assume, as the authors did, equal nucleotide frequencies and independence between nucleotides (memory-less sequence), the probability of finding a given pattern can be computed using a Markov chain model with one absorbing state (see Figure 1 illustrating the above-mentioned example).

This methodology can be extended to compute the probability of finding one of the 16 patterns using a Markov chain model with 16 absorbing states. Using the authors' parameters ($n = 3300$), we found a probability of 1.49×10^{-7}, approximately 10 times higher than the probability obtained by Ambati et al. (1.19×10^{-8}).

Search for a pattern in a large database

During their BLAST search, the authors queried a database of $L = 24{,}712$ sequences for the pattern that they had identified (5′-CTCCTCGGCGGGCACGTAG-3′). They approximated this experience as a binomial trial (statistically independent Bernoulli trials, with a success probability of $p = 1.19 \times 10^{-8}$ repeated 24,712 times) and computed the probability of finding exactly one sequence containing the pattern as $L \times p \times (1 - p)^{L-1}$. Several limitations can be reported:

1. This method assumes that all sequences in the database are independent.
2. It assumes that all sequences are 3330 nucleotides long.
3. It neglects the possibility of finding more than one sequence containing the pattern that they were looking for. The appropriate formula is $1 - (1 - p)^L$.
4. They should have considered the other possible extensions of the pattern and their reverse complement, leading to a success probability of $p = 1.49 \times 10^{-7}$.

The actualized computation performed under assumptions 1 and 2 leads to a final coincidence probability of 0.0037 (rather than 3.21×10^{-11}).

Lacking information regarding the database

The authors did not provide information regarding the sequence length distribution in their database, except that the

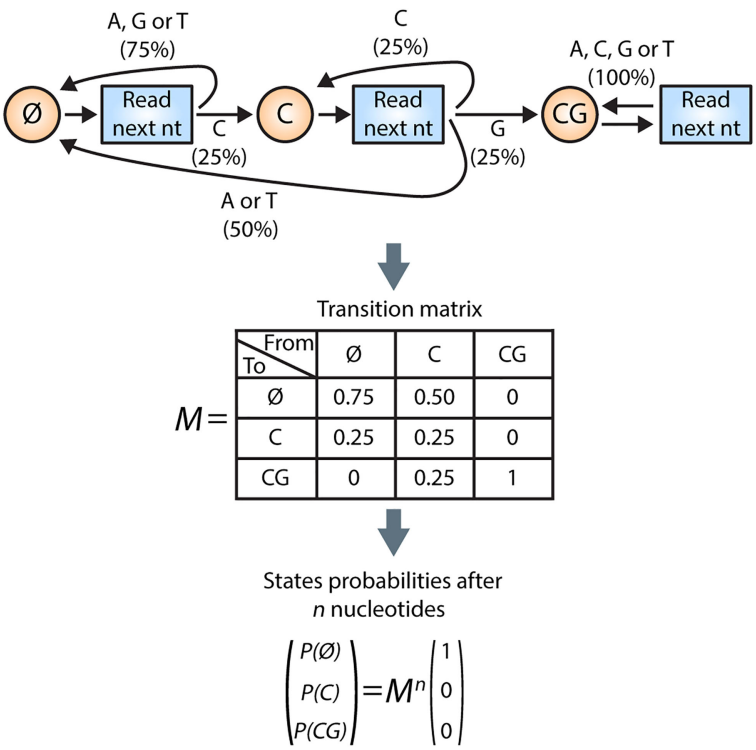

FIGURE 1
Illustration of a Markov chain to compute the probability to find the CG pattern in a sequence of length *n* . The Markov chain for this basic example contains three states: Ø, C, and CG. The chain starts in the Ø state and will read the nucleotides (nts) one by one. From state Ø, there is a 75% chance that the next nucleotide will not match the first nucleotide of the CG pattern. Therefore, from Ø state, the probability of reaching state C is only 25%, whereas the probability of remaining in state Ø is 75%. From state C, reading the next nucleotide gives three options (1): the next nucleotide is a G, leading to the CG state (with a probability of 25%) (2); the next nucleotide is again a C, i.e., we remain in the C state (with a probability of 25%); or (3) the next nucleotide is neither a C nor a G, resulting in a return to the Ø state (with a probability of 50%). When the CG state is reached, the pattern is found and, even if next nucleotides can be read, the chain is blocked in the CG state, a so-called absorbing state. This chain can be mathematically summarized by a transition matrix M. The probability of the three states after reading *n* nucleotides is given by the transition matrix at the power *n* multiplied by the initial conditions. Code to reproduce this basic example and the computation for the 19 nucleotides pattern is avalaible on OSF (https://osf.io/wsd5g/?view_only=0af888d0d29d452fa5dcb9cf769ae229).

median length was 3,300. In addition, they did not indicate whether or not their computation is robust when assumption 2 is not met. To assess the robustness of the computations, we simulated several databases with a median length set at 3,300 nucleotides but with different distribution shapes for sequence lengths. The results indicated that the distribution did not substantially affect the final probability so long as the median length was kept constant.

Unfortunately, the authors did not provide details of their BLAST research. The patent database that they used contained 24,712 sequences. Yet, by querying BLAST, we obtained a database of 46,121,617 patent sequences with an average length of 560 nucleotides. The authors should give more details and justification for their query, especially if they queried the full database but *a posteriori* restricted their computation. Of note, with such a large database, and despite the fact that the average sequence length decreased, the probability of finding at least one sequence containing one of the 16 patterns previously mentioned may rise to 68% under assumption 2.

Conclusion

Epidemiological studies support the conclusion that the SARS-CoV-2 pandemic originated in Huanan market, and was not the product of a laboratory accident (4–6). Moreover, *Sarbecovirus* phylogeny is still sparsely known, and the sequencing of new SARS-CoV-2 relatives could help us to understand the emergence of the FCS (2, 4). According to the current phylogeny, FCS appeared independently six times in the *Betacoronavirus* lineages, demonstrating that FCS insertion is compatible with natural evolution (2, 7, 8). The probabilities provided by Ambati et al. seem inexact, and their BLAST search

is not transparent enough. Based on our computations and BLAST research, the role of chance in this homology should not be dismissed.

