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Change in epidemiology and etiology of respiratory tract and gastrointestinal infections during COVID-19 pandemic

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Change in epidemiology and etiology of respiratory tract and gastrointestinal infections during COVID-19 pandemic

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Editorial: Change in epidemiology and etiology of respiratory tract and gastrointestinal infections during COVID-19 pandemic

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Editorial on the Research Topic
Change in epidemiology and etiology of respiratory tract and gastrointestinal infections during COVID-19 pandemic

The COVID-19 pandemic has profoundly impacted global health, not only through the direct effects of SARS-CoV-2 but also by altering the landscape related to other respiratory and gastrointestinal infections. As researchers have focused some attention to the understanding of these shifts, our Research Topic, "*Change in Epidemiology and Etiology of Respiratory Tract and Gastrointestinal Infections during COVID-19 Pandemic*," has gathered a diverse Research Topic of studies that shed light on these changes. This editorial aims to frame the significance of such research, to summarize key findings from the contributing articles, and to contextualize their implications for future public health strategies.

The COVID-19 pandemic imposed unprecedented public health measures at country scales, such as social distancing, lockdowns, and enhanced hygiene practices (Han et al., 2020). These measures not only influenced the transmission dynamic of SARS-CoV-2 but also had consequential effects on other infectious diseases (Kaba et al., 2021; Chow et al., 2023). Eight original articles (Pan et al.; Cao et al.; Zhou et al.; Guo et al.; Yokoyama et al.; Korsun et al.; Alemayehu et al.; Zhu et al.) and one review (He et al.) in this Research Topic explore these phenomena from various angles, providing valuable insights into the complex interplay between the pandemic and other pathogens.

Pan et al. offered a comprehensive analysis of how COVID-19 control measures impacted the prevalence of other respiratory viruses among 2,744 patients with acute respiratory tract infections in Shanghai, China. The authors found a significant reduction in the detection of common respiratory viruses, highlighting the collateral benefits of COVID-19 mitigation strategies on respiratory infection. Indeed, at the beginning of the

pandemic, there were no effective vaccines available globally, hence non-pharmaceutical interventions (NPIs) such as social distancing, wearing masks, practicing hand hygiene, and delaying the spring 2020 schools' semester were implemented to prevent the spread of SARS-CoV-2 (Li et al., 2022). These interventions impacted other respiratory viruses, including influenza, which was the most prevalent viral pathogen among the respiratory pathogens (Ye et al., 2017).

The COVID-19 pandemic particularly influenced the evolutionary dynamics of respiratory viruses (Chow et al., 2023). Guo et al. provided a comparative analysis of respiratory syncytial virus (RSV) genetic diversity before and during the pandemic using sequences collected from the NCBI GenBank Database. The study indicated shifts in evolutionary pressures, but no substitutions that altered the structural conformations of the antigenic sites. These findings suggested that the intensive NPIs during the COVID-19 pandemic did not influence the evolutionary patterns of RSV. The incidence of RSV after the main part of the pandemic was analyzed in another study conducted by Korsun et al. in Bulgaria. A considerable resurgence of this virus was reported during autumn 2022, following the lifting of NPIs for COVID-19.

In addition, Zhu et al. highlighted the re-emergence of *Mycoplasma pneumoniae* as COVID-19 restrictions were eased in Shanghai, China. Although these outbreaks may represent a return to the cyclical respiratory pathogens similar to the time before the COVID-19 pandemic (Upadhyay and Singh, 2024). They underscore the need for continuous vigilance and adaptability in public health responses to prevent and control outbreaks of other infectious diseases.

Another path to try understanding the COVID-19 impact was to determine the factors that influenced viral clearance. Cao et al. delved into the role of immune markers like IgG and IL-6 in predicting the duration of viral shedding. Their findings showed that age, IgG, and IL6 could potentially serve as useful predictors for SARS-CoV-2 RNA clearance exceeding 14 days in asymptomatic and mild COVID-19 patients in Tianjin, China. This information can assist in estimating which patients might have a shorter recovery period, offering valuable insights for clinical prevention and control strategies. In fact, achieving rapid clearance of SARS-CoV-2 during the early stages of infection is desirable, as it can help prevent viral spread within the body and minimize tissue damage and severe clinical outcome (Adhikari et al., 2020).

The COVID-19 pandemic also complicated the management of secondary infections. Zhou et al. provided a critical examination of the challenges faced by healthcare providers in managing secondary infections, and co-infections among vulnerable populations in Beijing, China. These authors found that predictors of secondary pulmonary infection and co-infection were as follow: severe COVID-19 disease, ICU admission within 48 h of hospitalization, PCT >0.5 ng/ml. and cerebrovascular diseases. Their study emphasized the importance of integrated care approaches and robust infection control practices in mitigating the impact of secondary infections. As they found that increased PCT was a strong prognostic factor, elevated PCT level could indicate antibiotic treatment need in these cases (Almulhim et al., 2024).

During the initial phase of COVID-19, patients often experience gastrointestinal symptoms (Mao et al., 2020). The severity of COVID-19 has been linked to changes in gut microbiota, with more pronounced dysbiosis observed in patients with more severe illness (Neag et al., 2022). This is thought to be due to the high expression of ACE2, which serves as the entry point for SARS-CoV-2 into the gastrointestinal system (Ni et al., 2020). Moreover, different SARS-CoV-2 variants might alter the gut microbiota and metabolites. This was illustrated in a Japanese study by Yokoyama et al. in this Research Topic. Fecal microbiome analysis showed that α-diversity was reduced in the order of the Omicron, Delta, and Alpha variants and that the Omicron and that Delta variants had markedly reduced propionic and lactic acid levels compared to the Alpha strain ($p < 0.05$). Other studies on the gut microbiota of COVID-19 patients reported that the abundance of some bacteria, such as *Faecalibacterium prausnitzii*, *Eubacterium rectale*, and various *Bifidobacteria* species, decreased as the disease progressed (Yeoh et al., 2021; Zhang et al., 2023). Additionally, metabolomic studies indicated a reduction in short-chain fatty acids and L-isoleucine production in the gut microbiota, which was linked to the severity of the disease (Zhang et al., 2023).

A narrative review conducted by He et al. explored the persistence and management of gastrointestinal symptoms in long-term COVID-19 patients. Such symptoms are caused by factors such as SARS-CoV-2 infection of intestinal epithelial cells, cytokine storm, gut dysbiosis, therapeutic drugs, psychological factors, and the worsening of pre-existing conditions. Interventions like probiotics, prebiotics, fecal microbiota transplantation, and antibiotics were shown to help maintaining the intestinal microecological balance and reducing gastrointestinal symptoms. This study's findings highlight the need for ongoing research into the mechanisms and treatments for such persistent symptoms.

Finally, in a study of Alemayehu et al. conducted in Ethiopia, a direct impact of NPIs against COVID-19, especially interventions in water, sanitation, and hygiene, was shown to significantly reduce the incidence of childhood diarrhea, suggesting that some pandemic-era practices could be beneficial if continued post-pandemic.

The collective findings of these studies underscore the multifaceted impact of the COVID-19 pandemic on respiratory and gastrointestinal infections. While the pandemic was associated with numerous challenges, it also provides that helped to understand the dynamics of other infectious diseases in unprecedented ways. As we move forward, the insights gained from this Research Topic will be valuable in shaping more effective public health strategies, enhancing our preparedness for future pandemics, and improving the overall management of respiratory and gastrointestinal infections. We extend our gratitude to all the contributing authors for their valuable research and to the reviewers for their insightful feedback. Together, these contributions have significantly advanced our understanding of the intricate interplay between COVID-19 and other infectious diseases.

Author contributions

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Conflict of interest

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Secondary pulmonary infection and co-infection in elderly COVID-19 patients during the pandemics in a tertiary general hospital in Beijing, China

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Background: Most people are infected with COVID-19 during pandemics at the end of 2022. Older patients were more vulnerable. However, the incidence of secondary bacterial, fungal or viral pulmonary infection and co-infection is not well described in elderly hospitalized COVID-19 patients.

Methods: We retrospectively reviewed the medical records of all elderly (≥65 years) hospitalized patients with laboratory-confirmed COVID-19 from December 1, 2022 to January 31, 2023. Demographics, underlying diseases, treatments, and laboratory data were collected. Univariate and multivariate logistic regression models were used to explore the risk factors associated with secondary bacterial, fungal or viral pulmonary infection and co-infection.

Results: A total of 322 older patients with COVID-19 were enrolled. The incidence of secondary bacterial, fungal or viral pulmonary infection and co-infection was 27.3% (88/322) and 7.5% (24/322), respectively. The overall in-hospital mortality of all patients was 32.9% (106/322), and the in-hospital mortality among patients who acquired with secondary pulmonary infection and co-infection was 57.0% (57/100). A total of 23.9% (77/322) of patients were admitted to ICU within 48 h of hospitalization. The incidence of secondary pulmonary infection and co-infection among patients admitted to the ICU was 50.6% (39/77) and 13.0% (10/77), respectively. The overall in-hospital mortality of ICU patients was 48.1% (37/77), and the in-hospital mortality of ICU patients acquired with secondary pulmonary infection and co-infection was 61.4% (27/44). A total of 83.5% (269/322) of the included patients received empirical antibiotic therapy before positive Clinical Microbiology results. Influenza A virus (the vast majority were the H3N2 subtype) was the most common community acquired pathogen for co-infection. While *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* were the common hospital acquired pathogens for co-infection and secondary pulmonary infection. The incidence of Carbapenem-resistant Gram-negative bacilli (CR-GNB) infections was high, and the mortality reached 76.9%. Predictors of secondary pulmonary infection and co-infection were ICU admission within 48 h of hospitalization, cerebrovascular diseases, critical COVID-19, and PCT > 0.5 ng/mL.

Conclusion: The prognosis for elderly hospitalized COVID-19 patients with secondary pulmonary infection or co-infection is poor. The inflammatory biomarker PCT > 0.5 ng/mL played an important role in the early prediction of secondary pulmonary infection and co-infection in COVID-19 patients.

KEYWORDS

COVID-19, elderly, co-infection, secondary infection, Carbapenem-resistant Gram-negative bacilli

1. Introduction

Coronavirus disease 2019 (COVID-19), was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It was first identified in December 2019 in Wuhan, China, then spread rapidly worldwide. By May 17, 2023, the WHO reported a total of 766,440,796 confirmed cases of COVID-19 globally, including 6,932,591 deaths.[1] In December 2022, the Chinese government announced that COVID-19 patients did not need to be quarantined. Within the next 2 months, a significant proportion of people were infected with SARS-CoV-2, especially elderly individuals. It posed formidable medical challenges for healthcare systems and clinicians.

Secondary bacterial, fungal or viral pulmonary infection and co-infection are a common and dangerous complication of COVID-19. A previous study showed that the incidence of co-infection and secondary infection in COVID-19 patients was generally low, but the incidence in Intensive Care Unit (ICU) patients was relatively high (Langford et al., 2020). It was significantly associated with poor clinical outcomes. According to existing report, 50% of COVID-19 deaths experienced secondary bacterial infection (Li et al., 2020). Several studies indicated that the majority of COVID-19 patients (>90%) received empirical antibiotics, which increased the risk of Multi-Drug Resistance (MDR) infection (Du et al., 2020; Wang Z. et al., 2020; Wu et al., 2020). International guidelines recommended empirical antibiotic therapy only for the possible occurrence of bacterial pneumonia in critically ill patients with COVID-19 (Poston et al., 2020).

Understanding the incidence of secondary bacterial, fungal or viral pulmonary infection and co-infection, and common pathogens in COVID-19 patients is crucial for appropriate antimicrobial therapy and improving outcomes. However, these data in older patients with COVID-19 have not been well characterized yet. There was only one relevant study on older patients (Wang L. et al., 2020). The objective of this study was to determine the incidence of secondary bacterial, fungal or viral pulmonary infection and co-infection in elderly COVID-19 patients, and to identify common pathogens. We also identified predictors independently associated with the development of infection.

2. Materials and methods

2.1. Study design and participants

We conducted a single-center, retrospective, observational study. It included all elderly (age ≥65 years) patients infected with COVID-19 who were consecutively hospitalized at Peking University First Hospital between December 2022 and January 2023 in Beijing, China. The exclusion criteria were as follows: (a) some key information was missing from the medical record; and (b) pathogens isolated from non-respiratory tract or non-bloodstream sources. The diagnosis of COVID-19 was made through Reverse Transcriptase real-time fluorescence Polymerase Chain Reaction (RT-PCR) in all cases from nasal or pharyngeal swabs. The primary outcome was the incidence of secondary bacterial, fungal or viral pulmonary infection and co-infection. We also assessed the risk factors associated with secondary bacterial, fungal or viral pulmonary infection and co-infection, and the impact of infection on clinical outcomes. Hospitalized patients with COVID-19 developed respiratory failure, required mechanical ventilation, was in shock or combined with other organ failure, they should be admitted to ICU for further treatment.

2.2. Data collection

Extracting clinical data from the electronic medical records, including patient demographics, underlying diseases, laboratory tests, clinical symptoms, treatments, microbiological results (blood culture, respiratory specimen culture, specific real-time fluorescence PCR test, urinary antigen test, and antimicrobial susceptibility testing), and clinical outcomes (in-hospital mortality, length of hospital stay, ICU admission, length of ICU stay, invasive mechanical ventilation, and the duration of invasive mechanical ventilation). All above data were entered into the computerized database for further statistical analyses.

2.3. Definitions

The severity of COVID-19 was defined according to the Chinese management guidelines for COVID-19 (version 10.0).[2]

1 https://covid19.who.int/

2 http://www.nhc.gov.cn/

In brief, the disease was defined as critical if the patient met one of the following conditions: respiratory failure occurred and required mechanical ventilation, shock, or other organ dysfunction requiring ICU monitoring and treatment. The definition of septic shock was based on the 2016 Third International Consensus Definition for Sepsis and Septic Shock (Singer et al., 2016). Pulmonary infection included either (a) co-infection, defined as patients with confirmed COVID-19 disease with other simultaneously co-infected pathogens (<48 h), or (b) secondary infection, defined as patients infected by other new pathogens after 48 h of hospital admission (Lansbury et al., 2020). Bloodstream infections were defined as at least one bottle of positive blood culture for a likely pathogen or ≥2 bottles of positive blood cultures for common skin colonizers (for example, Coagulase-negative *Staphylococci*, *Bacillus* spp., *Diphtheroids*, *Propionibacterium* spp., *Viridans group Streptococci*) (Ripa et al., 2021). Interleukin-6 (IL-6) or Janus kinase (JAK) inhibitors were tocilizumab and baricitinib.

2.4. Pathogen identification and antimicrobial susceptibility testing

The attending physicians decided to order microbiological tests for patients with suspected infection, and the clinical microbiologist handled with standard microbiological procedures. The cultured bacterial single colony was identified to species level by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) (Croxatto et al., 2012). Antimicrobial susceptibility testing was performed using VITEK 2 Compact automated system (Biomerieux, France) according to the manufacturer's instructions. Carbapenem-resistant bacteria were characterized by resistance to at least one kind of carbapenem (meropenem, imipenem, or ertapenem). The Minimal Inhibitory Concentrations (MICs) were determined and classified to susceptible, resistant, or intermediate according to breakpoints established by the Clinical and Laboratory Standards Institute (CLSI) (CLSI M100 31th) (Clinical and Laboratory Standards Institute, 2021).

2.5. Statistical analysis

Descriptive statistics included the median [interquartile range (IQR)] of continuous variables and n (%) of categorical variables. Mann-Whitney *U*-tests, $\chi 2$ tests or Fisher's exact tests were used to compare the differences between infection and non-infection, the survivors and non-survivors. Univariate and multivariate logistic regression models were used to explore the risk factors associated with infection. The variables identified as significant ($p < 0.05$) in the univariate analysis entered into the multivariate logistic regression models. Some laboratory findings, including serum ferritin, IL-6, lactate dehydrogenase, high-sensitivity cardiac troponin I, creatine kinase-MB, and D-dimer may not be available in some patients, therefore, only lymphocyte counts, procalcitonin (PCT), and hyper C-reactive protein were entered into the multivariate logistic regression models. All reported *p*-values were double-tailed. All analyses were performed using SPSS version 26 (IBM Corp, Armonk, NY, USA).

3. Results

There was a total of 516 inpatients with RT-PCR confirmed COVID-19 infection, 194 inpatients were excluded (20 inpatients were non-respiratory infection, and 174 patients were aged <65 years), thus, the remaining 322 elderly patients were eventually included for further analyses. A total of 65.5% of included patients were male. The median age was 81.0 (72.0-87.0) years. A total of 59.3% of patients had a Charlson comorbidity index ≥3. Hypertension (67.1%) was the most common underlying disease, followed by chronic cardiovascular diseases (52.5%), chronic respiratory diseases (38.5%), cerebrovascular diseases (37.3%), and diabetes mellitus (37.0%). The most common symptom of COVID-19 infection was fever (81.1%), followed by cough (72.7%) and expectoration (68.0%) (**Table 1**). A total of 23.9% (77/322) of these patients were admitted to the ICU within 48 h of hospitalization. A total of 36.3% (117/322) of patients were classified as critical COVID-19. A total of 83.5% (269/322) of patients received empirical antibiotic therapy prior to the first positive culture. A total of 69.6% (224/322) of patients received antiviral treatments (paxlovid or azvudine) (**Table 1**).

3.1. The clinical characteristics between bacterial infection and non-infection

The overall proportion of COVID-19 patients with secondary bacterial, fungal or viral pulmonary infection and co-infection was 31.1% (100/322). The distribution of age between patients with and without infection is shown in **Figure 1A**, which increased in parallel with age ($p = 0.002$).

The clinical characteristics between the two groups were compared (**Table 1**). Patients with chronic cardiovascular diseases and cerebrovascular diseases were more vulnerable to infection. In addition, Patients admitted to ICU within 48 h of hospitalization (44.0 vs. 14.9%, $p < 0.001$), classified as critical COVID-19 (65.0 vs. 23.4%, $p < 0.001$), received invasive mechanical ventilation (42.0 vs. 6.8%, $p < 0.001$) were more likely to be infected. More infected patients suffered from septic shock (43.0 vs. 5.4%, $p < 0.001$). Infected patients had higher in-hospital mortality (57.0 vs. 22.1%, $p < 0.001$), higher proportion of ICU admission (57.0 vs. 18.0%, $p < 0.001$), longer length of hospital stay (median days 23.0 vs. 14.0, $p < 0.001$), and longer length of ICU stay (median days 12.0 vs. 7.0, $p < 0.001$) than patients without infection.

Multivariate logistic regression analysis showed patients admitted to ICU within 48 h of hospitalization ($p < 0.001$, OR = 2.880, 95% CI, 1.520–5.457), with cerebrovascular diseases ($p = 0.022$, OR = 2.031, 95% CI, 1.109–3.719), classified as critical COVID-19 ($p < 0.001$, OR = 4.160, 95% CI, 2.245–7.707), and PCT > 0.5 ng/mL ($p = 0.046$, OR = 1.989, 95% CI, 1.014–3.902) were independent risk factors of secondary bacterial, fungal or viral pulmonary infection and co-infection (**Table 2**).