Author contributions

HL and YD contributed equally in the methodological considerations, probabilities computation, and the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Neutralizing antibodies from the rare convalescent donors elicited antibody-dependent enhancement of SARS-CoV-2 variants infection

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Currently, neutralizing antibody and vaccine strategies have been developed by targeting the SARS-CoV-2 strain identified during the early phase of the pandemic. Early studies showed that the ability of SARS-CoV-2 RBD or NTD antibodies to elicit infection enhancement *in vivo* is still controversial. There are growing concerns that the plasma and neutralizing antibodies from convalescent patients or people receiving vaccines mediate ADE of SARS-CoV-2 variants infections in immune cells. Here, we constructed engineered double-mutant variants containing an RBD mutation and D614G in the spike (S) protein and natural epidemic variants to gain insights into the correlation between the mutations in S proteins and the ADE activities and tested whether convalescent plasma and TOP10 neutralizing antibodies in our laboratory mediated the ADE effects of these SARS-CoV-2 variants. We found that one out of 29 convalescent plasma samples caused the ADE effect of pandemic variant B.1.1.7 and that the ADE effect of wild-type SARS-CoV-2 was not detected for any of these plasma samples. Only one antibody, 55A8, from the same batch of convalescent patients mediated the ADE effects of multiple SARS-CoV-2 variants *in vitro*, including six double-mutant variants and four epidemic variants, suggesting that ADE activities may be closely related to the antibody itself and the SARS-CoV-2 variants' S proteins. Moreover, the ADE activity of 55A8 depended on FcγRII on immune cells, and the introduction of LALA mutations at the Fc end of 55A8 eliminated the ADE effects *in vitro*, indicating that $55A8^{LALA}$ may be a clinical drug used to prevent SARS-CoV-2 variants. Altogether, ADE may occur in rare convalescent patients or vaccinees with

ADE-active antibodies who are then exposed to a SARS-CoV-2 variant. These data suggested that potential neutralizing antibodies may need to undergo ADE screening tests for SARS-CoV-2 variants, which should aid in the future design of effective antibody-based therapies.

KEYWORDS

antibody-dependent enhancement, neutralizing antibody, infection-enhancing antibodies, SARS-CoV-2 variants, receptor-binding domain (RBD)

Introduction

As SARS-CoV-2 strains continue to evolve, new variants have been increasing in the current global pandemic. Obviously, the SARS-CoV-2 variants causing the global pandemic are antigenically distinct from the previous prototype during the early phase of the pandemic. Current neutralizing antibodies and vaccine strategies were developed by targeting the prototype SARS-CoV-2 strain identified during the early phase of the pandemic (1–6). We also reported the isolation and characterization of several hundreds of RBD-specific mAbs from SARS-CoV-2-infected individuals (7), some of which could neutralize authentic SARS-CoV-2 variants (8). Previous studies have shown that some RBD or NTD antibodies mediate antibody-dependent enhancement (ADE) for wild-type SARS-CoV-2 *in vitro*, but that is still controversial *in vivo* (9, 10). Thus, a safety concern for the clinical use of neutralizing antibodies or plasma from convalescent patients is the ADE of these SARS-CoV-2 variant infections.

Antibody-dependent enhancement has been observed for coronaviruses, and it is often mediated by Fc receptors (FcRs) on different immune cells for immunoglobulin G (IgG) (11, 12). In SARS-CoV infection, studies have demonstrated FcR-IgG-mediated ADE in ACE2-negative cells (11, 13, 14). A novel mechanism for ADE in MERS-CoV has demonstrated that both the Fc and Fab portions of anti-MERS mAb are required for antibody-mediated viral entry, suggesting that the Fab-Spike complex was associated with ADE activity (15). Multiple studies have reported FcR-independent infection enhancement of wild-type SARS-CoV-2 *in vitro* but have not exhibited infection enhancement in animal model experiments (9, 10). Obviously, the antigenically of spike (S) protein on the surface of the worldwide SARS-CoV-2 variants differs from the previous wild type. Therefore, the ability of neutralizing antibodies to mediate the enhancement of new SARS-CoV-2 variant infection is unknown, but is a theoretical concern for COVID-19 antibody-based therapies development.

As SARS-CoV-2 strains continue to evolve, SARS-CoV-2 variants are replacing formerly dominant strains and sparking new COVID-19 epidemics (16), i.e., B.1.1.7 (broke out in the United Kingdom), B.1.351 (broke out in South Africa), and B.1.1.28 (broke out in Brazil). Most of these variants contain the D614G mutation and mutations of the receptor-binding domain (RBD) in the spike protein. Several studies have established that multiple of these mutations could increase the transmissibility of SARS-CoV-2 in ACE2-positive cells (17, 18). However, it has not been fully documented whether neutralizing antibodies or plasma from convalescent patients mediates ADE of these SARS-CoV-2 variant infections in immune cells. Therefore, we constructed engineered double-mutant variants containing an RBD mutation and D614G in the spike protein and natural epidemic variants to gain insights into the correlation between the mutations in the spike protein and the ADE of SARS-CoV-2 variant infection.

Here, we constructed a series of SARS-CoV-2 variants, including engineered double-mutant variants containing an RBD mutation and D614G in the spike protein and natural epidemic variants, and tested whether plasma samples and TOP10 neutralizing antibodies in our laboratory from convalescent patients mediated the ADE effects of these SARS-CoV-2 variants. We found that one out of 29 convalescent plasma samples caused the ADE effect, and one potential neutralizing antibody, 55A8, from the same batch of convalescent patients mediated the ADE effects for most of the SARS-CoV-2 variants *in vitro*. Furthermore, the ADE activity of 55A8 depended on FcγRII on immune cells, and the introduction of LALA mutations at the Fc end of 55A8 eliminated the ADE effects. The study demonstrated that the neutralizing antibodies from convalescent patients mediated the ADE of SARS-CoV-2 variant infection *in vitro*, providing additional evidence to better understand the biology of new SARS-CoV-2 variants for current antibody therapies and vaccine protection.

Materials and methods

Cell lines

HEK 293T cells, Daudi cells, Raji cells, and K562 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco™, USA) supplemented with 10% fetal bovine serum (FBS; Gibco,

Rockville, MD, USA), 100 mg/ml of streptomycin, and 100 units/ml of penicillin at 37°C in 5% CO2. HEK 293T cells transfected with human ACE2 (293T-ACE2) were cultured under the same conditions (7). Daudi, Raji, and K562 cell lines were cultured at 37 °C in Roswell Park Memorial Institute 1640 Medium (RPMI 1640 medium; Gibco™, USA) with 10% FBS.

Convalescent patients' plasma

Human plasma samples from 29 COVID-19 convalescent patients in Chongqing Medical University Affiliated Yongchuan Hospital were collected on 15 March 2020 and 16 March 2020 (7). All volunteers signed informed consent forms.

Plasmid construction and antibody expression

The pMD2.G plasmid encoding the wild-type SARS-CoV-2 spike (S) gene was generated as previously described[7]. The D614G plasmid and the double-mutant variant plasmids encoding the S gene were constructed by a Phusion site-directed mutagenesis kit (Thermo Scientific, USA), with the wild-type S gene plasmid as a template. Following the procedure of the mutagenesis kit to amplify variants by circular PCR, 15 to 20 nucleotides before and after the target mutation site were selected as forward primers, while the reverse complementary sequences were selected as reverse primers. Following site-directed mutagenesis PCR, the template chain was digested using *Dpn I* restriction endonuclease (NEB, USA). Afterward, the PCR product was directly used to transform *E. coli* Stbl3 competent cells; single clones were selected and then sequenced. The frequency of different variants in the epidemic population is shown in Supplementary Table 1, and the primers designed for the spike mutation sites are shown in Supplementary Table 3. The codon-optimized S gene variants encoding the newly epidemic SARS-CoV-2 were synthesized and cloned into pMD2.G vector by Tsingke Biotechnology (Beijing, China), including B.1.1.7, B.1.351, B.1.1.28, B.1.617, B.1.617.2, B.1.1.529.1, and B.1.1.529.2. The SARS-CoV-2 genome and lineage data were downloaded from the BIGD (https://bigd.big.ac.cn/ncov/) and GISAID (https://www.epicov.org/) databases with sample collection date and location information (19–21).

The neutralizing antibodies were generated from SARS-CoV-2 RBD-specific memory B cells using a single B-cell isolation and cloning strategy (7). The heavy chain and light chain plasmids were transiently co-transfected into HEK293 cells followed by purification with Protein A resin. The antibody $55A8^{LALA}$ was generated by introducing the LALA mutation (L234A and L235A) in the Fc region of IgG1 to abolish binding with FcγRs and prepared using the same protocol used for the generation of wild-type Ab.

Production of SARS-CoV-2 pseudoviruses

SARS-CoV-2 pseudotyped viruses were produced as previously described (7). Briefly, 1×10^6 HEK 293T cells were cotransfected with 3.8 μg psPAX2, 3.8 μg pWPXL luciferase, and 0.3 μg pMD2. G plasmid encoding SARS-CoV-2 S and mutations of S using Xfect Transfection Reagent (Takara, Japan) according to the manufacturer's instructions on 6-well-plates. The S and mutant S protein pseudotyped viruses in the supernatants were harvested 48 h after transfection, centrifuged at 300 g for 10 min, filtered through a 0.45 μm filter, and stored at −80°C. The titers of the pseudoviruses were calculated by determining the number of viral RNA genomes per ml of viral stock solution using a Lenti-X qRT-PCR Titration Kit (Takara, Japan).

ADE assays of pseudotyped SARS-CoV-2 infection

Antibody-dependent enhancement assays were performed using the Daudi, Raji, and K562 cell lines. Then, 100 μl of Daudi, Raji, and K562 cells at a density of 2×10^4 cells/ml were seeded 48 h before infection in a 96-well cell culture plate (NEST) coated with 0.01% poly-L-lysine in PBS[22,23]. Ten microliters of 4-fold serially diluted mAbs or 2-fold serially diluted convalescent plasma were mixed with 40 μl of supernatant containing 2×10^6 copies/μl of pseudovirus. The mixture was incubated for 1 h at 37°C and supplied with 5% CO_2. Then, the medium was replaced with 50 μl of fresh medium, and the cells were coincubated with mixtures of pseudoviruses and mAbs for 12 h. The mAb concentrations ranged from 0.12 to 8,000 ng/ml. Subsequently, 100 μl of supplemented fresh medium was added to each well for an additional 48 h of incubation. The relative luminescence units (RLU) were measured using luciferase assay reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol. To perform the ADE blocking experiment with the neutralizing antibody 55A8, Daudi cells were blocked with a fresh medium containing 4 μg/ml of purified mouse anti-human CD32 (Cat: 555447, BD Pharmingen) at 37°C for 1 h, and the other operating steps were in accordance with the ADE assay experiments.

Neutralization assay

Pseudoviruses were generated as previously described. The 50 μl serially diluted antibodies were incubated with pseudovirus (2.0×10^6 copies/μl, 50 μl) at 37°C for 1 h. The

mixture of viruses and purified antibodies was then added to a hACE2-expressing cell line (hACE2-293T cells). After 72 h of culture, the luciferase activity of infected hACE2/293T cells was measured by the Bright-Luciferase Reporter Assay System (Promega). The relative luminescence unit (RLU) of Luc activity was detected using the Thermo Fisher LUX reader. Half-maximal inhibitory concentrations (IC_{50}) were calculated using four-parameter logistic regression in GraphPad Prism 8.0.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8.0. Data are shown as the mean ± SEM. Two-group comparisons were performed by Student's *t*-test. All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

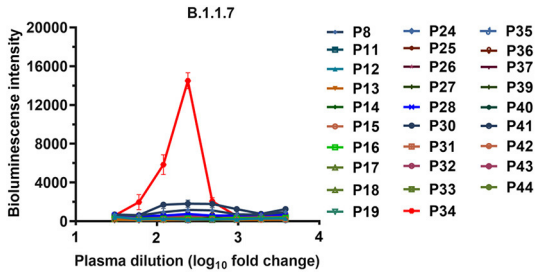

FIGURE 1
ADE activities of the plasma samples from the COVID-19 convalescent patients. The B.1.1.7 pseudovirus was preincubated with serially diluted plasma samples, and the mixtures were added to Daudi cells to evaluate their ability to enhance infection. Each curve represents an individual plasma sample. RLU values resulting from infection with variant pseudotyped viruses were quantified by a luminescence meter. Data for each plasma sample were obtained from a representative infectivity experiment of three replicates and presented as the mean values ± SEM.