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TABLE 1 Summary of clinical features and laboratory results between infection and non-infection groups.

Variables	Total (*n* = 322)	Infection (*n* = 100)	Non-infection (*n* = 222)	*p*
Gender, male, *n* (%)	211 (65.5%)	66 (66.0%)	145 (65.3%)	0.905
Age, median (IQR)	81.0 (72.0–87.0)	83.0 (75.3–88.0)	79.0 (71.0–86.0)	0.002
ICU admission within 48 h of hospitalization, *n* (%)	77 (23.9%)	44 (44.0%)	33 (14.9%)	<0.001
Underlying diseases, n (%)				
Hypertension	216 (67.1%)	70 (70.0%)	146 (65.8%)	0.454
Diabetes mellitus	119 (37.0%)	35 (35.0%)	84 (37.8%)	0.625
Chronic cardiovascular diseases	169 (52.5%)	61 (61.0%)	108 (48.6%)	0.040
Chronic respiratory diseases	124 (38.5%)	36 (36.0%)	88 (39.6%)	0.535
Chronic liver diseases	26 (8.1%)	11 (11.0%)	15 (6.8%)	0.196
Chronic kidney diseases	79 (24.5%)	24 (24.0%)	55 (24.8%)	0.881
Cerebrovascular diseases	120 (37.3%)	47 (47.0%)	73 (32.9%)	0.015
Peripheral vascular diseases	82 (25.5%)	30 (30.0%)	52 (23.4%)	0.210
Hemopathy	20 (6.2%)	5 (5.0%)	15 (6.8%)	0.546
Connective tissue diseases	13 (4.0%)	1 (1.0%)	12 (5.4%)	0.121
Peptic ulcer	26 (8.1%)	9 (9.0%)	17 (7.7%)	0.682
Hemiplegia	9 (2.8%)	5 (5.0%)	4 (1.8%)	0.213
Malignancy	87 (27.0%)	24 (24.0%)	63 (28.4%)	0.413
Charlson comorbidity index ≥3	191 (59.3%)	59 (59.0%)	41 (41.0%)	0.938
Exposure history, n (%)				
Smoking	97 (30.1%)	32 (32.0%)	65 (29.3%)	0.622
Drinking	49 (15.2%)	14 (14.0%)	35 (15.8%)	0.683
Vaccination	32 (9.9%)	10 (10.0%)	22 (9.9%)	0.308
Previous glucocorticoid use	24 (7.5%)	3 (3.0%)	21 (9.5%)	0.070
Previous invasive procedure within 3 months before hospital admission	52 (16.1%)	20 (20.0%)	32 (14.4%)	0.208
Empirical use of antibiotics before the first positive culture	269 (83.5%)	96 (96.0%)	173 (77.9%)	<0.001
Clinical symptoms, n (%)				
Fever	261 (81.1%)	90 (90.0%)	171 (77.0%)	0.006
Sore throat	48 (14.9%)	12 (12.0%)	36 (16.2%)	0.326
Cough	234 (72.7%)	76 (76.0%)	158 (71.2%)	0.368
Sputum	219 (68.0%)	75 (75.0%)	144 (64.9%)	0.071
Fatigue	93 (28.9%)	21 (21.0%)	72 (32.4%)	0.036
Myalgias	33 (10.2%)	11 (11.0%)	22 (9.9%)	0.765
Diarrhea	37 (11.5%)	14 (14.0%)	23 (10.4%)	0.343
Thoracalgia	11 (3.4%)	3 (3.0%)	8 (3.6%)	1.000
Respiratory failure	77 (23.9%)	45 (45.0%)	32 (14.4%)	<0.001
Laboratory findings, median (IQR)				
Write blood count × 10^9 per L	6.7 (4.7–9.8)	7.7 (5.2–11.7)	6.3 (4.3–9.1)	0.007
Neutrophil count × 10^9 per L	5.2 (3.3–8.5)	6.5 (4.2–9.9)	4.8 (2.8–7.6)	0.001
Lymphocyte count × 10^9 per L	0.6 (0.4–0.9)	0.5 (0.3–0.8)	0.6 (0.4–1.0)	0.007
Lymphocyte count < 0.8 × 10^9 per L, n (%)	199 (61.8%)	72 (72.0%)	127 (57.2%)	0.017
Plate count × 10^9 per L	155.0 (111.0–214.0)	145.0 (104.0–196.0)	158.0 (117.3–225.3)	0.058
Neutrophilic granulocyte percentage	81.0 (69.6–88.7)	86.2 (77.8–91.7)	79.0 (66.2–86.1)	<0.001

(Continued)

TABLE 1 (Continued)

Variables	Total (*n* = 322)	Infection (*n* = 100)	Non-infection (*n* = 222)	*p*
Lymphocyte percentage	10.2 (5.1–17.3)	6.7 (3.9–12.8)	11.9 (6.2–19.0)	<0.001
Prothrombin time, s	11.9 (11.3–13.0)	12.4 (11.6–14.7)	11.8 (11.2–12.8)	<0.001
Activated partial thromboplastin time, s	31.1 (28.3–34.4)	32.5 (28.9–37.2)	30.8 (27.9–33.2)	0.002
D-dimer, mg/L	0.6 (0.3–1.2)	0.6 (0.4–2.3)	0.5 (0.3–1.1)	0.012
High-sensitivity cardiac troponin I, ng/L	20.4 (9.6–59.4)	39.8 (18.0–145.3)	15.1 (7.8–36.8)	<0.001
Creatine kinase-MB, mg/mL	1.9 (1.1–3.8)	2.5 (1.3–7.2)	1.7 (1.0–2.8)	<0.001
Creatinine, μmol/L	91.3 (72.0–158.6)	106.7 (75.5–185.4)	89.8 (69.1–140.3)	0.057
Lactic dehydrogenase, U/L	271.0 (204.5–384.5)	311.0 (218.5–455.0)	255.0 (192.0–360.0)	0.001
Serum ferritin, ng/mL	617.1 (261.6–1053.8)	912.4 (399.7–1091.8)	517.4 (172.6–739.8)	0.033
IL-6, pg/mL,	49.7 (19.6–124.7)	65.7 (30.4–192.1)	41.1 (14.8–91.5)	0.001
PCT, ng/mL	0.2 (0.1–0.8)	0.5 (0.2–1.7)	0.1 (0.1–0.5)	<0.001
PCT > 0.5 ng/mL, *n* (%)	93 (28.9%)	48 (48.0%)	45 (20.3%)	<0.001
Hyper C-reactive protein, mg/dL	65.9 (24.5–113.1)	88.5 (44.4–135.3)	54.8 (19.0–105.0)	<0.001
Critical COVID-19	117 (36.3%)	65 (65.0%)	52 (23.4%)	<0.001
Antiviral treatment, *n* (%)	224 (69.6%)	81 (81.0%)	143 (64.4%)	0.003
Paxlovid	132 (41.0%)	58 (58.0%)	74 (33.3%)	<0.001
Azvudine	131 (40.7%)	45 (45.0%)	86 (38.7%)	0.290
Immunomodulatory treatment				
IL-6 or JAK inhibitors, *n* (%)	38 (11.8%)	20 (20.0%)	18 (8.1%)	0.002
Immunoglobulin therapy, *n (%)*	33 (10.2%)	19 (19.0%)	14 (6.3%)	0.001
Glucocorticoid treatment, *n* (%)	150 (46.6%)	64 (64.0%)	86 (38.7%)	<0.001
Time from onset of symptom to start of glucocorticoid treatment, days, median, (IQR)	8.0 (5.0–12.0)	8.5 (6.0–12.0)	8.0 (4.0–12.0)	0.593
Glucocorticoid treatment duration, median, (IQR)	6.0 (3.0–10.0)	5.0 (3.0–9.8)	7.0 (3.8–10.0)	0.526
ECMO, *n* (%)	3 (0.9%)	2 (2.0%)	1 (0.5%)	0.476
CRRT, *n* (%)	16 (5.0%)	10 (10.0%)	6 (2.7%)	0.005
Anticoagulant therapy	167 (51.9%)	70 (70.0%)	97 (43.7%)	<0.001
Outcomes				
ICU admission in hospitalization, *n* (%)	97 (30.1%)	57 (57.0%)	40 (18.0%)	<0.001
Length of ICU stay, days, median (IQR)	9.5 (6.0–17.3)	12.0 (8.0–25.0)	7.0 (3.5–10.0)	<0.001
Mechanism ventilation, *n* (%)	57 (17.7%)	42 (42.0%)	15 (6.8%)	<0.001
Duration of mechanism ventilation, hours, median (IQR)	122.0 (40.0–331.0)	211.5 (75.5–495.0)	38.0 (3.0–58.0)	<0.001
Septic shock, *n* (%)	55 (17.1%)	43 (43.0%)	12 (5.4%)	<0.001
In-hospital mortality, *n* (%)	106 (32.9%)	57 (57.0%)	49 (22.1%)	<0.001
Length of hospital stay from exposure to SARS-COV-2, days, median (IQR)	13.0 (7.0–21.0)	18.5 (11.0–29.0)	10.0 (6.0–18.0)	<0.001
Length of hospital stay, median (IQR)	16.0 (8.0–29.0)	23.0 (14.3–45.0)	14.0 (7.0–23.3)	<0.001

IQR, range interquartile; IL-6, interleukin-6; PCT, procalcitonin; JAK, Janus kinase; ECMO, extracorporeal membrane oxygenation; CRRT, continuous renal replacement therapy.

3.2. Incidence of secondary pulmonary infection and co-infection

The incidence of secondary bacterial, fungal or viral pulmonary infection and co-infection was 27.3% (88/322) and 7.5% (24/322), respectively. A total of 23.9% (77/322) of patients were admitted to ICU within 48 h of hospitalization. The incidence of secondary pulmonary infection and co-infection among patients admitted to the ICU was 50.6% (39/77) and 13.0% (10/77), respectively. In this study, 83.5% (269/322) of patients received empirical antibiotic therapy before positive Clinical Microbiology results, predominantly carbapenems (*n* = 112), followed by cefoperazone/sulbactam (*n* = 104), cephalosporin (*n* = 72), moxifloxacin (*n* = 46), piperacillin tazobactam (*n* = 30). In

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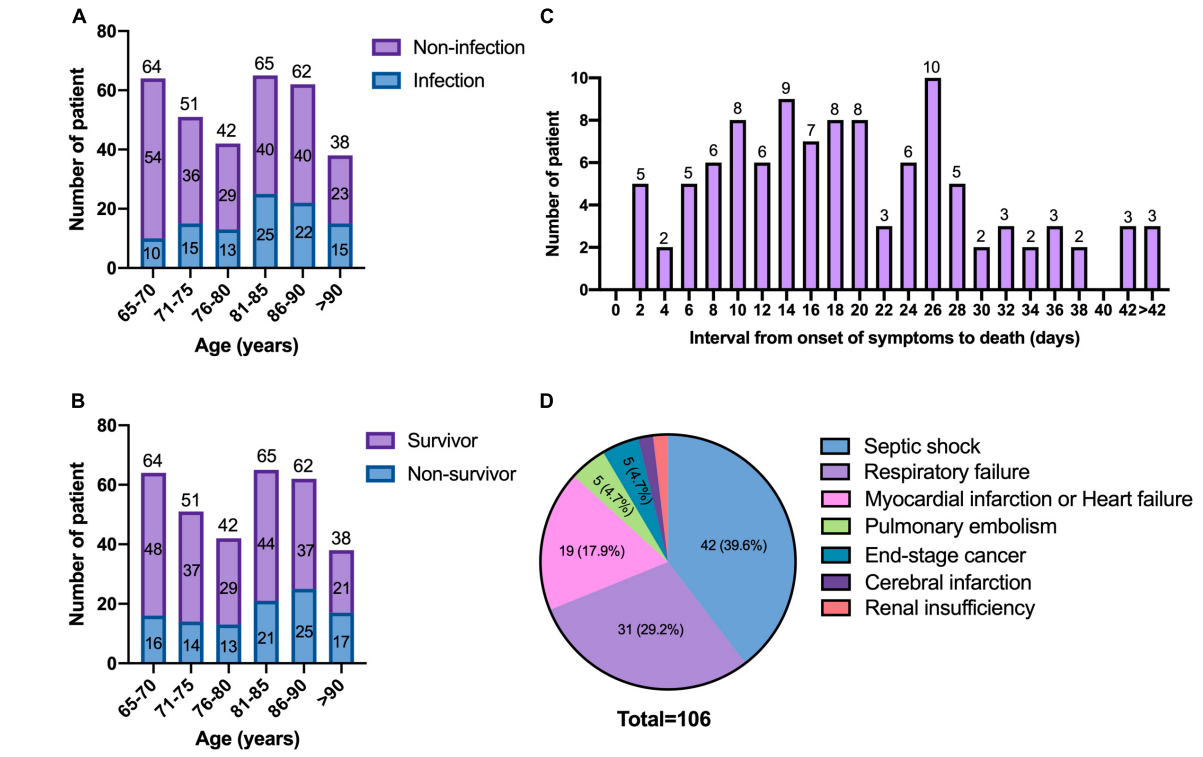

FIGURE 1
Age distribution and the causes of mortality. **(A)** Age distribution of patients between infection and non-infection; **(B)** age distribution of patients between the survivors and non-survivors; **(C)** interval from onset of symptoms to death of patients; **(D)** causes of mortality.

TABLE 2 Independent risk factors for pulmonary infection in COVID-19 patients.

	Univariate		Multivariate	
Variables	OR (95% CI)	*p*	OR (95% CI)	*p*
ICU admission within 48 h of hospitalization	4.50 (2.620–7.729)	<0.001	2.880 (1.516–5.449)	0.001
Chronic cardiovascular diseases	1.651 (1.021–2.669)	0.040	1.111 (0.605–2.038)	0.735
Cerebrovascular diseases	1.810 (1.117–2.932)	0.015	2.031 (1.109–3.719)	0.022
Empirical use of antibiotics before the first positive culture	6.798 (2.380–19.411)	<0.001	3.626 (0.786–16.726)	0.099
Critical COVID-19	6.071 (3.627–10.162)	<0.001	4.160 (2.245–7.707)	<0.001
PCT > 0.5 ng/mL	3.156 (1.872–5.318)	<0.001	1.989 (1.014–3.902)	0.046
Lymphopenia*	1.863 (1.116–3.109)	0.017	0.966 (0.510–1.832)	0.916
Hyper C-reactive protein	0.994 (0.991–0.998)	<0.001	1.000 (0.995–1.005)	0.973

*Refers to a lymphocyte count <0.8 × 10^9 per.

particular, 124 patients received empirical therapy with more than one antibiotic.

A total of 183 pathogens were isolated from microbiological tests in the 100 patients. The common pathogens for community acquired co-infection were Influenza A virus (50.0%, 4/8), the vast majority were the H3N2 subtype. The common pathogens for hospital acquired co-infection were *A. baumannii* (31.3%, 5/16), *K. pneumoniae* (25.0%, 4/16), and *P. aeruginosa* (12.5%, 2/16) (Figure 2A). The common pathogens for secondary infection were *A. baumannii* (21.4%, 34/159), *K. pneumoniae* (13.8%, 22/159), *Candida albicans* (9.4%, 15/159), and *P. aeruginosa* (7.5%, 12/159) (Figure 2B). There was a high incidence of CR-GNB infection. A total of 84.6% of *A. baumannii*, 50.0% of *K. pneumoniae*, and 35.7% of *P. aeruginosa* were resistant to carbapenems (Figures 2C–E). Particularly, 21.4% of *K. pneumoniae* were extended-spectrum β-Lactamase producers.

3.3. The clinical characteristics between the survivors and non-survivors

There were 106 (32.9%) patients who died in the hospital and 216 (67.1%) patients who discharged.

The in-hospital mortality among patients who acquired with secondary pulmonary infection and co-infection was 57.0% (57/100). The overall in-hospital mortality of ICU patients was

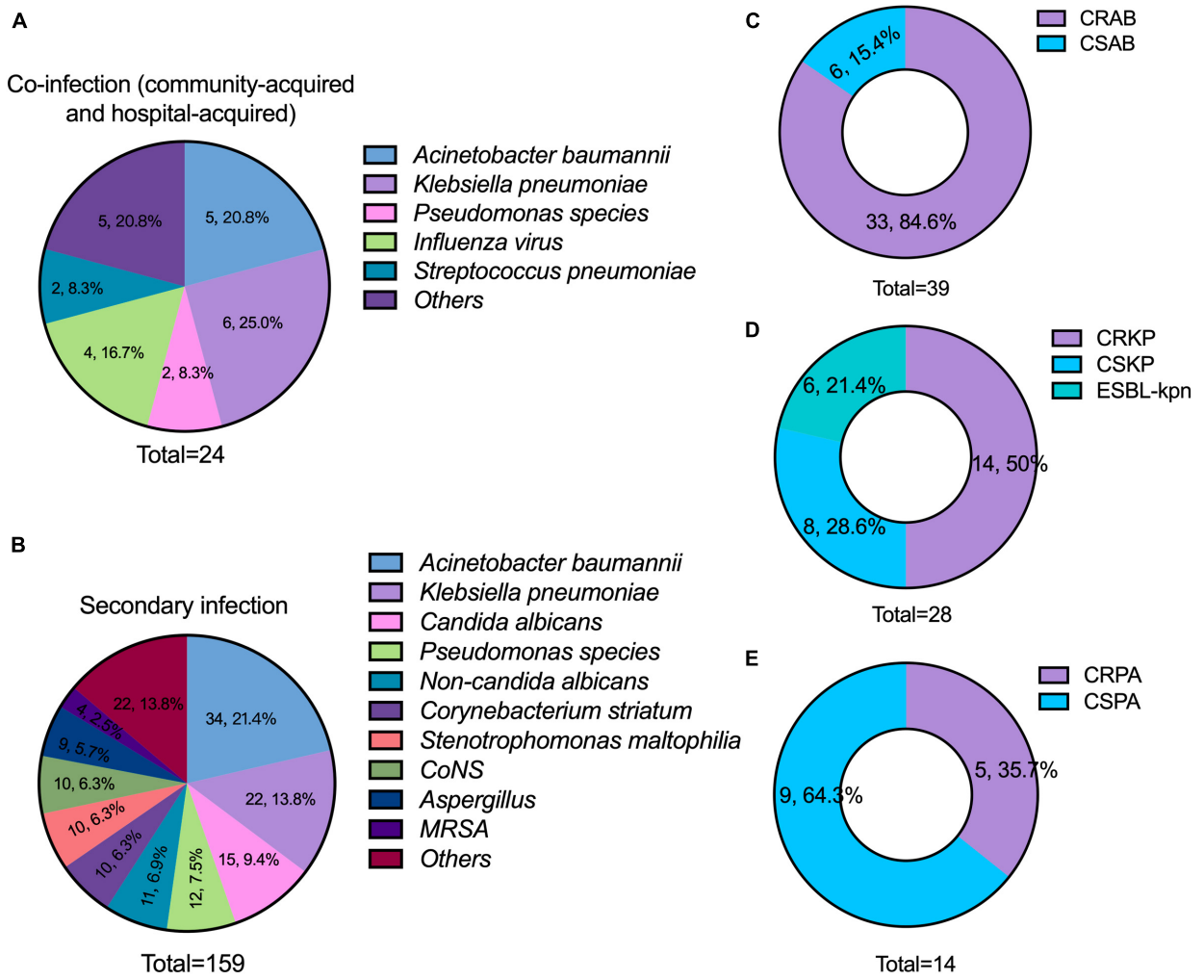

FIGURE 2
Distribution of pathogens and the results of antimicrobial susceptibility testing. **(A)** Major pathogens of co-infection (including community-acquired and hospital-acquired); **(B)** major pathogens of secondary infection; **(C–E)** incidence of carbapenem-resistant *A. baumannii* (CRAB), carbapenem-resistant *K. pneumoniae* (CRKP), and carbapenem-resistant *P. aeruginosa* (CRPA). CONS, coagulase-negative *Staphylococcus*; MRSA, methicillin-resistant *Staphylococcus aureus*; CSAB, carbapenem-susceptible A. *baumannii*; CSKP, carbapenem-susceptible *K. pneumoniae*; CSPA, carbapenem-susceptible *P. aeruginosa*.