Results

The plasma from convalescent patients against wild-type SARS-CoV-2 mediated the ADE effects of the epidemic variants *in vitro*

To assess whether the neutralizing antibodies obtained from the convalescent patients mediated the ADE effects of SARS-CoV-2, we first tested the enhancement of SARS-CoV-2 infection for the 29 convalescent plasma samples, which were from our previous research that screened neutralizing antibodies from the convalescent patients of COVID-19 (7). The ADE activities of these convalescent plasma samples were preliminarily screened and confirmed with three concentration dilutions using a magnetic chemiluminescence enzyme immunoassay (MCLIA) and a pseudovirus-based assay by the Wuhan-1 strain (GenBank: MN_908947, as a wild-type strain) and the variant B.1.1.7. Among the 29 convalescent plasma samples, none of the three dilutions mediated the ADE effects of wild-type SARS-CoV-2 (Supplementary Figure 1). However, the P34 plasma sample showed an enhancement for pseudotyped SARS-CoV-2 B.1.1.7 infection, which was indicated by the increase in luciferase expression in Daudi cells (Supplementary Figures 2, 3). We further detected the enhancement of the ADE effects for these 29 plasma samples at serial dilutions by the B.1.1.7. As shown in Figure 1, only the P34 sample showed a concentration-dependent enhancement of infection in Daudi cells, indicating that a small amount of convalescent plasma from convalescent patients mediated the ADE effects of the SARS-CoV-2 variants. Therefore, we speculated that a small number of neutralizing antibodies obtained from convalescent patients with COVID-19 may have ADE activities against SARS-CoV-2 variants.

Identification of neutralizing antibodies mediating the enhancement of SARS-CoV-2 variant infection

To investigate the ADE activities of the neutralizing antibodies for the SARS-CoV-2 variants, we analyzed mutants of the SARS-CoV-2 spike protein that determine the infectivity of the virus and its transmissibility in the host based on published genomic data (19). The analyses of variants showed that 81.79% of these variants contained the D614G mutant in the spike protein reported in the database in September 2020 (Supplementary Table 1). Therefore, using the codon-optimized S gene of wild-type SARS-CoV-2 (Wuhan-1 strain) as a template, we first constructed the D614G pseudotyped plasmid by site-directed mutagenesis, and the top 10 potential antibodies against D614G from our lab retained neutralizing activities identical or similar to those against wild-type SARS-CoV-2 (Supplementary Figure 4) (22). Next, we evaluated the enhancement of the D614G pseudotyped variant infection for the top 10 potential neutralizing antibodies from our lab by using Daudi cells (7). The results showed that only the neutralizing antibody 55A8 enhanced D614G variant infection at all three concentrations (Figure 2A). Moreover, the ADE activity of 55A8 showed a concentration-dependent enhancement for the D614G pseudotyped variant infection in Daudi cells and Raji cells (Figure 2B), suggesting that the neutralizing antibody 55A8 may be an ADE antibody of SARS-CoV-2 variants. According to our previous antibodies screening records, the neutralizing antibody 55A8 gene was isolated from a mixed memory B-cell sample containing the P8, P11, P12, P17, P18, P30, P31, P34, and P35 samples (7). Although the P34 plasma sample showed an ADE activity, it was difficult to

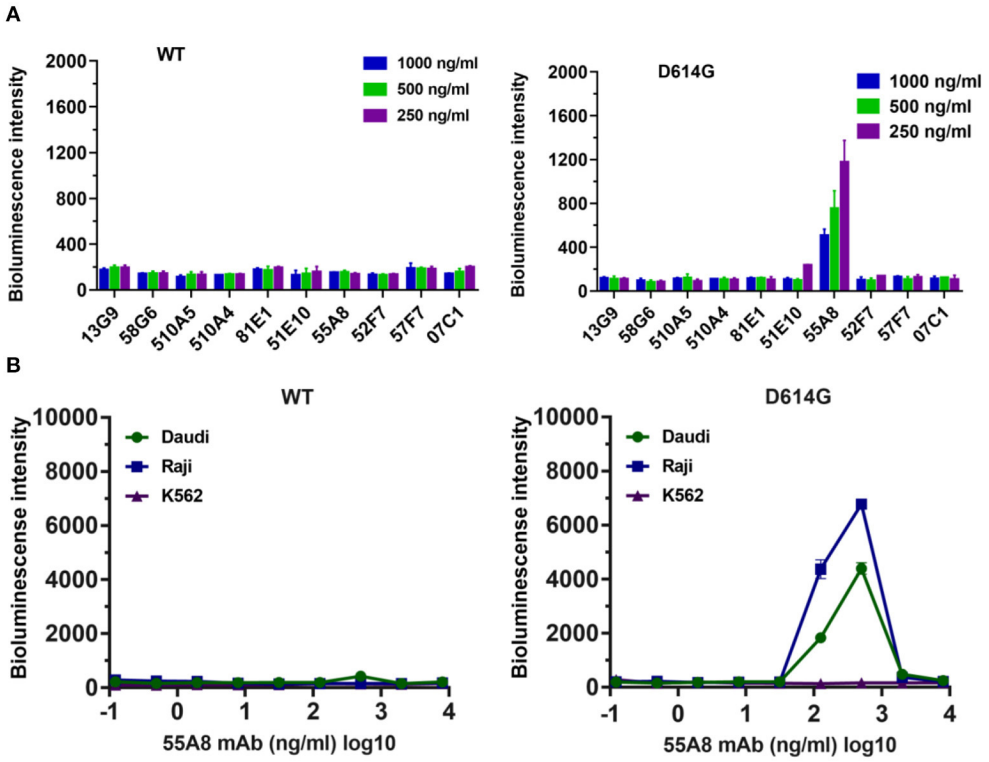

FIGURE 2
Identification of the neutralizing antibodies (Nabs)-mediated enhancement of SARS-CoV-2 variant infection. **(A)** Assessment of the ADE activities of 10 potent NAbs against SARS-CoV-2 WT and the D614G variant. Pseudoviruses were preincubated with 250, 500, and 1,000 ng/ml of NAbs, and these mixtures were added to Daudi cells to evaluate their ability to enhance infection. **(B)** Assessment of ADE activities of the neutralizing antibody 55A8 for SARS-CoV-2 WT and the D614G variant. Pseudoviruses preincubated with serial dilutions of 55A8 mixtures were added to Daudi, Raji, and K562 cells to evaluate their ability to enhance infection. RLU values resulting from infection with variant pseudotyped viruses were quantified by a luminescence meter. Data for each NAb were obtained from a representative infectivity experiment of three replicates and presented as the mean values ± SEM.

determine which patient the antibody 55A8 originated from as the neutralizing antibodies derived from memory B cells may be different from those obtained from the convalescent plasma. Additionally, we further analyzed the usage of antibody variable-gene segments for variable (V) genes (Supplementary Table 2). We found that the heavy chain of 55A8 was encoded by IGHV1-69, and the IGHV1-69 gene could pair with the light chain V gene IGKV1-5 (Supplementary Table 2), which was consistent with some ADE antibodies previously reported (23, 24). In summary, the neutralizing antibody 55A8 obtained from convalescent patients against wild-type SARS-CoV-2 mediated the ADE effects of the D614G variant.

The neutralizing antibody 55A8 mediated antibody-dependent enhancement of SARS-CoV-2 variant infection

As previously described, the early database showed that most of the RBD (neutralizing antibody-binding domain) mutants reported in September 2020 were found with the D614G mutant (Supplementary Table 1). We next selected two important mutants containing an RBD mutant and the D614G mutant to construct double-mutant pseudotyped viruses to confirm the ADE of SARS-CoV-2 variant infection mediated by 55A8. Therefore, 49 double-mutant variants were successfully constructed (Supplementary Figure 5). Of all 49 pseudotyped variants, only seven were determined to have high infectivity, and the RLU levels were 1.5-fold higher than those of the D614G strain, including D614G+P330S, D614G+F338L, D614G+A348T, D614G+N439K, D614G+G446V, D614G+T478I, and D614G+H519Q (Supplementary Figure 5). Among them, the results confirmed that the multiple mutations changed the infectivity of the SARS-CoV-2 variants, e.g., D614G, N439K, G446V, and H519Q, which was consistent with previous reports (25–28). Interestingly, the top 10 potential antibodies from our lab retained good neutralizing activities against these seven double-mutant SARS-CoV-2 variants (Supplementary Figure 5).

Then, we evaluated the enhancement of the seven highly infectious double-mutant variant infections for the top 10

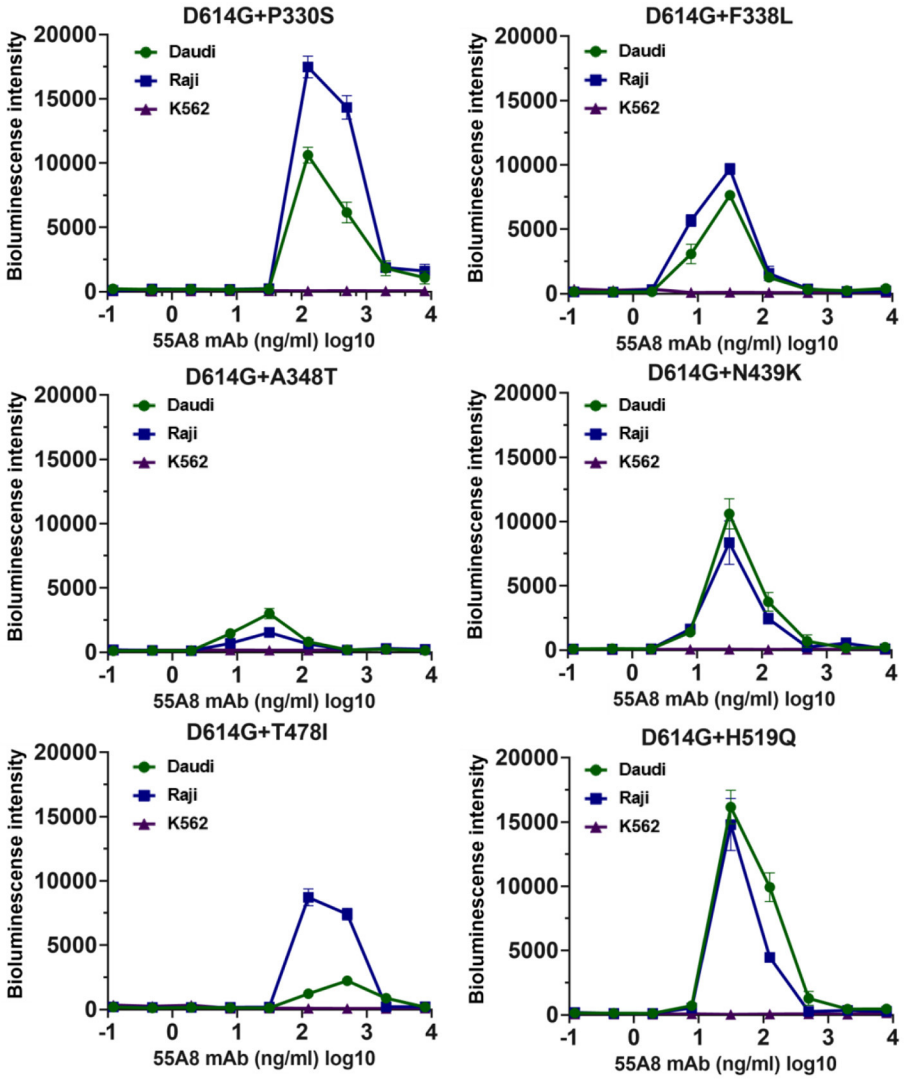

FIGURE 3
ADE activities of the neutralizing antibody 55A8 for the high-infective double-mutant variants. The highly infectious double-mutant variants included D614G+P330S, D614G+F338L, D614G+A348T, D614G+N439K, D614G+T478I, and D614G+H519Q. Pseudoviruses were preincubated with serial dilutions of 55A8 mAbs, and these mixtures were added to Daudi, Raji, and K562 cells to evaluate their ability to enhance infection. RLU values resulting from infection with variant pseudotyped viruses were quantified by a luminescence meter. Data for each NAb were obtained from a representative infectivity experiment of three replicates and presented as the mean values ± SEM.

potential neutralizing antibodies from our lab at three antibody concentrations by using Daudi cells. The results also showed that only the potential neutralizing antibody 55A8 showed an enhancement of the six high-infectivity double-mutant variant infections (including D614G+P330S, D614G+F338L, D614G+A348T, D614G+N439K, D614G+T478I, and D614G+H519Q), except SARS-CoV-2 D614G+G446V, as indicated by the increase in luciferase expression in Daudi cells (Supplementary Figure 6). We further detected the enhancement of the double-mutant variant infection for 55A8 at serial dilutions in Daudi, Raji, and K562 cells. The neutralizing antibody 55A8 showed a concentration-dependent enhancement of infection at six SARS-CoV-2 variants in Daudi cells and Raji cells (Figure 3), but the ADE effect of D614G+G446V was not observed (Supplementary Figure 7). The antibody concentration range of the 55A8 ADE activity was from 7.81 ng/ml to 2000.00 ng/ml, and the highest ADE levels of these variants containing different RBD mutation sites were different, suggesting that these mutations of RBD may be related to these variants' ADE activities. Taken together, these results confirmed that the neutralizing antibody 55A8 was an ADE antibody.