48.1% (37/77), and the in-hospital mortality of ICU patients acquired with secondary pulmonary infection and co-infection was 61.4% (27/44).

The distribution of age between the survivors and non-survivors is shown in Figure 1B. The age increased in parallel with death rates ($p = 0.008$). There was no significant difference in the frequency of underlying diseases between the two groups (Table 3). Compared with the survivors, non-survivors had a higher proportion of pulmonary infection (53.8 vs. 19.9%, $p < 0.001$), and more patients received empirical antibiotic therapy (95.3 vs. 77.8%, $p < 0.001$).

Abnormal laboratory parameters were commonly seen in the non-survivors. A total of 88.7% of patients classified as critical COVID-19 were died. Three patients were treated with extracorporeal membrane oxygenation (ECMO), and none survived. Death was associated with patients who received IL-6 or JAK inhibitors, systematic corticosteroids and intravenous immunoglobulin. The patients admitted to the ICU (23.1 vs. 44.3%, $p < 0.001$) during hospitalization, and suffered from septic shock (1.4 vs. 49.1%, $p < 0.001$) had a high mortality.

3.4. The causes of death

The survival time of the non-survivors was analyzed. The distribution of survival time from the symptoms onset to death showed two peaks, with the first one at approximately 14 days, and the second one at approximately 26 days (Figure 1C). The causes of death were analyzed (Figure 1D), the most common cause of death was septic shock, followed by respiratory failure. The risk factors for significantly higher 30-day mortality ($p \leq 0.001$) were summarized in Figures 3A–D, patients who were infected with CR-GNB and other bacteria, classified as critical COVID-19, received empirical antibiotic treatment and admitted to ICU during hospitalization had a high 30-day mortality.

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TABLE 3 Baseline characteristics, complications and prognosis of survivors and non-survivors.

Variables	Total (*n* = 322)	Non-survivors (*n* = 106)	Survivors (*n* = 216)	*p*
Gender, male, *n* (%)	211 (65.5%)	73 (68.9%)	138 (63.9%)	0.377
Age, years, median (IQR)	81.0 (72.0–87.0)	83.5 (75.0–89.0)	79.0 (71.0–86.0)	0.008
ICU admission within 48 h of hospitalization, n (%)	77 (23.9%)	37 (34.9%)	40 (18.5%)	0.001
Underlying diseases, n (%)				
Hypertension	216 (67.1%)	69 (65.1%)	147 (68.1%)	0.595
Diabetes mellitus	119 (37.0%)	41 (38.7%)	78 (36.1%)	0.654
Chronic cardiovascular diseases	169 (52.5%)	62 (58.5%)	107 (49.5%)	0.131
Chronic respiratory diseases	124 (38.5%)	39 (36.8%)	85 (39.4%)	0.657
Chronic liver diseases	26 (8.1%)	12 (11.3%)	14 (6.5%)	0.134
Chronic kidney diseases	79 (24.5%)	27 (25.5%)	52 (24.1%)	0.784
Cerebrovascular diseases	120 (37.3%)	46 (43.4%)	74 (34.3%)	0.111
Peripheral vascular diseases	82 (25.5%)	28 (26.4%)	54 (25.0%)	0.784
Hemopathy	20 (6.2%)	8 (7.5%)	12 (5.6%)	0.487
Connective tissue diseases	13 (4.0%)	3 (2.8%)	10 (4.6%)	0.639
Peptic ulcer	26 (8.1%)	11 (10.4%)	15 (6.9%)	0.288
Hemiplegia	9 (2.8%)	5 (4.7%)	4 (1.9%)	0.269
Immunocompromised	43 (13.4%)	16 (15.1%)	27 (12.5%)	0.520
Malignancy	87 (27.0%)	29 (27.4%)	58 (26.9%)	0.923
Charlson comorbidity index ≥ 3	191 (59.3%)	69 (65.1%)	122 (56.5%)	0.139
Exposure history, n (%)				
Smoking	97 (30.1%)	33 (31.1%)	64 (29.6%)	0.782
Drinking	49 (15.2%)	12 (11.3%)	37 (17.1%)	0.173
Vaccination	32 (9.9%)	7 (6.6%)	25 (11.6%)	0.135
Previous glucocorticoid use	24 (7.5%)	7 (6.6%)	17 (7.9%)	0.684
Previous invasive procedure within 3 months before hospital admission	52 (16.1%)	17 (16.0%)	35 (16.2%)	0.970
Empirical use of antibiotics before the first positive culture	269 (83.5%)	101 (95.3%)	168 (77.8%)	<0.001
Clinical symptoms, n (%)				
Fever	261 (81.1%)	90 (84.9%)	171 (79.2%)	0.217
Sore throat	48 (14.9%)	15 (14.2%)	33 (15.3%)	0.790
Cough	234 (72.7%)	78 (73.6%)	156 (72.2%)	0.797
Sputum	219 (68.0%)	77 (72.6%)	142 (65.7%)	0.212
Fatigue	93 (28.9%)	29 (27.4%)	64 (29.6%)	0.673
Myalgias	33 (10.2%)	12 (11.3%)	21 (9.7%)	0.657
Diarrhea	37 (11.5%)	9 (8.5%)	28 (13.0%)	0.237
Thoracalgia	11 (3.4%)	4 (3.8%)	7 (3.2%)	1.000
Respiratory failure	77 (23.9%)	51 (48.1%)	26 (12.0%)	<0.001
Laboratory findings, median (IQR)				
Write blood count × 10^9 per L	6.7 (4.7–9.8)	8.0 (5.2–11.9)	6.2 (4.4–8.9)	0.001
Neutrophil × 10^9 per L	5.2 (3.3–8.5)	6.7 (4.5–10.3)	4.6 (2.8–7.4)	<0.001
Lymphocyte × 10^9 per L	0.6 (0.4–0.9)	0.4 (0.3–0.7)	0.7 (0.5–1.0)	<0.001
Plate count × 10^9 per L	155.0 (111.0–214.0)	134.0 (96.5–193.3)	160.0 (122.0–227.0)	<0.001
Neutrophilic granulocyte percentage	81.0 (69.6–88.7)	88.3 (81.4–93.1)	77.9 (65.8–84.6)	<0.001

(Continued)

TABLE 3 (Continued)

Variables	Total (*n* = 322)	Non-survivors (*n* = 106)	Survivors (*n* = 216)	*p*
lymphocyte percentage	10.2 (5.1–17.3)	5.6 (2.8–11.5)	12.1 (7.0–18.7)	<0.001
Prothrombin time, s	11.9 (11.3–13.0)	12.7 (11.7–14.8)	11.7 (11.2–12.7)	<0.001
Activated partial thromboplastin time, s	31.1 (28.3–34.4)	32.2 (28.1–37.9)	30.9 (28.3–33.3)	0.050
D-dimer, mg/L	0.6 (0.3–1.2)	1.0 (0.4–3.6)	0.5 (0.3–0.9)	<0.001
High-sensitivity cardiac troponin I, ng/L	20.4 (9.6–59.4)	58.4 (21.2–335.8)	13.7 (7.0–29.7)	<0.001
Creatine kinase-MB, mg/mL	1.9 (1.1–3.8)	3.1 (1.6–8.6)	1.6 (0.9–2.5)	<0.001
Creatinine, μmol/L,	91.3 (72.0–158.6)	113.9 (77.0–191.1)	87.6 (66.5–133.8)	<0.001
Lactic dehydrogenase, U/L	271.0 (204.5–384.5)	372.0 (280.5–552.0)	236.5 (187.3–308.8)	< 0.001
Serum ferritin, ng/mL	617.1 (261.6–1053.8)	1048.2 (420.9–1566.0)	583.9 (174.4–770.5)	0.008
IL-6, pg/mL,	49.7 (19.6–124.7)	89.1 (38.7–212.3)	38.5 (14.6–81.7)	<0.001
PCT, ng/mL	0.2 (0.1–0.8)	0.6 (0.2–3.1)	0.1 (0.1–0.4)	<0.001
Hyper C-reactive protein, mg/dL	65.9 (24.5–113.1)	92.5 (55.5–139.8)	46.3 (18.0–98.7)	< 0.001
Infection				
Co-infection or secondary infection, n (%)	100 (31.1%)	57 (53.8%)	43 (19.9%)	<0.001
Critical COVID-19, *n* (%)	117 (36.3%)	94 (88.7%)	23 (10.6%)	<0.001
Anti-COVID-19 treatment, *n* (%)	224 (69.6%)	72 (67.9%)	152 (70.4%)	0.654
Paxlovid	132 (41.0%)	52 (49.1%)	80 (37.0%)	0.039
Azvudine	131 (40.7%)	37 (34.9%)	94 (43.5%)	0.139
Immunomodulatory treatment, n (%)				
IL-6 or JAK inhibitors	38 (11.8%)	26 (24.5%)	12 (5.6%)	<0.001
Immunoglobulin therapy	33 (10.2%)	23 (21.7%)	10 (4.6%)	<0.001
Glucocorticoid treatment	150 (46.6%)	63 (59.4%)	87 (40.3%)	0.001
Time from onset of symptom to start of glucocorticoid treatment, days, median (IQR)	8.0 (5.0–12.0)	8.0 (5.0–12.0)	9.0 (4.0–12.0)	0.861
Glucocorticoid treatment duration, median, (IQR)	6.0 (3.0–10.0)	5.0 (3.0–9.0)	7.0 (5.0–11.0)	0.013
Anticoagulant therapy	167 (51.9%)	65 (61.3%)	102 (47.2%)	0.017
ECMO, *n* (%)	3 (0.9%)	3 (2.8%)	0	0.035
CRRT, *n* (%)	16 (5.0%)	12 (11.3%)	4 (1.9%)	0.001
ICU admission in hospitalization, *n* (%)	97 (30.1%)	47 (44.3%)	50 (23.1%)	<0.001
Length of ICU stay, days, median (IQR)	9.5 (6.0–17.3)	11.0 (6.0–18.0)	9.0 (6.0–15.0)	0.654
Treated with invasive mechanical ventilation	57 (17.7%)	50 (47.2%)	7 (3.2%)	<0.001
Duration of mechanism ventilation, median (IQR)	122.0 (40.0–331.0)	119.0 (41.0–286.8)	130.0 (28.0–569.0)	0.422
Septic shock, *n* (%)	55 (17.1%)	52 (49.1%)	3 (1.4%)	<0.001
Length of hospital stay from exposure to SARS-COV-2, days, median (IQR)	13.0 (7.0–21.0)	10.0 (5.0–17.0)	14.0 (8.0–23.0)	0.001
Length of hospital stay, median (IQR)	16.0 (8.0–29.0)	14.0 (6.0–28.0)	18.0 (10.0–29.8)	0.012

IQR, range interquartile; IL-6, interleukin-6; PCT, procalcitonin; JAK, Janus kinase; ECMO, extracorporeal membrane oxygenation; CRRT, continuous renal replacement therapy.

4. Discussion

Our study confirmed that in a significant proportion of cases, secondary bacterial, fungal or viral pulmonary infection and co-infection could complicate the hospital course in elderly COVID-19 patients. In this study, we found the incidence of co-infection was 7.5%, and the incidence of secondary infection was 27.3%, which was slightly higher than previously published results (Lansbury et al., 2020; Ripa et al., 2021). The incidence of secondary pulmonary infection and co-infection among patients admitted to ICU was 50.6 and 13.0%, respectively, which was higher than previous study [early infection (the same definition as co-infection in this article) was 8.7%, late infection (the same definition as secondary infection in this article) was 41.1%] reported in ICU

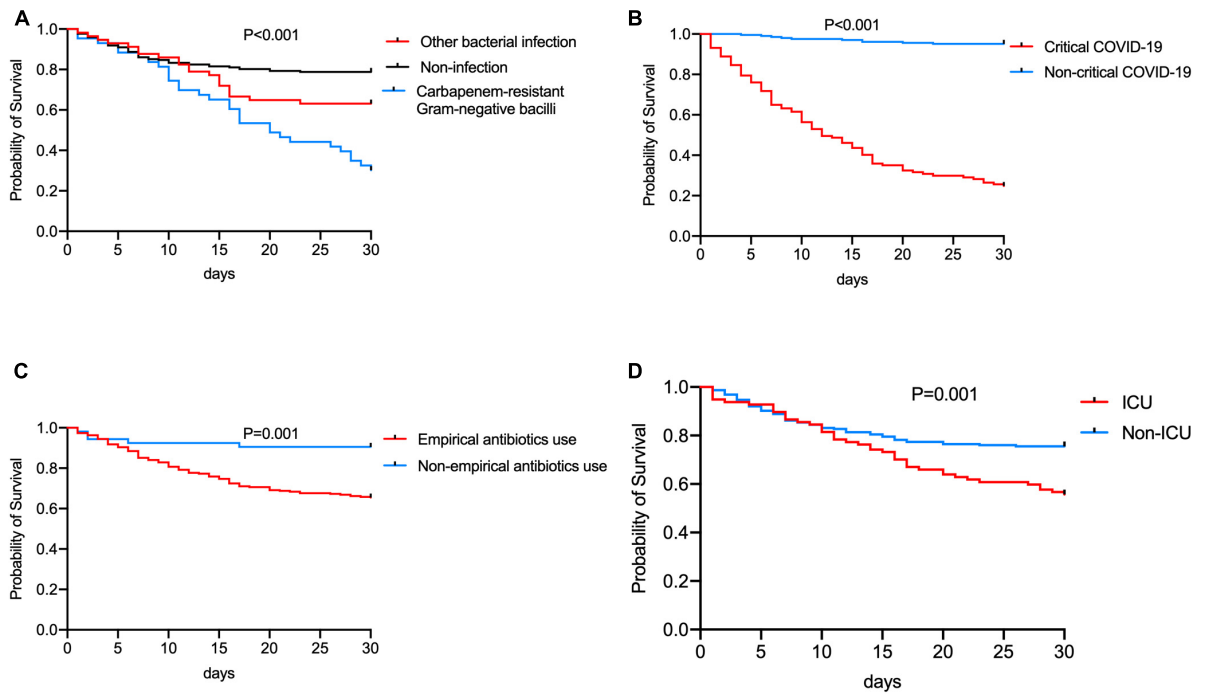

FIGURE 3
Comparation of the 30-day Kaplan-Meier survival curve between patients **(A)** infected by Carbapenem-resistant Gram-negative bacilli, other bacteria and non-infection, **(B)** with/without critical COVID-19, **(C)** with/without receiving empirical antibiotic treatment, and **(D)** with/without admitting to ICU in hospitalization.

patients (Pandey et al., 2022). The possible reason was that we focused on older people. Another study reported that 72.7% of the COVID-19 ICU patients had secondary infection (Akrami et al., 2023), which was higher than reported in this study. The possible reason might be due to widespread usage of mechanical ventilator that expose patients to infection.

Previous studies have identified critical COVID-19, oxygen saturation ≤94%, ferritin levels <338 ng/mL, PCT > 0.2 ng/mL, early need for ICU admission, respiratory failure, and severe lymphopenia as independent risk factors for secondary pulmonary infection and co-infection in COVID-19 patients (Feng et al., 2020; Ripa et al., 2021; Moreno-García et al., 2022). Our study showed some similar results. We identified predictors, including ICU admission within 48 h of hospitalization, cerebrovascular diseases, critical COVID-19, and PCT > 0.5 ng/mL. The proportion of critical cases in elderly patients with COVID-19 was high, most patients needed to be transferred to ICU for treatment. The high rate of invasive mechanical ventilation and central catheter placement in ICU may increase secondary bacterial, fungal or viral pulmonary infection and co-infection, resulting in high mortality. Previous study showed immune responses, such as lymphocyte count, T cells, were substantially decreased in COVID-19 patients, which made them at a high risk of infection (Lin et al., 2020). In the case of COVID-19, early detection of patients who are at high-risk of secondary bacterial or fungal pulmonary infection and co-infection can help guide management decisions and effectively combat this deadly respiratory pandemic.

Inflammatory biomarkers may help in early diagnose of secondary bacterial or fungal pulmonary infection and co-infection in COVID-19 patients (Wang L. et al., 2020). Despite the overlap of viral and bacterial symptoms, some typical biomarkers that indicate bacterial infection can still be found, including decreased lymphocyte count, elevated white blood cell count, neutrophil count, IL-6, PCT, and hyper C-reactive protein. Elevated PCT is often used as a biomarker for bacterial infections, within the normal range in viral infections (Schuetz et al., 2011). In this study, we found PCT > 0.5 ng/mL could predict secondary bacterial pulmonary infection and co-infection. This was consistent with previous result (Malik et al., 2021). Lymphocytes play a key role in the adaptive immunity against viral infection (Wang F. et al., 2020). COVID-19 patients often developed lymphocytopenia, the immunodeficient state that made patients more vulnerable to be infected with other respiratory pathogens. In this study, we found patients with secondary pulmonary infection and co-infection had lower lymphocyte counts than patients without infection. Several studies also showed that one of the typical characteristics of COVID-19 patients was lymphocytopenia, which was significantly associated with poor prognosis (Huang and Pranata, 2020; Yang et al., 2020).

Previous study identified the common bacteria in co-infection were *M. pneumonia*, *P. aeruginosa*, and *H. influenzae* (Lansbury et al., 2020), it was different from this study, the most common pathogen of community acquired co-infection was Influenza A virus. The high incidence of Influenza A virus may be a dual seasonal pattern in China, with the prevalence in northern China following a winter pattern (Shu et al., 2010). The common pathogens of hospital acquired co-infection and secondary infection were *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*, which were consistent with common pathogens frequently identified in hospital acquired pneumonia (Jones, 2010).

The results of antimicrobial susceptibility testing showed that most of *A. baumannii* and *K. pneumoniae* were resistant to carbapenems, the patients infected with CR-GNB had a considerably high mortality. Previous study showed in a COVID-19 respiratory sub-intensive care unit, carbapenem-resistant *A. baumannii* (CRAB) infections occurred in almost half of patients and were associated with high mortality (Iacovelli et al., 2023). Thus, these findings suggested that infection control measures should be enhanced to prevent the transmission of CR-GNB in healthcare settings among older patients. Firstly, routine infection control measures including environmental cleaning, staff education, hand hygiene, microbiological capacity need to be strengthened; Secondly, identification of "at-risk" carriers of CR-GNB should be performed on admission, pre-emptively isolated in a single room; Thirdly, active screening for CR-GNB by obtaining swabs from rectal or perirectal areas, and any other site that is either actively infected or colonized. If the screening test result is positive for CR-GNB, patient isolation and contact precautions are continued. Finally, antimicrobial stewardship should be implemented. Antibiotic usage is a primary driver of antibiotic resistance. Previous study showed that antibiotic exposure (cephalosporins, fluoroquinolones, and carbapenems) was a risk factor for CR-GNB infection (Çölkesen et al., 2023).

In our study, the majority of patients (83.5%) received empirical antibiotic therapy before the first positive culture result, which was consistent with a previous study (60.0–100%) (Chong et al., 2021). The reason for the overuse of antibiotics was the difficulty in early identification of bacterial or fungal co-infections. Moreover, the possibility of secondary bacterial or fungal pulmonary infection should be considered. In this study, the main empirical antibiotic prescription was carbapenems, followed by cefoperazone/sulbactam, cephalosporin, moxifloxacin, and piperacillin tazobactam. In particular, 124 patients received empirical therapy with more than one antibiotic. However, overuse of antibiotics can lead to emergence of MDR pathogens. Implementing strategies, such as empirical therapy should take into account local epidemiological data and/or individual risk factors, in-time de-escalation of antibiotic therapy according to culture data, using high-dose, susceptible antibiotics, are essential in appropriate antibiotic usage. A variety of studies have demonstrated that optimizing antimicrobial use could improve patient safety, minimize antimicrobial resistance, reduce the side effects of antibiotics, decrease the usage of unnecessary antibiotics, shorten hospital stay, and cut down economic cost (Owens, 2009; Campion and Scully, 2018; Majumder et al., 2020). The COVID-19 guidelines recommended that antibiotics should be considered for patients who may only have bacterial infection, comorbidities, or at high risk of complications from untreated bacterial infection (Poston et al., 2020).

In the meta-analysis of clinical trials of patients hospitalized for COVID-19, administration of IL-6 antagonists, compared with placebo or usual care, was associated with lower 28-day all-cause mortality (Shankar-Hari et al., 2021). In this study, a small number of patients were treated with IL-6 antagonists, and we did not find that the use of IL-6 antagonists could significantly improve patients' survival. There was inconsistency between different studies. More patients received IL-6 antagonist treatment in this study acquired bacterial or fungal pulmonary infection, the infected patients had high mortality. In addition, the association of IL-6 antagonists with lower 28-day all-cause mortality was significant among patients who did not require invasive mechanical ventilation at randomization (Shankar-Hari et al., 2021). However, most patients received invasive mechanical ventilation treatment in this study. The patients received systematic corticosteroids and intravenous immunoglobulin had a high mortality. There were some possible reasons. Firstly, more severe patients were treated with these treatments; Secondly, patients received systematic corticosteroids and intravenous immunoglobulin may predispose to secondary bacterial or fungal pulmonary infection and co-infection. Emerging evidence has unveiled infection as one of the mortal causes of post-SARS-CoV-2 infection (Patton et al., 2023). Thirdly, these treatments may be associated with declined immune responses. Owing to relatively small sample size and retrospective nature of this study, there may also be a selection bias when identifying factors that influence the clinical outcomes. A larger cohort study of patients with COVID-19 pneumonia are needed to further explore.

In this study, we did not find that treatment with paxlovid could improve patients' survival. Previous study reported that paxlovid was highly effective in reducing the risk of severe COVID-19 or mortality in the era of Omicron and in real world setting (Najjar-Debbiny et al., 2023). The reasons for the inconsistent results may be the majority of patients treated with paxlovid in this study were severely or critically ill, and more likely to develop secondary or co-occurring bacterial, fungal, and viral infections. Considering older patients have more basic diseases, and there is a special situation of multi-drug sharing, paxlovid has a high potential to cause clinically important drug–drug interactions with other concurrent medications. In many settings, paxlovid will be primarily prescribed by practitioners who may lack in-depth knowledge of managing these complex drug–drug interactions associated with paxlovid, leading to unintended denial of therapy or the occurrence of serious adverse events. However, these results should be interpreted with caution owing to potential bias and residual confounding in this observational study. Double-blinded randomized clinical trials should be conducted to validate these results.

In this study, the overall in-hospital mortality of COVID-19 patients was 32.9%, and the in-hospital mortality of patients who acquired secondary pulmonary infection or co-infection was 57.0%. The mortality was higher than previous study (Zhou et al., 2020), the possible reason was that our study only focused on elderly patients. Additionally, we found that patients with bacterial, fungal or viral pulmonary infections had increased rates of ICU admission and prolonged hospital stay. The overall in-hospital mortality of ICU patients was 48.1%, and the in-hospital mortality of ICU patients acquired with secondary pulmonary infection and co-infection was 61.4%. Previous study showed the mortality among adults with COVID-19 admitted to the ICU who acquired secondary infection was 83% (Pourajam et al., 2022). The mortality was higher than in this study. The possible reason was that more ICU patients were infected with CR-GNB in the previous study. In this study, the mortality of patients infected by CR-GNB was 76.9%. The result was much closer. However, this was unadjusted to baseline patient characteristics and cannot be completely attributed to bacterial, fungal or viral pulmonary infections.

There are some limitations in this study. First, this is a single-center retrospective study, the results of our analysis are limited to short-term follow-up, and the long-term effects of infection on

these patients are unknown. Second, not all patients with suspected infection had ordered microbiological testing due to unprecedented circumstances and immense pressure on hospital systems during the COVID-19 pandemics. However, the results of this study are still representative and reflect the real-world situation of elderly COVID-19 patients in China during the pandemics.

5. Conclusion

We described the incidence and predictors of secondary bacterial, fungal or viral pulmonary infection and co-infection in elderly COVID-19 patients during the Omicron BA.7 and BA.5.2 variant pandemics in a Chinese tertiary general hospital, showing a high burden of infection caused by CR-GNB. The inflammatory biomarker PCT > 0.5 ng/mL played an important role in the early prediction of secondary bacterial pulmonary infection and co-infection in COVID-19 patients, this could provide a reference for the rational use of antibiotics.

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 humans were approved by the Ethics Committee of Peking University First Hospital, Beijing, China (Approval No. 2023-yan-090). The studies were conducted in accordance with the local legislation and institutional requirements. The Ethics Committee/Institutional Review Board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because Due to the retrospective observational study and the confidentiality of patient information.

Author contributions

CZ: Formal analysis, Data curation, Writing – original draft. YJ: Writing – review and editing, Data curation, Writing – review and editing. HL: Conceptualization, Writing – review and editing. XL: Conceptualization, Funding acquisition, Writing – review and editing. LH: Data curation, Funding acquisition, Writing – review and editing.

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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

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Comparative analysis of human respiratory syncytial virus evolutionary patterns during the COVID-19 pandemic and pre-pandemic periods

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The COVID-19 pandemic has resulted in the implementation of strict mitigation measures that have impacted the transmission dynamics of human respiratory syncytial virus (HRSV). The measures also have the potential to influence the evolutionary patterns of the virus. In this study, we conducted a comprehensive analysis comparing genomic variations and evolving characteristics of its neutralizing antigens, specifically F and G proteins, before and during the COVID-19 pandemic. Our findings showed that both HRSV A and B exhibited an overall chronological evolutionary pattern. For the sequences obtained during the pandemic period (2019–2022), we observed that the HRSV A distributed in A23 genotype, but formed into three subclusters; whereas the HRSV B sequences were relatively concentrated within genotype B6. Additionally, multiple positively selected sites were detected on F and G proteins but none were located at neutralizing antigenic sites of the F protein. Notably, amino acids within antigenic site III, IV, and V of F protein remained strictly conserved, while some substitutions occurred over time on antigenic site Ø, I, II and VIII; substitution S389P on antigenic site I of HRSV B occurred during the pandemic period with nearly 50% frequency. However, further analysis revealed no substitutions have altered the structural conformations of the antigenic sites, the vial antigenicity has not been changed. We inferred that the intensive public health interventions during the COVID-19 pandemic did not affect the evolutionary mode of HRSV.

KEYWORDS

human respiratory syncytial virus, antigenic site, evolutionary pattern, tertiary structure, positive selection

Introduction

Human respiratory syncytial virus (HRSV) is a leading cause of acute lower respiratory tract infections in young children and poses a major risk to elderly individuals, imposing a substantial burden on healthcare systems worldwide (Shi et al., 2020; Li et al., 2022). Although HRSV infection can manifest as mild upper respiratory tract illness that typically resolves within 7–10 days without complications, severe lower respiratory tract infections such as bronchiolitis or pneumonia may occur. Infants and individuals with underlying medical conditions or

weakened immune systems are particularly vulnerable, which can potentially result in mortality (Falsey and Walsh, 2000; Perk and Ozdil, 2018).

HRSV belongs to the *Orthopneumovirus* genus of the *Paramyxoviridae* family, with a genome consisting of approximately 15,000 nucleotides that encode for 11 viral proteins (Collins et al., 2013). The non-structural proteins NS1 and NS2 play crucial roles in evading the host's antiviral innate system (Sedeyn et al., 2019), while the attachment glycoprotein (G), a structural protein, acts as a primary target for neutralizing antibodies and plays a critical role in facilitating initial attachment to host cells. Meanwhile, the fusion glycoprotein (F) mediates viral entry by inducing fusion between the viral envelope and cell membranes, and during this process, F undergoes a conformational change from the pre-fusion to post-fusion form (McLellan et al., 2013a,b). The G protein is highly variable and consists of two hypervariable regions flanking a mostly conserved central region. Although HRSV has only one serotype, it can be divided into two subtypes: A and B. Furthermore, based on the second hypervariable region (HVR2) of the G protein, HRSV can be further divided into 13 genotypes, while HRSV B can be classified into 37 genotypes (Muñoz-Escalante et al., 2019, 2021; Kim et al., 2023). The F protein demonstrates conservation across both HRSV subgroups A and B, with multiple major antigenic sites shared between them. Among these sites, site ∅ and V are exclusively present in the pre-fusion form, while site I is only found in the post-fusion form. However, other sites such as site II and IV exist in both forms (McLellan et al., 2013a; Mousa et al., 2017; Bin et al., 2019). Currently, the most prevalent genotypes of HRSV circulating worldwide are ON1 for HRSV A and BA9 for HRSV B (Vieira et al., 2017; Al-Sharif et al., 2020; Song et al., 2023). HRSV tends to be prevalent during winter months lasting 4 or 5 months depending upon different geographical locations, it can also remain sustained throughout the year (Janet et al., 2018). Approximately 33 million cases of acute lower respiratory infections were associated with HRSV, leading to over 100 million deaths annually worldwide among children under 5 years old (Shi et al., 2020; Li et al., 2022).

In late 2019, the COVID-19 pandemic emerged, leading to the implementation of various public health measures. These included wearing facial masks, placing restrictions on public gatherings, and closing borders. Like SARS-CoV-2, HRSV primarily spreads through direct physical contact with infected individuals or via droplets from an infected person. Consequently, the preventive measures had a significant impact on the transmission pattern of HRSV: during the early phase of the COVID-19 pandemic, there was a decline in HRSV-associated hospitalizations due to strict adherence to the preventive measures. However, after their relaxation or lifting in some regions of the world, there has been a resurgence in transmission leading to unexpected peak times for infection (Olsen et al., 2021a; Chow et al., 2023; Ludlow, 2023). For example, in the United States, after lockdown measures were eased, the number of RSV cases was unexpectedly higher during 2022–2023 (Goya et al., 2023). Similarly, in Japan, the Tokyo metropolitan area experienced its highest surge in HRSV cases in 2021 since the establishment of HRSV surveillance in 2003 (Ujiie et al., 2021).

To understand whether changes in HRSV transmission patterns have affected its evolutionary mode, we conducted a comparative evolutionary genomics analysis between pre-pandemic and during-pandemic periods for HRSV circulating globally by using public genomic data sources in this study.

Materials and methods

Sequence retrieval and selection

To gather a comprehensive dataset of HRSV sequences, we conducted a search in the NCBI GenBank Database[1] using "human respiratory syncytial virus" as keywords. Nearly complete HRSV sequences spanning its entire history from 1956 to late December 2022 were downloaded. Important genes such as F, G, and L were truncated from the raw nearly-complete sequences data. In order to ensure high accuracy of the dataset, we excluded sequences that contained low-quality regions with Ns or gaps, as well as those with insertions causing frameshift mutations. Additionally, any sequences that had been manually modified were also omitted from this study.

Sequence alignment, annotation and distance clustering

The MAFFT 7 online version[2] was utilized to align all the sequences, followed by manual adjustment of the alignment using MEGA 7 software (version 7.0.26). Additionally, each sequence was annotated with relevant information including the GenBank number, subtype, collection time, and location. Nucleotide pairwise distances between HRSV subtype A and B were calculated by employing the Kimura 2-parameter model in MEGA software. The resulting distance matrix was then subjected to a Multidimensional Scaling Analysis (MDS) algorithm from the R cmdscale package (R version 4.1.2).

Root-to-tip divergence

TempEst (version 1.5.3, formerly called Path-P-Gen) was implemented to plot the root-to-tip divergence of nucleotide sequences for the G and F genes of HRSV against sampling time, revealing their pattern of divergence and clock-likeness with phylogenetic trees (Rambaut et al., 2016). The trees were constructed by IQTREE software (version 2.1.3) under a general time-reversible (GTR) model that accounted for inter-site rate variation with a discrete gamma distribution. HRSV F and G genes were included to calibrate the molecular clock applied for the analysis. Finally, Prism software was used to process the generated data and create the final graph.

Phylogenetic analyses

The aligned complete genome sequences of HRSV A and B were separately used for phylogeny analysis. The phylogenetic trees were performed by MEGA 7.0.26 software using maximum likelihood method with Kimura 2-parameter substitution model. Different circulating time and sub-clusters of sequences collected during the

1 http://www.ncbi.nlm.nih.gov/genbank

2 https://mafft.cbrc.jp/alignment/software/

COVID-19 period were marked, and proportions of each continent's contribution to the sub-clusters were calculated.

SNP calling and selective pressure analysis

Single nucleotide polymorphisms (SNPs) calling was developed by R codes deposited in Github as previously described (Zhang et al., 2022). Statistically supported positively selected sites were localized through Hyphy package. The mixed-effects model of evolution (MEME), a fast unbiased Bayesian approximation (FUBAR), and single-likelihood ancestor counting (SLAC) models were applied for the analysis, and the results from the three models were merged.

Prediction of tertiary structure for F proteins

Amino acid substitutions on the key antigenic sites of the F proteins were analyzed. Furthermore, the tertiary structures of the proteins with modified amino acids were predicted and aligned to the ones of the prototypes. The online SWISS-MODEL service platform[3] was utilized to construct the models of the F proteins based on template models derived from RSV F. PYMOL 2.5.1 was used for visualizing and labeling the models. The key antigenic sites of the F protein were plotted on the models.

Results

Sequence information

A total of 4,646 nearly complete genome sequences were initially downloaded. After filtering out low-quality and manually modified sequences, there remained 3,886 nearly full-length sequences. Out of these, HRSV A accounted for 2,167 sequences while HRSV B had 1,719. In addition to the full-length sequences, truncation from the raw sequence data resulted in high-quality F and G gene datasets comprising of 4,382 and 4,520 sequences, respectively. Specifically, there were 2,531 and 2,637 high-quality F and G gene sequences, respectively, identified as belonging to HRSV A, and there were a total of 1,851 and 1,883 truncated high-quality F and G genes, respectively, for HRSV B.

To understand the evolutionary characteristics of the virus during the COVID-19 pandemic (2020–2022), we divided sequences dating back to 2008 into 3-year stages. As there were limited sequences available before 2008, we separated them into two periods: from 2000 to 2007 and before 2000. The results revealed that HRSV B had a greater abundance of sequences during the stages of 2014–2016 and 2017–2019, whereas HRSV A dominated in all other time periods with noticeably higher numbers of sequences (Supplementary Figure S1).

In terms of geographical distribution, South America, Oceania, Europe and Africa had approximately equal sequence counts except for Asia with relatively few sequences and North America with large numbers. Specifically, in Africa, the sequence number of HRSV B was slightly higher than that of HRSV A; in Europe, there were roughly equal numbers of HRSV A and B sequences. On all other continents except for North America, where there were over twice as many HRSV A sequences compared to HRSV B (918 versus 435) (Supplementary Figure S1).

HRSV A and B overall showed chronological specificities, but some sequences in HRSV A were not

To investigate the genetic characteristics of HRSV A and B, we analyzed their nucleotide distance matrix of the G genes and generated multidimensional scaling (MDS) plots. The results showed that over time, HRSV A underwent evolutionary changes forming a large cluster on the top and two small clusters at the bottom (Figure 1A). The sequences in these two small clusters were primarily associated with North American sequences prior to 2007 (Figure 1B). The big cluster, centered around early pre-2000 sequences, further evolved into two sub-clusters in opposite directions. These sub-clusters did not show any specific regional patterns, although the left sub-cluster demonstrated a chronological evolution. Notably, all HRSV A sequences from the three-year COVID-19 epidemic (2020–2022) were found within the left sub-cluster (Figure 1A). Similarly, HRSV B presented a chronological evolutionary trend where genetic distances between different time periods formed separate clusters (Figure 1C). Sequences before 2000 had more dispersed genetic distances due to prolonged duration and greater diversity during the period. Spatially, these dispersed sequences originated mainly from North America. Furthermore, genetic distances among HRSV B sequences from other continents were closely clustered without clear regional specificity, particularly for those from Africa which had a substantial number of sequences but remained tightly clustered together (Figure 1D).