Additionally, studies on ADE effects using 55A8-mediated new epidemic SARS-CoV-2 variants (including B.1.1.7, B.1.351,

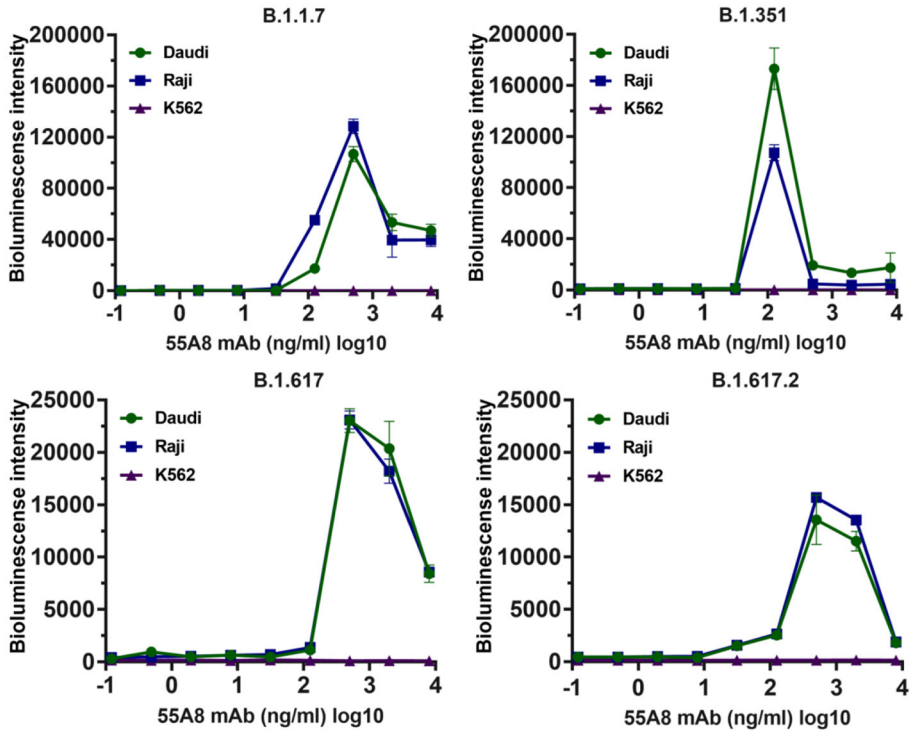

FIGURE 4
The ADE activities of 55A8 for the newly emerged variants. New epidemic SARS-CoV-2 variants (including B.1.1.7, B.1.351, B.1.617, and B.1.617.2) were preincubated with serially 55A8, and their mixtures were added to Daudi, Raji, and K562 cells to evaluate their ability to enhance infection. RLU values resulting from infection with variant pseudotyped viruses were quantified by a luminescence meter. Data for each NAb were obtained from a representative infectivity experiment of three replicates and presented as the mean values ± SEM.

B.1.1.28, B.1.617, B.1.617.2, B.1.1.529.1, and B.1.1.529.2) were also evaluated. As shown in Figure 4, the assay results indicated that the diluted neutralizing antibody 55A8 mediated the ADE effects of four epidemic SARS-CoV-2 variants (including B.1.1.7, B.1.351, B.1.617, and B.1.617.2), but the ADE effect of the B.1.1.28, B.1.1.529.1, and B.1.1.529.2 strains was not observed (Supplementary Figure 7). Therefore, these results have shown that the neutralizing antibody 55A8 can mediate the ADE effects of most double-mutant variants and the epidemic variants, indicating that ADE activity may be closely related to the antibody characteristics.

The neutralizing antibody 55A8 caused ADE effects depending on the Fc receptors

Fc receptors (FcRs) have been shown to enhance antibody-dependent infectivity in several viral infections, including dengue fever virus, severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS) coronavirus infections (11, 29, 30). Recent studies have shown that the ADE antibodies of SARS-CoV-2 have at least two mechanisms (9, 31): RBD-specific antibodies depend on Fc-FcγRII, while NTD-specific antibodies depend on changing the conformation of the S protein, which affects the binding of the S protein to the receptor ACE2. Therefore, the RBD-specific antibody 55A8 was used to confirm whether the enhancements of these new SARS-CoV-2 variant infections were mediated by Fc-FcγRII. In this study, we used anti-CD32 (blocking FcγRII) to block the cell surface FcγR receptor to evaluate the engagement of the FcγR receptor in promoting SARS-CoV-2 variants infection. As shown in Figure 5A, the addition of the blocking anti-FcγRII antibody eliminated the enhancement of B.1.1.7 and B.1.351 infection by the neutralizing antibody 55A8, which is similar to the ADE of coronavirus infections, including SARS-CoV, MERS-CoV, Zika, and dengue viruses (32). Additionally, previous studies have shown that the introduction of a LALA mutation at the Fc end of the antibody can eliminate the ADE effects without decreasing its neutralizing activity (9). We also introduced the LALA mutation to the Fc region of 55A8 ($55A8^{LALA}$) to decrease the engagement of 55A8 with FcγRs. Interestingly, no ADE activities were detected for $55A8^{LALA}$ on all two new SARS-CoV-2 variants, including B.1.1.7 and B.1.351 (Figure 5B). Therefore, these results showed that the ADE activity mediated

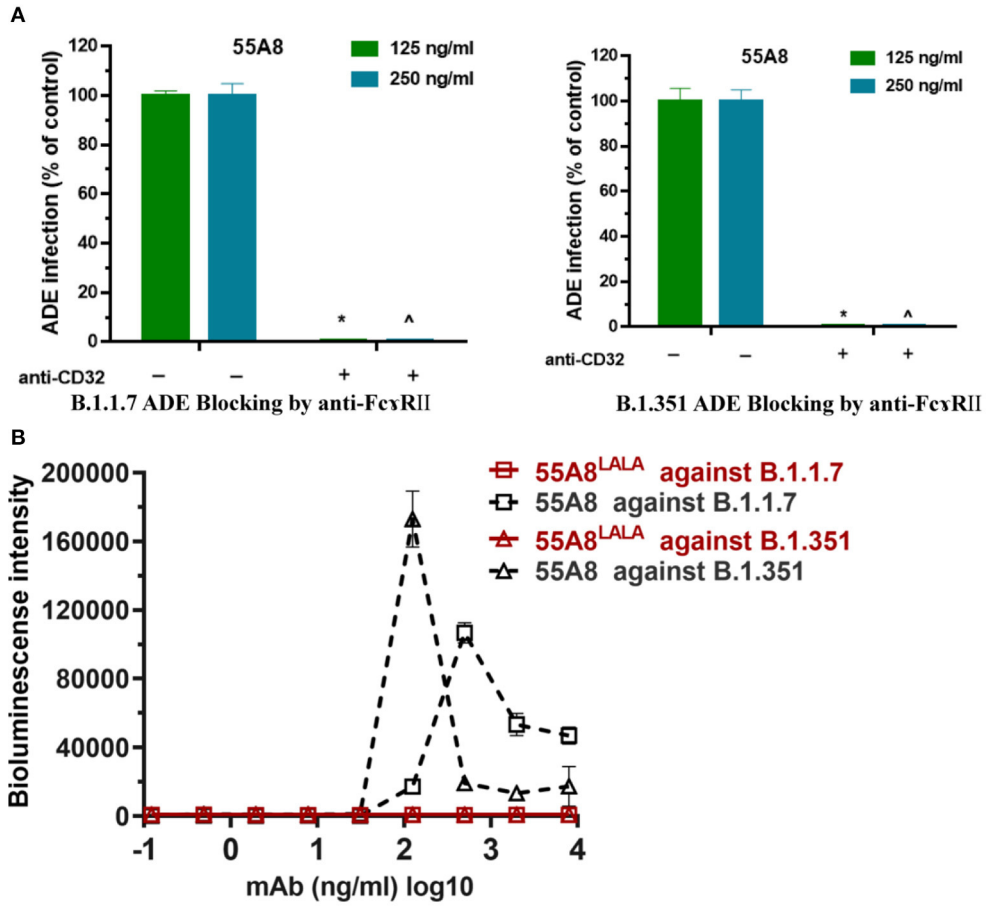

FIGURE 5
The ADE effects mediated by the neutralizing antibody 55A8 depend on FcγRII. **(A)** Assessment of ADE activities of the neutralizing antibody 55A8 for the epidemic variants (including B.1.1.7 and B.1.351) with or without anti-CD32 (blocking FcγR II). The SARS-CoV-2 B.1.1.7 and B.1.351 pseudoviruses were preincubated with 125 ng/ml and 250 ng/ml of 55A8, and their mixtures were added to Daudi cells to evaluate their ability to enhance infection. The percentage of control-represented ADE infection was quantified by luminescence meter and normalized to the reference groups without anti-CD32 produced in parallel, and the anti-CD32 was diluted to 40 ng/ml. **(B)** Assessment of ADE activities of $55A8^{LALA}$ for epidemic variants, including B.1.1.7 and B.1.351. Pseudoviruses were preincubated with serially diluted 55A8 or $55A8^{LALA}$, and their mixtures were added to Daudi cells to evaluate their ability to enhance infection. RLU values resulting from infection with variant pseudotyped viruses were quantified by a luminescence meter. Data for each NAb were obtained from a representative infectivity experiment of three replicates, presented as the mean values ± SEM, and *p*-values were calculated via two-sided Student's *t*-test. *$p < 0.05$ vs. the 125 ng/ml of 55A8 group without blocking anti-FcγRII antibody, and ^$p < 0.05$ vs. the 500 ng/ml of 55A8 group without blocking anti-FcγRII antibody.

by 55A8 was dependent on FcγRII expressed in immune cells, and the neutralizing antibody $55A8^{LALA}$ could still be used to prevent SARS-CoV-2 variants.

Discussion

The SARS-CoV-2 variants are of concern because of their rapid increase to dominance as well as their unusually large number of mutations in the spike protein, which could lead to changes in mAb therapies and vaccine protection. There are growing concerns that the ADE of SARS-CoV-2 variant infection may affect the safety of therapeutic Abs agents and vaccines. In this study, we described the ADE of SARS-CoV-2 variant infection mediated by plasma samples and neutralizing antibodies obtained from convalescent patients.

Currently, strategies for neutralizing antibodies and vaccines have been developed by targeting the prototype SARS-CoV-2 Wuhan-Hu-1 strain. Notably, the new SARS-CoV-2 variants circulating around the world are antigenically distinct from the previous type. For example, the ongoing Omicron variants have been increasing in the current global pandemic. In this study, we constructed two types of SARS-CoV-2 variants, including engineered double-mutant variants containing an RBD mutation and D614G in the spike protein

and natural epidemic variants, to assess the ADE activities for the convalescent plasma samples and neutralizing antibodies. Interestingly, we found that one out of 29 convalescent plasma samples caused the ADE effects of pandemic variant B.1.1.7, suggesting that the rare convalescent plasma from convalescent patients mediated the ADE effects of the SARS-CoV-2 variants. Moreover, we also detected the enhancement of B.1.1.7 infection for the 17 vaccinees' plasma samples. Notably, among the 17 vaccinee**s'** plasma samples, the results showed that one vaccinee plasma sample showed an enhancement for pseudotyped B.1.1.7 infection (Supplementary Figure 8). Thus, these results showed that the rare ADE activities can be observed *in vitro* in the convalescents and vaccinee**s,** indicating that the neutralizing antibodies obtained from convalescent patients or vaccinee**s** may have ADE activities against SARS-CoV-2 variants.

Then, our further study found that only one potent neutralizing antibody obtained from convalescent patients, 55A8, triggered the ADE effects for most of the engineered double-mutant variants except D614G+G446V, and it also mediated the ADE of four of seven new epidemic SARS-CoV-2 variant infections, including B.1.1.7, B.1.351, B.1.617, and B.1.617.2, which was consistent with previously described engineered double-mutant variants. These results indicated that the ADE effects may be closely related to the characteristics of the antibody itself. In addition, the antibody 55A8 retained neutralizing activities against Omicron strains (B.1.1.529.1 and B.1.1.529.2)[34], and a previous study reported that the Omicron variants (B.1.1.529.1 and B.1.1.529.2), B.1.1.7 and B.1.351 contained multiple shared key mutation sites in the SARS-CoV-2 S protein (33–35). Thus, it is necessary to clarify whether the antibody 55A8 mediates the ADE effects of the Omicron strains. Interestingly, we found that 55A8 was unable to mediate the ADE effects of Omicron variants. Therefore, the relationship between the ADE effects mediated by the 55A8 and the shared key mutations of spike was not clear, and we speculate that it may be related to the binding conformations of this antibody and these S proteins.

In our previous study (22), we performed epitope mapping for the top 20 neutralizing antibodies (including 55A8) in our laboratory via competitive ELISA. The results showed that 16 out of these 20 neutralizing antibodies were grouped into the epitopes recognized by a neutralizing antibody 13G9 (13G9e), but the neutralizing antibody 55A8 had no competition with 13G9. Structural analysis revealed that 13G9 recognizes the steric region $S^{470-495}$ in the wild-type RBD[24], while 55A8 recognizes $S^{345-352}$ and $S^{440-450}$ in the Omicron RBD[34]. The previous study reported that peptides from the S1 region, including $S^{304-323}$, $S^{364-383}$, $S^{364-403}$, $S^{454-473}$, $S^{484-503}$,$S^{544-563}$, $S^{564-583}$, and $S^{574-593}$, dramatically blocked the ADE of patient plasma by peptide scanning (36), but these epitopes don't contain the antigen mutation epitopes recognized by the ADE-inducible antibody 55A8. Therefore, these results suggested that some new antigen mutation epitopes recognized by the antibody 55A8 may be associated with ADE activities.