G genes had higher evolutionary rates than F, and HRSV A evolving slightly faster than HRSV B

Root-to-tip linear regression analyses were performed to examine the genetic divergence of G and F genes in HRSV A and B by utilizing the best-fitting root. The results showed that phylogeny for both G and F sequences of HRSV B exhibited a stronger association ($R^2 = 0.83$ and 0.85, respectively) compared to those of HRSV A ($R^2 = 0.67$ and 0.68, respectively), while the association between different subtypes of the same gene (G or F) was similar. All four datasets exhibited positive correlations, indicating their suitability for molecular clock analysis (Figures 2A–D). The evolutionary rates of G genes in both HRSV A and B subtypes were notably higher than those of F genes, with rates of 2.05×10^{-3} versus 7.76×10^{-4} and 1.81×10^{-3} versus 7.28×10^{-4} substitutions/site/year, respectively. Furthermore, it was observed that the evolutionary rates of G and F genes was slightly higher in HRSV A than in HRSV B (Figures 2A–D). During the three-year COVID-19 epidemic period, there was an average divergence between sequences of F and G genes when compared to other sequences. Additionally, a small cluster of sequences deviated from others within the G gene

3 https://swissmodel.expasy.org/

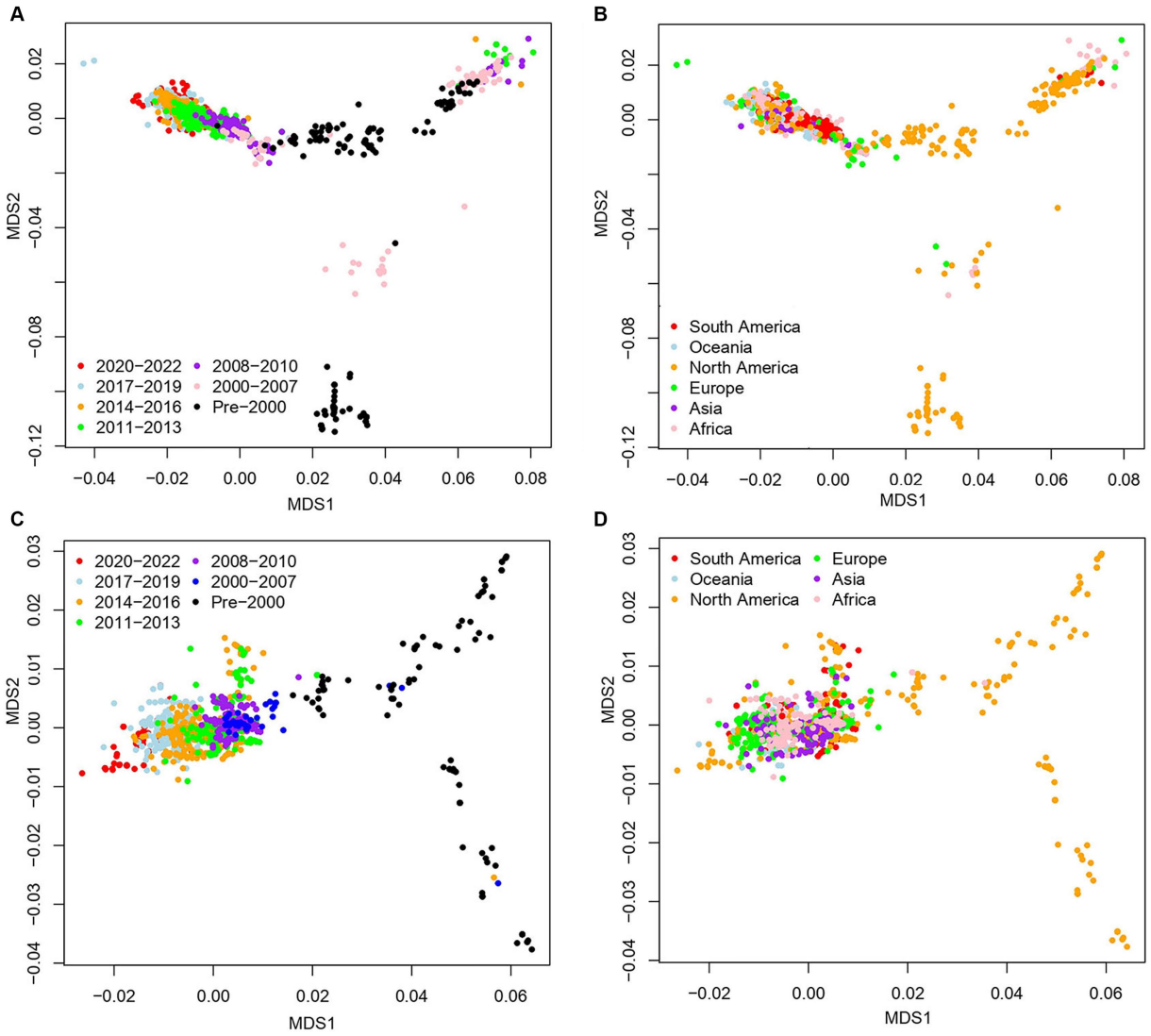

FIGURE 1
Sequence clustering of G genes for the HRSV A and B by multidimensional scaling (MDS) analysis. **(A,B)** Spatial–temporal distributions of the sequences for HRSV A; **(C,D)** spatial–temporal distributions of the sequences for HRSV B. The sequences overall exhibited time specificity but presented no spatial specificity.

sequence in HRSV A, further analysis revealed that these sequences all from the United States spanning years from 2003 to 2006.

HRSV A and B exhibited chronological evolutionary pattern and sequences during COVID-19 pandemic presented geographical specificity

The maximum likelihood trees were constructed based on the complete genomes of both HRSV serotypes A and B. The results showed a consistent chronological evolutionary trend for both serotypes, where early sequences (pre-2000) clustered at the bottom of the phylogenic trees, while later sequences grouped towards the top. However, there were instances where different subclades co-circulated within the same time period. Notably, from 2011 to 2013 onwards, A23 genotype was predominant for HRSV A, whereas from the year 2000, sequences were mostly B6 genotype for HRSV B. In terms of sequences obtained during 2020–2022, HRSV A and B exhibited a different distribution pattern: the HRSV A formed intro three relatively scattered subclusters; whereas the HRSV B sequences were concentrated (Figures 3A,B). Furthermore, we found that HRSV A sequences during the pandemic period presented a geographical specificity: Oceania-associated sequences distributed in A-I; Asian- and North American-related sequences were found both in A-II and A-III; European sequences distributed predominantly in A-II (Figure 3C).

The G gene had more SNPs and the G protein had more positively selected sites

In order to understand the genetic variations of HRSV A and B, SNP callings were performed on their genomes, specifically focusing

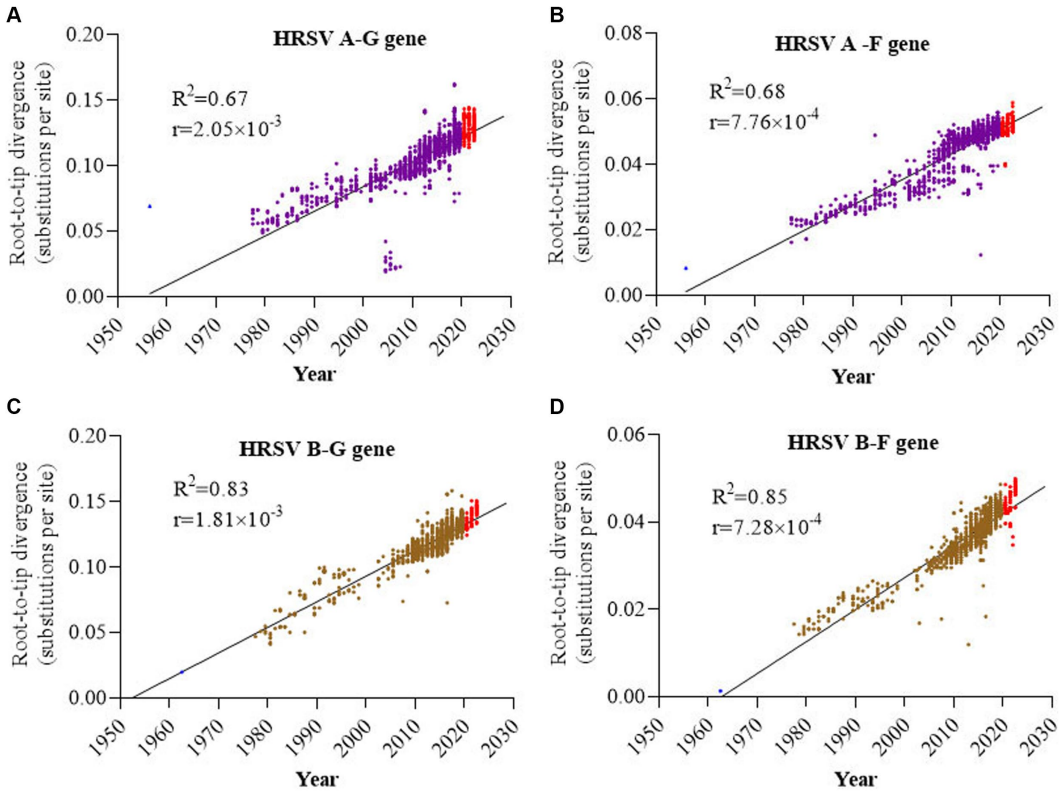

FIGURE 2
Root-to-tip divergence of the HRSV F and G genes. **(A)** G gene of HRSV A; **(B)** F gene of HRSV A; **(C)** G gene of HRSV B; **(D)** F gene of HRSV B. G genes had higher evolutionary rates than F genes, and HRSV A F and G both had higher evolutionary rates than HRSV B F and G.

on the G, F and L genes. An SNP was defined as a site with a mutation frequency greater than 1%. The analysis revealed that HRSV A had more SNPs across the entire genome and in all three genes compared to HRSV B. The majority of observed SNPs occurred at frequencies less than 10%, accounting for approximately 50% of all SNPs; relatively rare were those occurring between 10% to 50% (Figure 4A). On average, HRSV A had 17 SNPs per every 100 nucleotides in its complete genome, while HRSV B had only 12. Although the SNP count per 100 nucleotides for the F genes of both HRSV A and B was equivalent to their full-length sequences, it was lower for their L genes. Notably, the G genes had a considerably higher number of SNPs (Figure 4A), which were distributed throughout the entire sequences with high density in Mucin-like regions I and II, but low density in the central conserved region. Furthermore, an insertion consisting of either a segment of 72-nucleotide or one of 60-nucleotide was found in HRSV A and B, respectively. The occurrence of SNPs in the G gene of HRSV B was found to be less frequent than in HRSV A, particularly at the N-terminus and transmembrane region (Figures 4B,C). Moreover, we further conducted selective pressure analysis on the F and G proteins of both HRSV A and B. Positively selected sites were defined as those supported by all three methods (p-value of <0.1 in MEME and SLAC, posterior probabilities of >0.9 in FUBAR). Our results showed a higher number of positive selection sites for the F and G proteins in HRSV A compared to those observed in HRSV B. In particular, the G protein exhibited a markedly greater number of positive selection sites compared to the F protein, with these sites mainly located within the Mucin regions. Notably, none of the amino acids at major antigenic sites of the F protein underwent positive selection. Additionally, there was no simultaneous positive selection observed at the same site for both HRSV A and B regarding their respective F and G proteins (Figure 4D).

Multiple amino acid substitutions on major antigenic sites of F protein increased over time, but did not alter structural conformation

The F genes of HRSV A and B had 33 and 37 nonsynonymous mutations, respectively. Among these mutations, three (amino acid (Aa)169, 276 and 384) were situated at the major antigenic sites in HRSV A, while eight (Aa68, 172, 173, 206, 209, 276, 380 and 389) were located at the major antigenic sites in HRSV B. The amino acids on the antigenic sites III, IV and V were strictly conserved, with no changes observed in both HRSV A and B. Out of the 11 identified nonsynonymous mutation sites in this study, five displayed a relatively high frequency of occurrence: four belonged to HRSV B (Aa172, 61.6%; Aa173, 58.6%; Aa206, 23.6%; Aa209, 27.9%), while only one was found in HRSV A (Aa276, 75.7%). On the other hand, the remaining six mutations showed a low frequency of occurrence (less than 5%). Further analysis revealed that amino acid changes at the five high-frequency occurring sites mentioned above, as well as one site with low occurring frequency (i.e., Aa389), exhibited notable temporal features over time. For instance, in HRSV A, Aa276 was

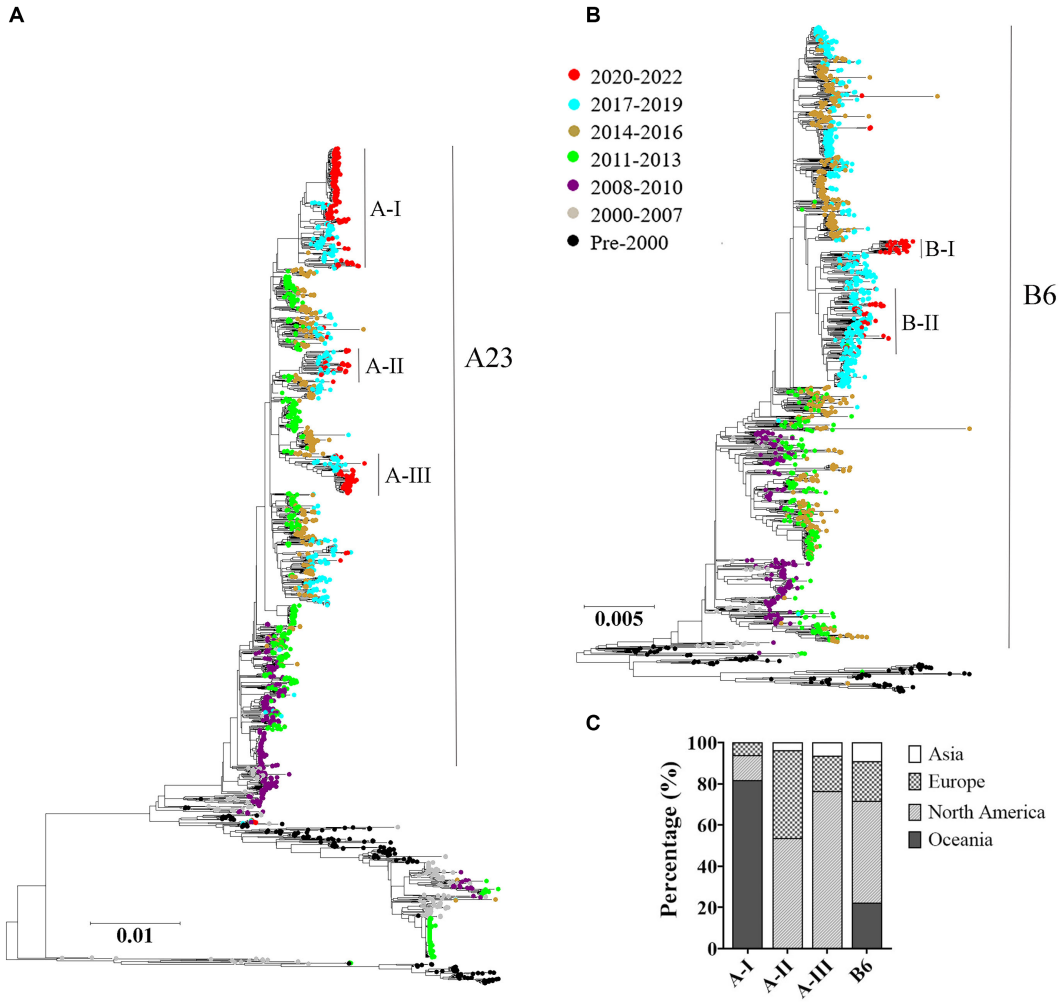

FIGURE 3
Temporal distributions of evolutionary clusters based on the HRSV complete genome using maximum likelihood method. **(A)** HRSV A phylogenetic tree; **(B)** HRSV B phylogenetic tree; **(C)** geographical distributions of the sub-clusters in COVID-19 pandemic. The genotypes of HRSV A and B overall exhibited a chronological sequential emergence. HRSV A sequences from the pandemic period displayed a geographical specificity.

exclusively composed of N before time period of 2008–2010, and starting from 2014 to 2016, it underwent a significant replacement with S almost entirely replacing N. While in HRSV B, S was predominated in this position (Aa276), with only a small percentage being N (at 1.73% occurring frequency). Additionally, Aa172 and Aa173 in HRSV A were strictly conserved as L and S, respectively. However, in HRSV B, these two sites experienced substitutions to Q and to L, respectively. By the period of 2017–2019, they had undergone complete changes. Similarly, residue I at Aa206 and residue K at Aa209 were strictly conserved in HRSV A. In contrast, they were L and Q, respectively, in HRSV B but simultaneously changed to M and R since the period of 2017–2019. Concerning the residue at position Aa389, it was P for HRSV A and S for HRSV B with both residues being well-conserved. However, during the COVID-19 epidemic period, a large proportion of sequences in HRSV B changed from S to P (Figure 5).

To investigate the potential impact of amino acid substitutions at major antigenic sites in F proteins of HRSV A and B on their spatial configuration and subsequent antigenic properties, tertiary structures of the F proteins were generated and compared. Our findings revealed that despite substitutions at Aa276 (N to S) in antigenic site II in HRSV A, as well as Aa172 (L to Q) and Aa173 (S to L) in antigenic site VIII, Aa206 (I to M) and Aa209 (Q to R) in antigenic site Ø, and Aa389 in antigenic site I in HRSV B, no changes were observed in the three-dimensional structures of the F proteins (Figure 6). Furthermore, other neutralizing antigenic sites (III, IV, and V) exhibited high conservation across both HRSV A and B, with no discernible amino acid substitutions.

Discussion

The COVID-19 outbreak has resulted in notable modifications to the incidence pattern of HRSV, impacting both viral prevalence and seasonal trends (Olsen et al., 2021b). Following the lifting of local COVID-19 restrictions, there was a notable surge in HRSV activity, particularly among young children (Falsey et al., 2022; Riepl et al., 2023). This study aimed to delineate the similarities and differences in evolutionary patterns of HRSV genomes, with a specific focus on the F and G genes during pre-pandemic and pandemic periods.

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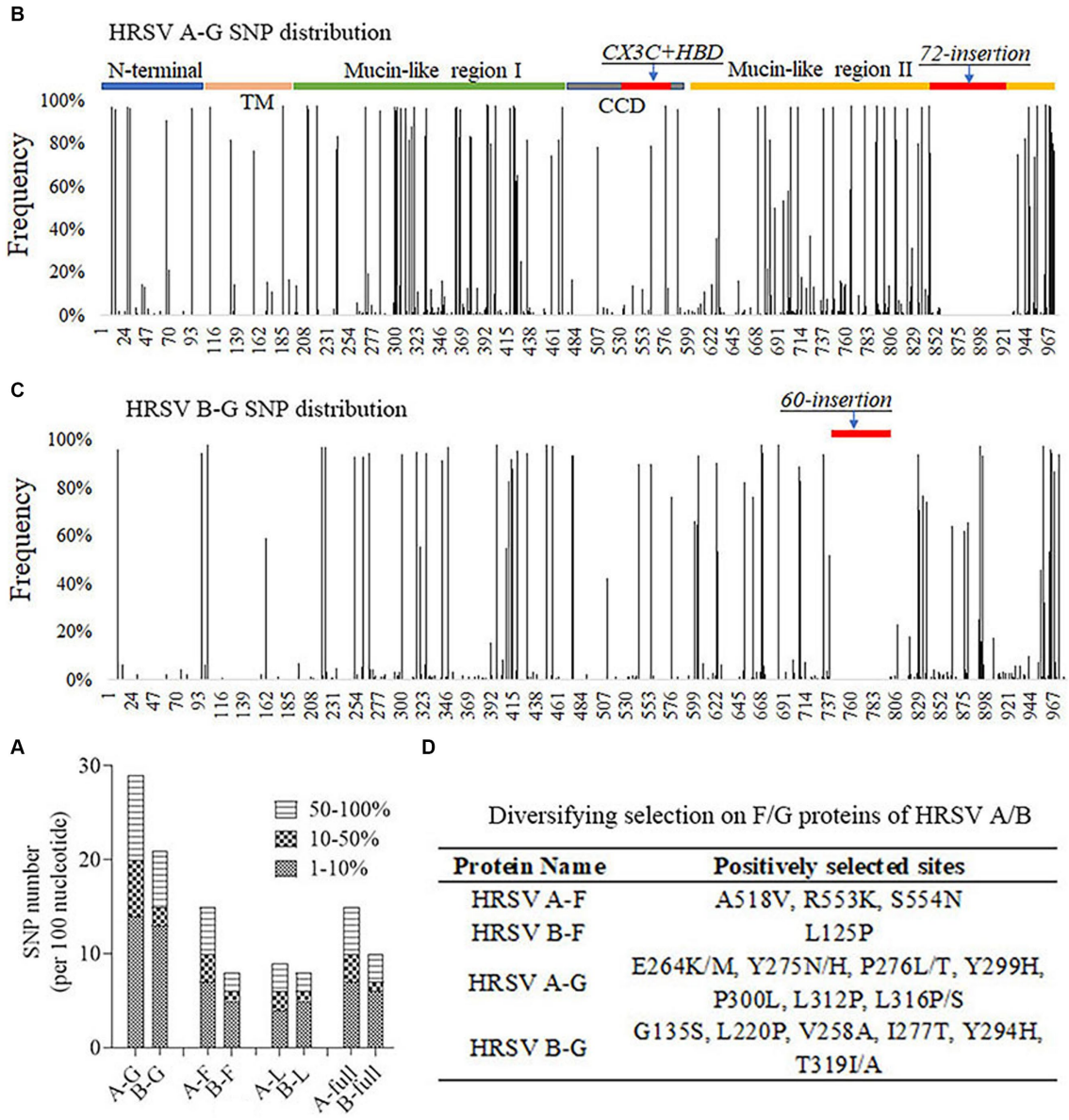

Protein Name	Positively selected sites
HRSV A-F	A518V, R553K, S554N
HRSV B-F	L125P
HRSV A-G	E264K/M, Y275N/H, P276L/T, Y299H, P300L, L312P, L316P/S
HRSV B-G	G135S, L220P, V258A, I277T, Y294H, T319I/A

FIGURE 4
Single nucleotide polymorphism (SNP) distribution and selective pressure analysis. **(A)** The number of SNPs per 100 nucleotides on the viral genomic and important genes. G gene was the most variable. **(B,C)** SNP distributions on the G gene of the HRSV A and B. Mucin-like regions had the greatest number of SNPs and CCD region was relatively conserved. **(D)** Positively selected sites on F and G proteins of HRSV A and B. None sites were located at key neutralizing antigenic sites. TM, Transmembrane region. CCD, Central conserved domain. HBD, Heparin binding domain.

It has been documented that the circulation patterns of HRSV encompass continuous seasons where HRSV A prevails, as well as alternating periods with a predominance of either HRSV A and B (Cantu-Flores et al., 2022). Furthermore, the correlations between HRSV and weather conditions have exhibited variations across different geographic locations (Haynes et al., 2013). In this study, we observed disparities in the proportions of HRSV A and B during distinct time periods. Specifically, sequences belonging to HRSV B were more prevalent than those attributed to HRSV A in the 2014–2016 and 2017–2019 periods. Conversely, in other time intervals, including the COVID-19 pandemic, there was a higher prevalence of HRSV A compared to HRSV B. However, it is important to consider possible sampling bias due to limitations in sample size and geographical coverage.

This study revealed substantial genetic divergence in the G genes of HRSV A and B, especially evident in early sequences predating 2000. Post-2000 sequences exhibited a clear chronological evolution pattern for HRSV B, while HRSV A diverged into two distinct subsets. One subset exhibited temporal order, while the other indicated the simultaneous presence of multiple subclusters. This finding was consistent with a previous study demonstrating that different lineages of ON1 genotype co-circulate without apparent temporal or geographical distribution tendencies (Song et al., 2023). Geographic analysis demonstrated that North America had the highest diversity

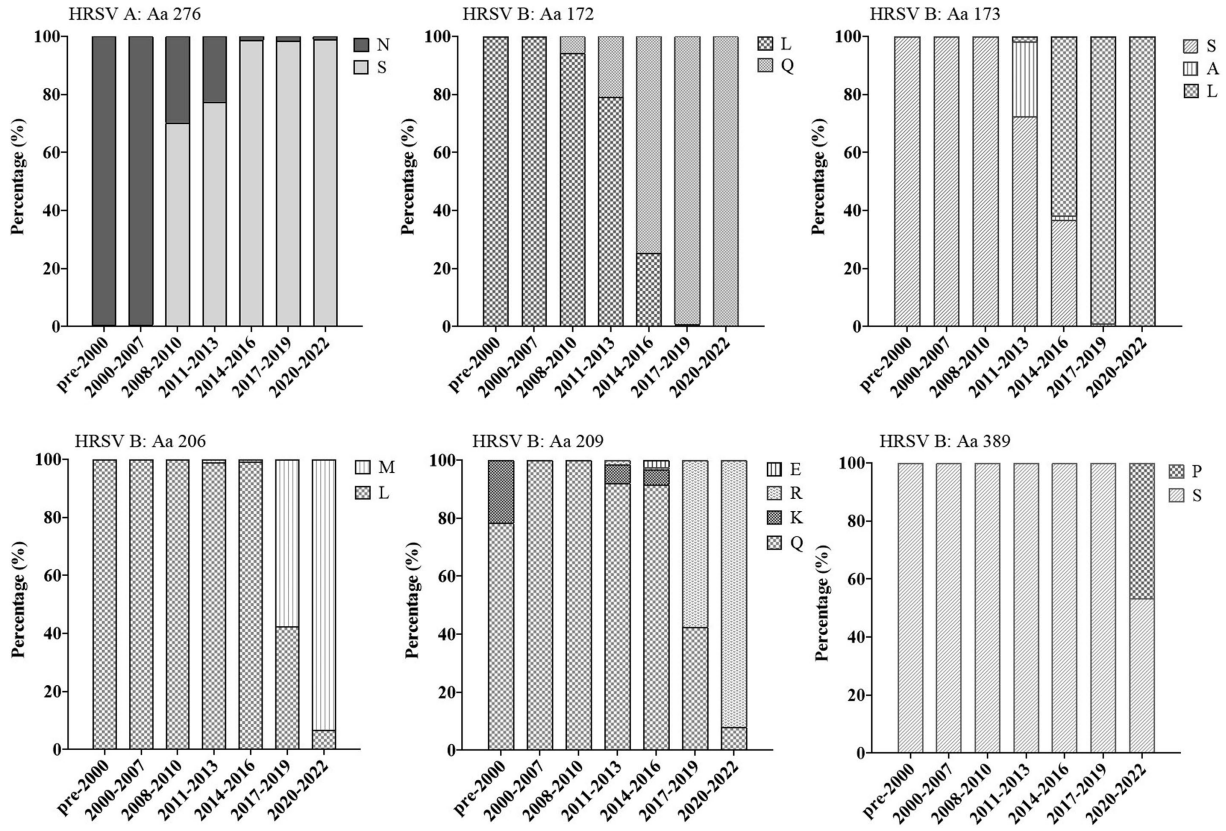

FIGURE 5
Proportions of substitutions located at the key antigenic sites on F proteins at different time periods. Multiple sites had substitutions and their proportion increased over time. Substitution of S389P occurred during the COVID-19 pandemic period. Aa, amino acid.

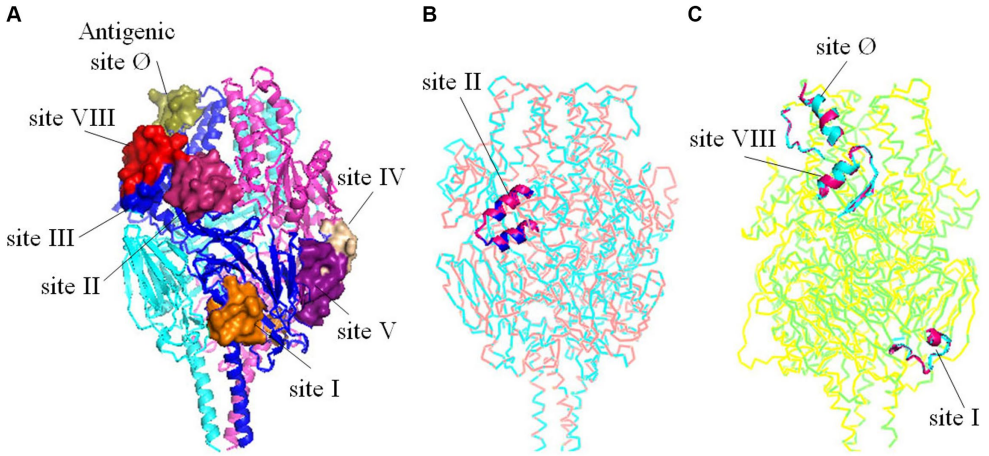

FIGURE 6
Tertiary structures comparisons of the trimeric F proteins. **(A)** Three-dimensional structure diagrams of the major antigenic sites (Ø-V, VIII) on the prototype of F protein. **(B)** Structural alignment between the mutant with substitutions on the antigenic site II and the prototype of HRSV A. **(C)** Structural alignment between the mutant with substitutions on the antigenic site Ø, I, VIII and the prototype of HRSV B. The substitutions did not alter the tertiary structures of the key antigenic sites.

in terms of sequence variations due to its larger sample size compared to other regions.

Previous studies have shown that the discriminatory power of partial or complete sequences from the highly variable G gene was limited in characterizing HRSV transmission, it has been suggested to use whole genome data in defining HRSV genotypes (Agoti et al., 2015; Ramaekers et al., 2020; Chen et al., 2022). Therefore, in this study, we employed full-genome sequences for

phylogenetic analysis, and used the newly established criteria for defining genotypes within HRSV-A and B (Ramaekers et al., 2020). The result revealed a chronological sequential emergence of both HRSV A and B genotypes throughout history. During the COVID-19 pandemic period, HRSV A sequences scattered in three different genotypes and exhibited geographical specificity, while HRSV B sequences all centered in one genotype. This observation is not surprising since previous studies have revealed an apparent pattern of geographical clustering among HRSVs (Robertson et al., 2021). The geographical-specific distribution of HRSV may be associated with different climatic conditions in various areas, as previous studies have indicated that climate may play a crucial role in driving viral epidemics (Pitzer et al., 2015; Baker et al., 2019).

Studies have estimated that the evolutionary rate of HRSV B was higher than that of HRSV A, with the G gene evolving at a faster pace compared to the F gene (Bose et al., 2015; Chi et al., 2019; Yu et al., 2021). During the COVID-19 pandemic period, it has been reported that both HRSV A and B continue to evolve, but with a faster evolutionary rate observed in HRSV B compared to HRSV A (Yan et al., 2023). However, our study found that the mean evolutionary rates for both G and F genes were slightly faster in HRSVA compared to those in HRSV B. Additionally, we identified an approximately 2.5 times faster evolutionary rate for the G gene when compared to the F gene (Chi et al., 2019). The discrepancy between our findings and previous studies regarding evolutionary rates may be attributed either to larger sample sizes or biases introduced through different calculation models used. Furthermore, we observed certain deviating sequences from other regions and time periods with HRSV A sequences from the United States between 2003 and 2006. However, these particular sequences were not detected in any other regions or subsequent time periods. We hypothesized that this observation could be due to founder effects. Interestingly, sequence divergence during the COVID-19 pandemic period was comparable to those from other time periods, indicating preventive measures such as mask-wearing and social distancing did not introduce additional pressures on the evolution of HRSV.

RNA viruses are known to have a higher tendency for mutation compared to DNA viruses due to the lack of proofreading activity in their RNA-dependent RNA polymerases (Peck and Lauring, 2018). In particular, the G gene of HRSV is considered the most variable region, and it contains two highly variable mucin-like domains flanking the central region, making it a valuable characteristic for studying viral evolution (McLellan et al., 2013b). Our study found a remarkably higher mutation rate in the G genes of both HRSV A and B. Specifically, we found that there were 2–3 times more SNPs occurring per 100 nucleotides in the G genes compared to the full genome length, as well as the F and L genes. Moreover, these SNPs were mainly located within the mucin-like domains of the G genes. Further selective pressure analysis suggested that positive selection acting on proteins (F and G) containing neutralizing antigenic sites in both HRSV A and B. It is worth noting that different studies may employ various models for RSV evolution when conducting selective pressures analysis, resulting in slightly different outcomes (Pretorius et al., 2013; Tan et al., 2013). Our study took a conservative approach by defining positive selection sites only when they were detected by all three models (MEME, FUBAR and SLAC). The presence of positively selected sites at specific codon positions suggested that HRSV has been evolving over time through selective processes favoring new variants.

The F protein of HRSV is highly conserved and plays a crucial role in membrane fusion and infection (McLellan et al., 2013a). It exists in two primary conformations: a pre-fusion form (preF), which is metastable, and a post-fusion form (post F), which is stable (Crank et al., 2019). The F protein contains seven major neutralizing antigenic sites (Ø-V, VIII) with sites Ø, V, and VIII exclusively present on the pre-F. Site III is predominantly located in the preF, while the remaining sites are found in both preF and postF forms (Graham, 2017; Jones et al., 2019). Neutralizing antibodies targeting the F protein are critical for developing RSV vaccines aiming to prevent infection (Ruckwardt et al., 2019). Recent neutralization assays conducted in China revealed that pediatric patients infected with different subtypes/genotypes of HRSV exhibited varying antibody titers against different subtypes/genotypes of HRSV (Zhou et al., 2023). Our study identified amino acids changes at antigenic sites Ø, I, II and VIII within the F protein. These alterations have potential implications for viral antigenicity. Therefore, we conducted further predictions and annotation of tertiary structures for F proteins to clarify the impact of these amino acid changes at important sites on major antigenic sites. The results showed that none of the observed substitutions led to changes in spatial structure within the identified antigenic sites, indicating there were no alterations in the antigenic properties of the F protein due to these specific amino acid changes. It was speculated that those substitutions may not be driven by host immune pressure but rather related more to viral fitness.

In conclusion, this study investigated the evolving characteristics of HRSVs during pre-pandemic and COVID-19 pandemic periods. Our findings revealed that although there were certain differences in genetic variation and prevalence features between HRSV A and B, their evolutionary patterns remained similar before and during the pandemic. Notably, amino acid substitutions at nuetralizing antigenic sites of F protein exhibited temporal specificity. However, these substitutions did not result in any discernible alterations in viral biological properties but may have been associated with viral adaptability. It is speculated that the stringent mitigation measures implemented during the pandemic effectively controlled COVID-19 incidence and excess mortality without placing additional immune pressure on HRSV. Nonetheless, continuous monitoring of genomic variations within HRSV remains crucial to generate scientific data for designing effective vaccines.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

C-yG: Data curation, Formal analysis, Methodology, Writing – original draft. YZ: Data curation, Formal analysis, Methodology,

Software, Writing – original draft. Y-yZ: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. WZ: Data curation, Formal analysis, Methodology, Validation, Writing – original draft. X-lP: Data curation, Formal analysis, Resources, Software, Writing – review & editing. Y-pZ: Data curation, Formal analysis, Methodology, Writing – review & editing. Y-hF: Data curation, Funding acquisition, Investigation, Software, Writing – review & editing. J-mY: Conceptualization, Project administration, Supervision, Visualization, Writing – review & editing, Writing – original draft. J-sH: Conceptualization, Funding acquisition, Project administration, Validation, Visualization, Writing – review & editing.

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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/fmicb.2023.1298026/full#supplementary-material

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The value of age IgG and IL6 in estimating time of viral clearance in asymptomatic or mild patients with COVID-19

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Background: The aim of this study was to investigate the relationship between Age, immunoglobin G (IgG), immunoglobin M (IgM), procalcitonin (PCT), and interleukin-6 (IL6), and the time to clear viral nucleic acids in asymptomatic and mild coronavirus disease 2019 (COVID-19) patients, as well as evaluated the predictive value of these biochemical indicators.

Methods: We performed a retrospective analysis on 1,570 individuals who were admitted to Tianjin First Central Hospital and diagnosed with asymptomatic or mild cases. Laboratory data were collected, including age, gender, levels of IgG, IgM, PCT and IL6, as well as results of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) nucleic acid test. These data were statistically analyzed using SPSS software, version 24.0.

Results: The results indicated that among mild patients, Age, IgG, and the time to clear viral nucleic acids were higher than asymptomatic patients ($p < 0.05$). And the time to clear viral nucleic acids was significantly correlated with Age, IgG, IgM, PCT, and IL6 ($p < 0.05$), IgG ($r = -0.445$, $p < 0.001$) showed moderate correlations. Using logistic regression analysis, we identified older age, high IL6 levels, and low IgG levels were risk factors for nucleic acid clearance exceeding 14 days ($p < 0.05$). When combining these three indicators to predict the probability of nucleic acid clearance exceeding 14 days in the 1,570 patients, the AUROC was found to be 0.727.

Conclusion: Age, IgG, and IL6 could potentially serve as useful predictors for nucleic acid clearance exceeding 14 days in asymptomatic and mild COVID-19 patients.

KEYWORDS

COVID-19, RT-PCR, nucleic acid clearance, IgG, IL6

Introduction

The Coronavirus disease 2019 (COVID-19) is an infectious disease caused by a novel strain of viruses that belong to a family responsible for various illnesses ranging from the common cold to severe acute respiratory syndrome (SARS). The COVID-19 disease progression could lead to an overactive inflammatory response and cytokine storm, which can cause organ damage (Chen et al., 2020; Lopes-Pacheco et al., 2021). Studies have reported that high levels of viral

RNA can be detected shortly after the onset of symptoms in COVID-19 patients, and these levels may persist for an extended period (Zou et al., 2020). There is growing concern that patients with prolonged viral infection may experience further organ or tissue injury as a result of the SARS-CoV-2 virus itself or ongoing inflammatory processes (Wang and Yang, 2022). Exploring the factors that influence virus clearance in patients is crucial for addressing chronic COVID-19 infection.

Recent studies have identified several risk factors for chronic viral infection, including older age (Crook et al., 2021; Notarte et al., 2022c), decreased humoral immune responses (Notarte et al., 2022a,b), and excessive production of inflammatory factors (Merad et al., 2022). As COVID-19 progresses, the levels of inflammatory factors in the body can significantly change, impacting the formation of immune responses during the early stages of the disease. Neutralizing immunoglobulin (IG) is capable of minimizing viral RNA replication and reducing the chances of disease progression by responding to the SARS-CoV-2 spike protein and/or its receptor-binding domain like the primary receptor angiotensin-converting enzyme 2 (ACE2) (Tegenge et al., 2021). Moreover, diminishing serum neutralizing activity or decreased antibody sensitivity has been linked to higher frequencies and longer time of COVID-19 infections (Gruell et al., 2022). Therefore the time it takes for SARS-CoV-2 nucleic acid to turn negative may be associated with cytokine storms and antibody levels induced by the viral infection.

Most published findings on COVID-19 have focused on critical patients, however there have been limited reports on patients with mild or asymptomatic cases, which make up the majority of infections. Asymptomatic or mild patients who experience long-term chronic viral infection are at a potential risk of developing severe symptoms. The objective of this article was to investigate relationship between important factors, including IgG, IgM, PCT, and IL6, and the time to clear viral nucleic acids. Additionally, we sought to assess the predictive value of these factors in determining the probability of nucleic acid clearance exceeding 14 days.