Moreover, the ADE activities analysis of multiple nAbs with clear epitope information, such as 55A8, 58G6, and 13G9, were completed in this study. Barnes et al. (37) classified the neutralizing antibodies (NAbs) targeting the RBD into four classes (classes 1–4) according to their neutralizing mechanisms. The previous study reported that the ADE-inducible neutralizing antibody 7F3 (class 2 NAb) bound to the spike proteins with one "up" and two "down" RBD domains (36), while the antibody 55A8 (class 3 NAb) has no ADE activity when it against the Omicron variants in this study, which also contained the RBD accessibility epitopes in "up/down" conformations (38). This study also revealed that the neutralizing antibodies 58G6 (class 1 NAb) and 13G9 (class 1 NAb) are bound to the spike protein with three "up" RBD domains (22), which have no ADE activities. Therefore, we speculated that there were other factors that influenced ADE activities in addition to RBD accessibility epitopes in the "up/down" conformation, and the specific mechanisms still need to be further studied.

Previous studies have shown that ADE antibodies against SARS-CoV-2 have at least two mechanisms (9, 31): RBD-specific ADE antibodies rely on Fc-FcγRII, while NTD-specific ADE antibodies affect the binding of S proteins to the receptor ACE2, altering the conformation of S proteins. Our previous study showed that the neutralizing antibody 55A8 is an RBD-specific antibody (22), and the results of this study demonstrated that 55A8 mediated the enhancement of SARS-CoV-2 pseudovirus by FcγRII expression in leukocyte lines, which was consistent with the mechanism of the SARS-CoV-2 monoclonal antibody 7F3 (36). Most importantly, our results also confirmed that the introduction of LALA mutations at the Fc end of 55A8 eliminated the ADE effects, indicating that 55A8 may be used as a clinical drug to prevent SARS-CoV-2 variants.

Interestingly, we noted that the variable genes of 55A8 were transcribed from IGHV1-69 and IGKV1-5, while the variable genes of the non-ADE antibodies in this study tended to be distributed in other gene clusters (Supplementary Table 2). According to the data analysis from a previous report, these two variant regions were also genetically responsible for a panel of ADE Abs (23, 24). However, this observed phenomenon with the IGHV1-69 and IGKV1-5 germline genes in ADE Abs could not be demonstrated due to insufficient data; in other words, its correlation with ADE activities remains unknown.

Additionally, another study from our team confirmed that the 55A8 reduced Omicron viral replication and prevented disease symptoms without causing additional distress in hamsters (38), indicating that the 55A8 antibody could not

mediate the ADE effect of the Omicron variant *in vivo*, which was consistent with the results using the pseudoviruses system *in vitro* (Supplementary Figure 7). Since antibody-enhancing infection *in vitro* does not necessarily herald enhanced infection *in vivo*, one additional improvement that may be integrated into our study is to provide more validation experiments *in vivo* using authentic SARS-CoV-2 variants. Taken together, although rare enhanced infection was observed in the neutralizing antibodies and plasma samples, it is difficult to predict whether this phenomenon will occur in the setting of human infection or vaccination. If the rarely enhanced immunopathology was observed *in vivo*, it will be important to continue to monitor ongoing COVID-19 vaccination and neutralizing antibody drugs.

In conclusion, the rare plasma and neutralizing antibodies from convalescent patients mediated the ADE of SARS-CoV-2 variants infection *in vitro*. Thus, ADE may occur in a minority of people who have ADE antibodies and are then exposed to a newly emerged SARS-CoV-2 variant. These data suggested the ongoing neutralizing antibody drugs from convalescent patients who may need to undergo an ADE screening test by SARS-CoV-2 variants, which may benefit the safety of antibody-based therapies in future. Additionally, the potent neutralizing antibody 55A8 mediated the ADE effects depending on FcγRII, and the ADE effects of this antibody could be eliminated after Fc segment modification, indicating that this neutralizing antibody could still be used to prevent SARS-CoV-2 variants. This work also provides a reference for the development of approaches for the treatment of COVID-19 based on potential neutralizing antibodies.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the application of antibody tests patients infected with SARS-CoV-2. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AJ and YinW conceived and designed the study. YH, RW, and YinW constructed the plasmids of SARS-CoV-2 variants. FL and JH were responsible for antibody expression and purification. SS, YH, SM, and YiW performed SARS-CoV-2 variant ADE assays. SM, SS, YinW, XH, TL, CH, SL, MS, WW, and AJ generated figures and tables and take responsibility for the integrity and accuracy of data presentation. YinW, WW, and AJ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.952697/full#supplementary-material

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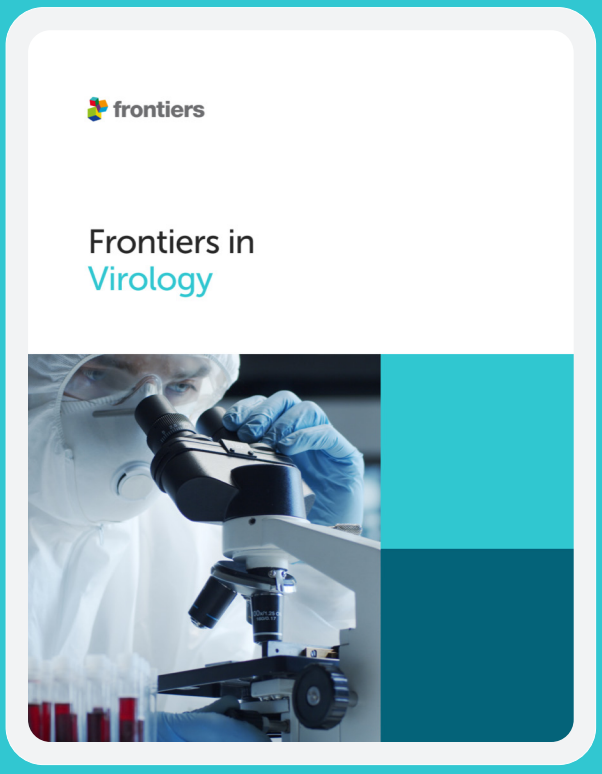

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