Materials and methods

Participants and data source

This study was conducted at Tianjin First Central Hospital in Tianjin, China, from September 2022 to June 2023. Inclusion criteria for this study were: (1) confirmed asymptomatic or mild COVID-19 cases, (2) nasopharyngeal swab test been performed more than twice within hospital stay, and the interval time > 24 h. Exclusion criteria for this study were: (1) patients with liver and/or kidney dysfunction, (2) patients with serious inflammatory diseases such as chronic pulmonary disease, digestive system disease, immunological diseases, et al., (3) patients with cerebral infarction or cardiovascular at the time of admission, or (4) patients with missing data. A total of 1,570 COVID-19 patients were included, with 743 being asymptomatic and 827 being mild cases. Data collection included recording symptoms at admission, laboratory results, and nucleic acid test outcomes from the COVID-19 rehabilitation ward at Tianjin First Central Hospital. The diagnosis of asymptomatic or mild cases was based on guidelines provided by the National Health Commission of China.

Serum IgG/IgM against the SARS-CoV-2 protein were evaluated by magnetic particle chemiluminescence (Biology and Science, China), PCT and IL6 were evaluated by quantitative electrochemiluminescence immunoassay (Ren mai, China). During the observation period, nasopharyngeal swabs were taken from all patients daily to minimize the occurrence of false-negative results. Each patient's sample was tested using commercial kits for SARS-CoV-2 provided by two different manufacturers (Sheng Xiang/Bo Jie, China). The Ct value for each sample was calculated according to the manufacturer's instructions, with a threshold Ct value of 40 set as positive according to the China Technical Guidelines for Laboratory Testing for COVID-19. The date of diagnosis was defined as the day when the first sample tested positive for SARS-CoV-2 by RT-PCR. Discharge criteria followed WHO guidance, requiring two consecutive negative PCR swabs taken more than 24 h apart, with no clinical symptoms.

According to the ninth edition of the China Novel Coronavirus Pneumonia Prevention and Control Protocol, COVID-19 patients were advised to be isolated for at least 14 days. So we set limit at 14 days, the patients were then divided into two groups: one group included patients whose SARS-CoV-2 nucleic acid turned negative within 14 days ($n = 624$), and the other group included patients whose SARS-CoV-2 nucleic acid turned negative after exceeding 14 days ($n = 946$). Demographic information, laboratory results, and chest CT scans were performed for all inpatients. These data were obtained from electronic medical records and reviewed by specialized physicians. This study was reviewed and approved by the Medical Ethics Committee of Tianjin First Central Hospital (Ethics Committee archiving No. 2022N052KY) and conformed to the principles outlined in the Declaration of Helsinki.

Statistical analysis

Statistical analysis was performed with SPSS 24.0 for Windows software (SPSS, Chicago, IL). Normally distributed data were expressed as the mean ± standard deviation. We applied the t-test for between-group comparisons of variables that showed normal distribution of data with homogeneous variances. Non-normally distributed data are represented by the median (upper quartile and lower quartile) and we used the non-parametric Mann–Whitney U test for between-group comparisons of non-normally distributed data or data with heterogeneous variance. The categorical characteristics were described as counts and percentage (%). The relationship between biochemical indicators and prognosis were assessed by using Pearson or Spearman test. Risk factors were analyzed by univariate logistics regression, which described the direction of the relationship between predictor and the response variable, p value and odds ratio (OR) with its 95% confidence interval (CI). Significance corresponded to $p < 0.05$ and 95% CI for OR; 95% CI > 1 indicated risk of the event; whereas, 95% CI < 1 indicated protection. The performance of prognostic indicators were evaluated by the receiver operating characteristic (ROC) curves analysis. The value of AUROC closed to 1.0 indicates a high diagnostic accuracy. According to the principle that maximum of Youden index access to the best sensitivity and specificity. $p < 0.05$ was considered statistically significant, and $p < 0.001$ was considered highly statistically significant.

Results

In total of 1,570 patients with COVID-19 (743 were asymptomatic patients and 827 were mild patients) were enrolled in the study. As shown in Table 1, the demographic and clinical characteristics of these patients were compared. The proportion of males were 52.22% in asymptomatic patients and 48.25% in mild patients. Significant difference was observed in age distribution between asymptomatic and mild patients, with mild patients being slightly older (37.52 ± 0.57 vs. 35.78 ± 0.65) ($p < 0.05$). There were no statistically significant differences in IgM, PCT, or IL6 levels between the two groups ($p > 0.05$). However, mild patients had higher levels of IgG and longer time to clear viral nucleic acids compared to asymptomatic patients, and these differences were statistically significant ($p < 0.05$).

We analyzed the correlation between biochemical indicators and days of nucleic acid turn negative. The correlation between time to clear viral nucleic acids and corresponding indicators in patients that are shown in Table 2. The results showed that correlation statistics of these indicators are statistically significant ($p < 0.05$). Age, IgM, PCT, and IL6 were weakly correlated with the time to clear viral nucleic acids ($r = -0.144$–0.151), however IgG was moderately correlated with the time to clear viral nucleic acids ($r = -0.445$). Based on the days for nucleic acid clearance, the patients were divided into two groups: those whose nucleic acid turned negative within 14 days (624 patients) and those whose nucleic acid turned negative after 14 days (946 patients). The data showed that patients whose nucleic acid turned negative within 14 days had lower age, lower IL6 levels, and higher IgG levels ($p < 0.05$). There were slight differences in PCT and IgM levels, but they were not statistically significant ($p > 0.05$) (Table 3).

We then used logistic regression analysis to evaluate the risk posed by these significant parameters in predicting the time to clear viral nucleic acids. The main factors influencing nucleic acid clearance exceeding 14 days were assessed (outcome of logistic regression: predictors and protectors of nucleic acid turned negative exceed 14 days). The results showed that IgG acted as a protective factor associated with nucleic acid clearance exceeding 14 days (OR = 0.786) (Table 4), while age, IgM, PCT, and IL6 were risk factors (OR = 1.516, 1.018, 1.307, and 1.617) (Table 4), and OR values calculation of Age, IgG, and IL6 were statistically significant ($p < 0.05$). A multivariate logistic regression model was built using these three variables, and it showed better predictive ability with an AUROC of 0.727 for predicting nucleic acid clearance exceeding 14 days.

TABLE 1 Laboratory characteristics of 1,570 asymptomatic or mild patients (mean ± SD).

Variables	Asymptomatic patients	Mild patients	*t* value	*p* value
Cases, *n*	743	827	/	/
Male, *n* (%)	388 (52.22%)	399 (48.25%)	/	/
Age (year)	35.78 ± 0.65	37.52 ± 0.57	−2.028	0.043
IgG (g/L)	18.69 ± 1.49	25.76 ± 1.71	−3.079	0.002
IgM (g/L)	0.49 ± 0.12	0.44 ± 0.08	0.360	0.719
PCT (ng/mL)	0.065 ± 0.0037	0.059 ± 0.0024	1.348	0.178
IL6 (U/ml)	12.37 ± 0.33	12.19 ± 0.33	0.386	0.700
Days of nucleic acid turn negative	15.86 ± 0.18	16.54 ± 0.19	2.641	0.008

TABLE 2 Correlation analysis of serum markers with the time of viral clearance.

Variables	Days of nucleic acid turned negative	
	P value	Correlation coefficient
Age (year)	<0.001	0.100
IgG (g/L)	<0.001	−0.445
IgM (g/L)	<0.001	−0.144
PCT (ng/mL)	0.034	0.054
IL6 (U/ml)	<0.001	0.151

TABLE 3 Characteristics differences of 1,570 COVID-19 patients between different groups.

Variables	Nucleic acid turned negative ≤14 days	Nucleic acid turned negative >14 days	*t* value	*p* value
Cases, *n*	624	946	/	/
Male, *n* (%)	329 (52.72%)	458 (48.41%)	/	/
Age (year)	34.24 ± 0.64	38.32 ± 0.57	−4.675	<0.001
IgG (g/L)	37.17 ± 2.50	12.68 ± 0.82	10.800	<0.001
IgM (g/L)	0.58 ± 0.15	0.38 ± 0.06	1.339	0.181
PCT (ng/mL)	0.058 ± 0.0031	0.064 ± 0.0029	−1.319	0.187
IL6 (U/ml)	11.11 ± 0.32	13.05 ± 0.33	−4.048	<0.001

Discussion

SARS-CoV-2 is a novel SARS-related coronavirus with high transmissibility. During the early stage of illness, patients may underestimate their symptoms and delay seeking medical attention. Recent reports have shown that the median time to clear viral nucleic acids in COVID-19 patients is around 20 days, but it can be as long as 37 days (Zhou et al., 2020). Although many patients show improvement in clinical and radiographic manifestations over time, their viral load may still remain high (Magleby et al., 2021), prolonged viral clearance has been associated with unfavorable outcomes and organ injury (Al-Aly et al., 2022). Therefore, achieving rapid clearance of SARS-CoV-2 is desirable during the early stage of infection as it can help halt the dissemination of the virus in the body and limit tissue damage. In this analysis, we studied a large number of asymptomatic or mild COVID-19 patients and explored factors that influence the time to clear viral nucleic acids. We observed that the mild group tended to be older compared to the asymptomatic group. Additionally, they also took more days for their nucleic acid to turn negative. Through logistic regression analysis, we found that patients whose nucleic acid turned negative within 14 days had lower age and IL6 levels, as well as higher levels of IgG. When combining age, IgG, and IL6 as predictive factors, we were able to predict the likelihood of nucleic acid conversion exceeding 14 days roughly, with an AUROC value of 0.727.

TABLE 4 Influence factors associated with the time of viral clearance (univariate logistics regression).

Variables	Regression coefficient	Wald value	OR (95%CI)	*p* value	AUROC
Age	0.016	24.167	1.516 (1.510–1.523)	<0.001	0.595
IgG	−0.014	73.244	0.786 (0.783–0.789)	<0.001	0.626
IgM	0.018	0.880	1.018 (0.980–1.058)	0.348	0.480
PCT	0.268	0.158	1.307 (0.849–2.898)	0.691	0.530
IL6	0.017	6.407	1.617 (1.504–1.631)	0.011	0.610
Age + IgG + IL6	0.416	65.096	1.916 (1.614–2.215)	<0.001	0.727

Cytokines play a crucial role in regulating immune and inflammatory responses. Excessive production of certain cytokines, such as IL-6, has been identified as a major factor contributing to the inflammatory response observed in COVID-19 (Rabaan et al., 2021). It is produced by various cell types, including immune cells, endothelial cells, keratinocytes and tumor cells. IL6 serves as a proinflammatory cytokine capable of activating the intracellular Janus kinase (Jak)/signal transducer and activator of transcription (STAT) cascade and perpetuating inflammation through a STAT3-mediated positive feedback loop in non-immune cells. Clinical trials and reports had shown that IL-6 played an important role in the pathogenesis and severity of COVID-19 (Pelaia et al., 2021), inhibiting the IL6 receptor could effectively reduce mortality or the side effects of COVID-19 (Gupta et al., 2021). Our results demonstrated that high IL-6 levels was closely related with long time to clear viral nucleic acids, potentially due to the reduced efficiency of virus clearance caused by a cytokine storm. PCT is a marker commonly used to indicate bacterial infection and may be more effective than other clinical indicators such as C-reactive protein (CRP) and white blood cell count (WBC) (Becker et al., 2010). However, in our research, we found no significant difference in PCT levels between the mild patient group and the asymptomatic group. Additionally, PCT performed poorly in predicting virus clearance within the studied population ($p > 0.05$).

Neutralizing antibodies play a crucial role in providing protection against SARS-CoV-2 and are considered a key factor induced by COVID-19 vaccines. These antibodies have also been utilized in convalescent plasma therapy for antiviral treatment (Corti et al., 2021). Elevated levels of IgG antibodies can inhibit the inflammatory response in COVID-19 patients. They bind to the viral S-protein and interfere with its interaction with the ACE2 receptor, thus neutralizing the activity of SARS-CoV-2. Even low levels of IgG can be highly effective in the early stages of COVID-19 by opsonizing the virus and facilitating its clearance via Fc receptors (Marconato et al., 2022). Our finding aligns with this, revealing that patients with high serum IgG levels have a shorter viral clearance time. Higher IgG levels in patients during the early infection stage effectively suppressed viral replication and facilitated faster viral clearance. Interestingly, IgM did not demonstrate similar abilities. We supposed that IgG antibodies primarily play a significant role in viral clearance, particularly benefiting individuals in the early stages of infection. Antibody activities like IgG-driven neutralization and antibody-dependent cellular cytotoxicity had a greater impact on the rapid viral clearance than IgM.

In our study, we found that Age, IL6, and IgG levels have prognostic value in predicting prolonged time for nucleic acid conversion. This information can help us roughly determine which patients may take a shorter period of time to recover, providing valuable insights for clinical prevention and control strategies. However, this study has several limitations. Firstly, it is a retrospective study conducted in a single medical center. Secondly, viral mRNA was detected using a qualitative assay, lacking quantitative analysis for viral load calculation. Lastly, there are limited relevant studies available, and our research solely explored the correlation between several markers (IgG, IgM, PCT, and IL6) and the time of nucleic acid conversion. Future studies should comprehensively analyze additional laboratory test results.

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 humans were approved by Medical Ethics Committee of Tianjin First Central Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XC: Conceptualization, Writing – original draft, Writing – review & editing, Data curation. Y-LX: Investigation, Software, Writing – original draft. C-lZ: Investigation, Software, Writing – original draft. HM: Project administration, Supervision, Writing – review & editing, Writing – original draft.

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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

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Development and management of gastrointestinal symptoms in long-term COVID-19

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Background: Emerging evidence reveals that SARS-CoV-2 possesses the capability to disrupt the gastrointestinal (GI) homeostasis, resulting in the long-term symptoms such as loss of appetite, diarrhea, gastroesophageal reflux, and nausea. In the current review, we summarized recent reports regarding the long-term effects of COVID-19 (long COVID) on the gastrointestine.

Objective: To provide a narrative review of abundant clinical evidence regarding the development and management of long-term GI symptoms in COVID-19 patients.

Results: Long-term persistent digestive symptoms are exhibited in a majority of long-COVID patients. SARS-CoV-2 infection of intestinal epithelial cells, cytokine storm, gut dysbiosis, therapeutic drugs, psychological factors and exacerbation of primary underlying diseases lead to long-term GI symptoms in COVID-19 patients. Interventions like probiotics, prebiotics, fecal microbiota transplantation, and antibiotics are proved to be beneficial in preserving intestinal microecological homeostasis and alleviating GI symptoms.

Conclusion: Timely diagnosis and treatment of GI symptoms in long-COVID patients hold great significance as they may contribute to the mitigation of severe conditions and ultimately lead to the improvement of outcomes of the patients.

KEYWORDS

long-term effects of COVID-19 (long-COVID), SARS-CoV-2, gastrointestinal symptoms, prognosis, therapeutics, gut microbiota, immune responses

1 Introduction

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is primarily recognized as an acute respiratory infectious disease (Zhu et al., 2020). SARS-CoV-2 has unique virological characteristics, associated with the rapid spread of SARS-CoV-2, which brings new challenges for the prevention and control of the long term outcomes of the epidemic (Hakim et al., 2021). Despite it remains challenging to determine exact number of individuals infected with COVID-19 over the last three or more years, evaluating the population affected by long-COVID is relatively easier to

assess (Baroni et al., 2023). Long-COVID is defined as post-COVID-19 symptoms or post-acute sequelae of COVID-19 that involve illnesses associated with both direct and indirect impacts of SARS-CoV-2 infection (Levine, 2022). The World Health Organization defines "post-COVID-19 symptoms" as those of individuals with probable or confirmed SARS-CoV-2 infection who continue to have symptoms 3 months after infection, persisting for at least 2 months, and with no other obvious cause. The US Centers for Disease Control and Prevention uses a definition of persistent symptoms or health problems 4 weeks after infection, while the UK uses a 12-week time standard (Mansell et al., 2022; Soriano et al., 2022). In September 2022, leading data assessed that approximately 17 million people in Europe have been suffered from long-COVID and potentially millions may have to endure its effects for several consecutive years (Baroni et al., 2023). Three years after the onset of irresistible COVID-19, we have characterized major of its features, but we are still exploring about the potential prognosis and long-term outcomes of COVID-19 (Wang Z. et al., 2022).

Patients who have afflicted with COVID-19 for a prolonged duration predominantly experience persistent appearance of general influenza, including fever, cough, myalgia, and fatigue along with digestive manifestations such as abdominal pain, vomiting, anorexia, nausea, diarrhea, and elevated transaminase (Ren et al., 2021). Huang et al. (2020) also reported instances of "atypical" COVID-19 patients presenting with digestive symptoms as either their primary or sole manifestation (Chen N. et al., 2020). In addition, it has been reported that patients exhibiting digestive symptoms experience prolonged hospitalization and a less favorable prognosis compared with patients without gastrointestinal (GI) symptoms (Pan et al., 2020). Therefore, it is critical to investigate characteristics and underlying mechanisms of digestive system in long-COVID patients. In this review, we highlighted the prognosis and mechanism of intestinal symptoms with long-COVID more than 3 years since the outbreak onset, providing valuable and comprehensive evidence on the enduring effects of COVID-19. These endeavors aimed at monitoring the long-term outcomes of COVID-19 are critical for better understanding these clinical digestive manifestations and thus alleviating their consequences effectively.

2 Long-term GI effects of long-COVID

The spike protein of SARS-CoV-2 targets and attacks GI cells by specifically binding with the angiotensin-converting enzyme 2 receptor (ACE2) present on the surface of intestinal epithelial cells (Liang et al., 2020). Huang et al. (2020) revealed that 76% of hospitalized patients experienced at least one symptom that continues at least 6 months after infection of SARS-CoV-2, with intestinal manifestations being among those reported (Zhou et al., 2017; Cheung et al., 2020; Ong et al., 2020; Xiao et al., 2020; Joshee et al., 2022). Liang et al. (2020) identified the expression of ACE2 in various tissues showing that the small intestine bears the highest localization of ACE2, rendering IECs are more susceptible to SARS-CoV-2 infection (Carfì et al., 2020). SARS-CoV-2 is detected in feces of nearly half (48.1%) of COVID-19 patients (Cheung et al., 2020). A total of 70.3% of the patients with respiratory samples negative are notably stool samples positive for SARS-CoV-2 (Dhar and Mohanty, 2020; Pan et al., 2020; Xiao et al., 2020). The persistent existence of the SARS-CoV-2 genome in feces suggests that the virus continues to interact with the GI cells and cause prolonged clinical symptoms over time, with diarrhea being the primary manifestation (Zhou et al., 2017; Cheung et al., 2020; Ong et al., 2020; Xiao et al., 2020). Researchers detected the level of fecal SARS-CoV-2 RNA in mild-to-moderate COVID-19 patients 10 months after diagnosis and found that viral RNA shedding in feces was positively correlated with GI adverse conditions in patients with long COVID (Natarajan et al., 2022). A number of additional studies showed a similar result (Zollner et al., 2022; Upadhyay et al., 2023), suggesting there is an important biological significance for the continuous presence of SARS-CoV-2 in feces.

Zonulin (pre-Haptoglobin 2) is the precursor of haptoglobin (Hp)-2, whose uncleaved form detected in human serum is considered as a biomarker of increased gut permeability (Fasano, 2011). It has been reported that SARS-CoV-2 spike stimulates the expression of zonulin, leading to the increase of gut permeability (Llorens et al., 2021). Moreover, it was found that levels of zonulin in patients dying of severe COVID-19 are higher than those in patients recovered from the disease (Giron et al., 2021; Palomino-Kobayashi et al., 2022). These data suggest that zonulin might be associated with poor outcomes in COVID-19.

The majority of acute COVID-19-associated GI manifestations are mild and self-limiting and include anorexia, acute diarrhea, nausea, vomiting and abdominal pain/discomfort (Han et al., 2020; Pan et al., 2020). However, Weng et al. (2021) followed up 117 patients after Covid-19 for 90 days and found that patients with long COVID exhibit loss of appetite (24%), nausea (18%), acid reflux (18%), diarrhea (15%), and abdominal distension (14%) with < 10% of patients reporting belching, vomiting, and bloody stools. GI symptoms in long COVID are partially related to psycho-psychological factors, such as stress, anxiety, and depression, after recovery from COVID-19 (Freedberg and Chang, 2022). GI symptoms may not be evident during or prior of infection, but they can arise after a certain period or even manifest months after initial infection. Patients who have developed severe pneumonia followed by diminished blood oxygen saturation values are at higher risk of developing GI sequelae. This phenomenon could be attributed to the presence of multi-organ dysfunction syndrome that can exhibit in severe COVID-19 infection, particularly under the condition of septic shock (Luo et al., 2020; Wan et al., 2020). Notably, it is reported that GI manifestations during hospitalization for severe COVID-19 can lead to malnutrition, and this has been associated with raised mortality rates in patients (Zhang P. et al., 2021; Afrisham et al., 2023). Moreover, supplemental nutrition plays a pivotal role not only during hospitalization but also in alleviating GI sequelae effectively.

3 Mechanisms of long-term GI injury caused by long-COVID

3.1 SARS-CoV-2 infection of IECs

Intestinal organoid culture plays an important role in researches bridging the cell culture and *in vivo* animal work

for disease models. SARS-CoV-2 is highly infectious in primates (including humans, rhesus monkeys, and crab eating monkeys), but has low transmission in wild-type mice, which greatly limits the research of SARS-CoV-2 in animals. SARS-CoV-2 is mainly thought to infect the lungs. In order to determine that SARS-CoV-2 can infect and replicate in the intestine, Zhou et al. (2020) established intestinal organoids from humans, a "mini-gut" cultured in a dish, and demonstrated that the virus can infect and replicate in intestinal cells of human intestinal organoids (Lamers et al., 2020), supporting the notion that the gut could be another crucial target organ for SARS-CoV-2. Moreover, researchers found that intestinal cells are susceptible to SARS-CoV and SARS-CoV-2 infection using human small intestinal organoids (hSIOs), and demonstrated that the intestinal epithelium supports SARS-CoV-2 replication (Lamers et al., 2020). Han et al. (2021) used intestinal organoids as a disease model for studying SARS-CoV-2 infection and screened inhibitors of SARS-CoV-2, providing valuable resources for drug screening and identifying candidates for COVID-19 therapeutics, arguing that intestinal organoids can indeed serve as an experimental model for coronavirus infection and therapeutic studies.

Multiple reports suggesting that viral nucleic acids are detected in anal/rectal swabs and stool samples from patients with mild and severe coronavirus pneumonia (Chen Y. et al., 2020; Liang et al., 2020; Lin et al., 2020; Wu et al., 2020; Xiao et al., 2020; Xu et al., 2020; Young et al., 2020), and the typical particles of SARS-CoV-2 are observed in IECs (Qian et al., 2021), indicating that SARS-CoV-2 actively replicates in the intestine. There are also studies suggesting that the presence of SARS-CoV-2 viral RNAs in GI tissue may be related to more severe cases (El Hajra Martínez et al., 2020). Massive infiltration of plasma cells and lymphocytes is observed in the lamina propria of the stomach, duodenum, and rectum, accompanied by the SARS-CoV-2 shell throughout the GI lumen, indicating that virus infection has triggered the inflammatory response in intestine (Liang et al., 2020; Xiao et al., 2020). Plasma VEGF level is markedly increased in patients with GI symptoms and positively correlated with intestinal inflammation. Mechanistically, Zeng et al. (2022) found that the spike of SARS-CoV-2 contributes to VEGF production in the duodenum of mice through activating the Ras-Raf-MEK-ERK pathway in IECs, but not in endothelium, and leading to an increase of permeability and inflammation. Moreover, epithelial-enteric neuronal crosstalk plays a vital role in SARS-CoV-2-induced inflammation. SARS-CoV-2 infection in enterocyte results in endoplasmic reticulum (ER) stress and the release of damage-associated molecular patterns (DAMPs) that induces the expression and release of vasoactive intestinal peptides (VIP) by enteric neurons (EN), hence disrupts gut electrolyte homeostasis. These findings highlight the role of epithelial-enteric neuronal crosstalk in COVID-19-related GI symptoms (Balasubramaniam et al., 2023). In addition, overexpression of interleukin (IL)-1β, IL-6 and TNF-α are also important in SARS-CoV-2-induced inflammation (Hoffmann et al., 2020; Villapol, 2020; Triana et al., 2021), supporting the notion that virus infection has triggered the inflammatory response in intestine.

Angiotensin-converting enzyme 2, as the main receptor for SARS-CoV-2 invasion, predominantly has higher expression and activity in the intestine. Therefore, the high expression of ACE2 in the gut is the key factor for SARS-CoV-2 to enter into host cells and induce GI symptoms (Du et al., 2020; Xiao et al., 2020; Zou et al., 2020). Structural biology and biochemical analysis reveal that SARS-CoV-2 is highly homologous to SARS-CoV, both belonging to β coronaviruses. The S protein of SARS-CoV-2 shares similarities with that in SARS-CoV, and the RBD structure of the SARS-CoV-2 S protein enhances its binding affinity to ACE2 on IECs. Proteins such as transmembrane serine protease 2 (TMPRSS2), endoprotease (Furin) and cathepsin L (CTSL) activate the S protein of SARS-CoV-2 to facilitate the fusion of SARS-CoV-2 and IECs membranes, thereby enhancing virus infection and leading to GI symptoms (D'Amico et al., 2020; Hoffmann et al., 2020; Lu et al., 2020; Wang M. Y. et al., 2020; Zhong et al., 2020). It can be inferred that SARS-CoV-2 adheres to ACE2-expressing cells and enters into IECs through receptor recognition, protease cleavage activation and membrane fusion, resulting in infiltration of plasma cells and lymphocytes and GI interstitial edema (**Figure 1**). The infected intestinal cells are thus damaged, leading to long-term malabsorption and intestinal inflammation.

3.2 Cytokine storm

Cytokine storm refers to the uncontrolled and excessive release of inflammatory factors due to immune dysregulation after stimulation. After SARS-CoV-2 infection, the immune system is disordered, resulting in cytokine storm (Chen N. et al., 2020). During conditions like COVID-19, homeostasis

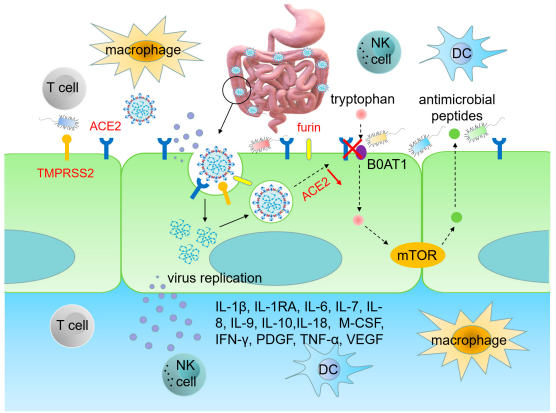

FIGURE 1
SARS-CoV-2 infects intestinal epithelial cells. The RBD region of S protein enhances the ability of SARS-CoV-2 to bind to ACE2 on intestinal cells. TMPRSS2 and furin promote RBD binding to ACE2 in IECs, thereby enhancing viral infection and causing GI damage. Upon infecting IECs, SARS-CoV-2 disrupts immune system, leading to an upsurge of pro-inflammatory cytokines, such as IL-1β, IL-1RA, IL-6, IL-7, IL-8, IL-9, IL-10, IL-18, M-CSF, IFN-γ, PDGF, TNF-α, and VEGF, which triggers cytokine storms. Infection of IECs by SARS-CoV-2 also leads to gut dysbiosis. The homeostasis of gut microbiota is related to the amino acid transport function of ACE2, which binds to the neutral amino acid transporter (B0AT1). Tryptophan, a neutral amino acid, regulates the expression of antimicrobial peptides, which maintain the flora of small intestine and large intestine in a stable state. RBD, receptor binding domain; ACE2, angiotensin converting enzyme 2; TMPRSS2, transmembrane serine protease 2; DC, dendritic cell; M-CSF, macrophage colony-stimulating factor; PDGF, platelet derived growth factor; VEGF, vascular endothelial growth factor.

of intestinal microbiota is essential to maintenance of balanced immune reactions preventing an array of excessively detrimental inflammatory responses. The microbe-associated molecular patterns (MAMPs) of gram-negative bacteria activate inflammatory cells in COVID-19 conditions. Plasma levels of LPS produced by gram-negative bacteria are positively correlated with the severity of intestinal permeability in SARS-CoV-2 infection. The small intestine contains a significant amount of lymphoid tissues and numerous activated immune cells (Wang et al., 2023). Researchers have found the association of LPS with activated T cell and increased pro-inflammatory responses in the intestine (Clerbaux et al., 2022). Intestinal dysbiosis in some COVID-19 patients may contribute to the translocation of LPS from intestine into the portal circulation, hence stimulating the Kupffer cells in the liver and leading to the activation of NF-κB signaling and release of IFN-β and TNF-α (Li et al., 2015; Zuo et al., 2020b). When LPS enters into the systemic circulation, aforementioned response leads to an elevated hepatic and systemic inflammation (Kawaratani et al., 2013). Moreover, researchers found that low dose of LPS circulates in the plasma of patients with intestine dysbiosis and endotoxemia may act as a predictive cofactor in accelerating the severity of cytokine storm in COVID-19 patients (Szeto et al., 2008; Vignesh et al., 2020). In addition, IECs infected with SARS-CoV-2 release massive inflammatory mediators and chemokines, leading to the accumulation of neutrophils and further promoting the inflammatory response (Chen N. et al., 2020). When SARS-CoV-2 infects IECs, cytokines are excessively released, leading to long-term GI symptoms including predominantly diarrhea. According to Huang et al. (2020), levels of IL-1β, IL-1RA, IL-6, IL-7, IL-8, IL-9, IL-10, macrophage colony-stimulating factor (M-CSF), IFN-γ, platelet-derived growth factor (PDGF), TNF-α and vascular endothelial growth factor (VEGF) in COVID-19 patients were much higher as compared to healthy individuals, indicating that cytokine storm is being associated with the occurrence of extrapulmonary multiple organ dysfunction during the progression of COVID-19 (Effenberger et al., 2020; Ojetti et al., 2020).

Tao et al. (2021) followed 173 COVID-19 patients after post recovery and discharge and found that 52.3% of patients exhibit a mucosal immune response primarily driven by IgA, accompanied by increased serum creatinine, deteriorating proteinuria, and elevated levels of pro-inflammatory cytokines, notably IL-18 (Zhang P. et al., 2021). Plasma levels of IL-2, IL-7, IL-10, granulocytic stimulator, IP-10, human monocyte chemotactic protein 1, and human macrophage inflammatory protein 1α in ICU patients with COVID-19 are higher than those in mild patients, indicating strong correlation between cytokine storm and disease severity (Ma et al., 2020; Neurath, 2020; Au et al., 2021; Kumar et al., 2021). Virus-induced cytokine storm leads to rapid deterioration in COVID-19 patients with GI diseases, so immune-mediated cytokine storm plays a crucial role in the progression and development of COVID-19 (**Figure 1**).

3.3 Gut dysbiosis during long-COVID exposure

Researchers analyzed the composition of gut microbiota in feces from COVID-19 patients in acute, convalescent and post-discharge periods, and found that while the abundance of gut commensals in COVID-19 patients gradually increases as the patient showed clinical improvement and recovery, the profusion of flora remains significantly lower than that of healthy subjects at the three time points (**Table 1**). Even after the clearance of SARS-CoV-2 (confirmed by throat swabs) and resolution of respiratory symptoms, commensal depletion and gut dysbiosis persist and contribute to long-term (up to 30 days after clearance of SARS-CoV-2) GI complications (Zuo et al., 2020b; Yeoh et al., 2021). The baseline abundance of *Clostridium ramosum*, *Coprobacillus*, and *Clostridium hathewayi* is positively correlated with the severity of COVID-19 while disease severity inversely correlates with abundance of *Faecalibacterium prausnitzii, Eubacterium rectale* and *Bifidobacteria*, which possesses anti-inflammatory properties (**Figure 2**). *Bacteroides dorei, Bacteroides thetaiotaomicron, Bacteroides massiliensis*, and *Bacteroides ovatus*, which downregulate expression of ACE2 in murine intestine, are negatively correlated with SARS-CoV-2 load in fecal samples from COVID-19 patients during hospitalization. However, the abundance of *Granulicatella* and *Rothia mucilaginosa* in oral and intestinal samples of COVID-19 patients shows a positive correlation with the presence of SARS-CoV-2 (Zuo et al., 2020b; Gou et al., 2021; Wu et al., 2021; Yeoh et al., 2021). Tao et al. (2021) tracked and monitored 173 discharged COVID-19 patients, and observed that the level of virus-specific IgA antibody in serum of relapsed patients increases, while the diversity of gut microbiota decreases, but the abundance of conditional pathogens such as *Streptococcus* is amplified (Zhang P. et al., 2021). In addition, SARS-CoV-2 leads to dysbiosis of multiple microbiota. The abundance of bacteria, such as *Coprobacillus*, *Clostridium ramosum*, *Clostridium hathewayi*, *Erysipelotrichaceae*, *Actinomyces*, *Enterobacteriaceae*, *Parabacteroides*, *Alistipes_indistinctus*, *Fusobacterium*, *Streptococcus*, *Morganella*, *Neisseria*, *Burkholderia*, *Desulfovibrionaceae*, *Granulicatella*, and *Rothia mucilaginosa* is positively correlated with disease severity in COVID-19 patients (**Table 2**). Whereas the abundance of *Bifidobacterium*, *Dorea*, *Bacteroides*, *Anaerostipes*, *Lachnospiraceae*, *Roseburia*, *Alistipes_onderdonkii*, *Faecalibacterium*, *Blautia*, *Ruminococcus*, *Coprococcus*, *Eggerthella*, *Akkermansia*, and *Eubacterium rectale* is negatively correlated with disease severity in COVID-19 patients (**Table 2**).

The homeostasis of gut microbiota is associated with the amino acid transport function of ACE2, which binds to the neutral amino acid transporter protein (B0AT1) on the luminal surface of IECs. Tryptophan, a neutral amino acid, is a key regulator of gut microbiota and inflammatory response. Tryptophan regulates the expression of antimicrobial peptides through the B0AT1-mTOR pathway to maintain the homeostasis of small intestinal and colonic flora (Hashimoto et al., 2012). After SARS-CoV-2 infects intestinal cells, IECs become dysfunctional, with some even necrotic and shedding, resulting in a reduction in the quantity of ACE2 (Trottein and Sokol, 2020; Verdecchia et al., 2020; Guo et al., 2021; Rajput et al., 2021). In addition, SARS-CoV-2 also co-internalizes with ACE2 through endocytosis, leading to a decreased expression of ACE2 and impaired transport of neutral amino acids, such as tryptophan, and inhibits the expression of antibacterial peptides, which contributes to gut dysbiosis and heightens

TABLE 1 Alteration of gut microbiota in patients with acute Covid or long Covid.

	Acute Covid	References	Long Covid	References
Reduced relative abundance of gut microbiota	*Bifidobacterium adolescentis*	Yeoh et al., 2021; Venzon and Cadwell, 2022; Zhang et al., 2022	*Bifidobacterium adolescentis*	Yeoh et al., 2021; Venzon and Cadwell, 2022; Zhang et al., 2022
	Ruminococcus bromii	Schult et al., 2022; Venzon and Cadwell, 2022; Zhang et al., 2022	*Ruminococcus bromii*	Yeoh et al., 2021; Schult et al., 2022; Vestad et al., 2022; Zhang et al., 2022
	Faecalibacterium prausnitzii	Zuo et al., 2020b; Yeoh et al., 2021; Schult et al., 2022; Venzon and Cadwell, 2022; Zhang et al., 2022	*Faecalibacterium prausnitzii*	Tian et al., 2021; Yeoh et al., 2021
	Fusicatenibacter	Schult et al., 2022	*Eubacterium rectale*	Zuo et al., 2020b; Tian et al., 2021; Yeoh et al., 2021; Vestad et al., 2022
	Blautia	Schult et al., 2022	*Lachnospiraceae*	Vestad et al., 2022; Zhang et al., 2022
	Lactobacillus	Vodnar et al., 2020	*Erysipelotrichaceae*	Tian et al., 2021; Vestad et al., 2022
	Lachnospiraceae bacterium	Zuo et al., 2020b; Zhang et al., 2022	*Coprococcus*	Vestad et al., 2022
	Eubacterium rectale	Zuo et al., 2020b; Yeoh et al., 2021	*Barnesiella*	Vestad et al., 2022
	Ruminococcus obeum	Zuo et al., 2020b; Zhang et al., 2022	*Corynebacterium*	Vestad et al., 2022
	Dorea formicigenerans	Zuo et al., 2020b	*Subdoligranulum*	Vestad et al., 2022
	Actinobacteria	Yeoh et al., 2021	*Fusicatenibacter*	Vestad et al., 2022
	/	/	*Oscillospira*	Vestad et al., 2022
	/	/	*Clostridia*	Vestad et al., 2022
	/	/	*Bifidobacterium longum*	Yeoh et al., 2021
	/	/	*Collinsella*	Tian et al., 2021
	/	/	*Coriobacteriia*	Tian et al., 2021
Increased relative abundance of gut microbiota	*Bacteroides ovatus*	Zuo et al., 2020b; Yeoh et al., 2021; Zhang et al., 2022	*Bacteroides thetaiotaomicron*	Zuo et al., 2020b; Yeoh et al., 2021; Zhang et al., 2022
	Bacteroides dorei	Zuo et al., 2020b; Yeoh et al., 2021; Zhang et al., 2022	*Bacteroides caccae*	Zuo et al., 2020b; Yeoh et al., 2021; Zhang et al., 2022
	Bacteroides thetaiotaomicron	Zuo et al., 2020b; Yeoh et al., 2021; Zhang et al., 2022	*Agathobacter*	Newsome et al., 2021
	Peptoniphilus	Newsome et al., 2021	*Blautia*	Newsome et al., 2021
	Corynebacterium	Newsome et al., 2021	*Granulicatella*	Newsome et al., 2021
	Campylobacter	Newsome et al., 2021	*Klebsiella*	Newsome et al., 2021
	Finegoldia	Newsome et al., 2021	*Lactobacillus ruminis*	Yeoh et al., 2021
	Comamonas	Newsome et al., 2021	*Phocea*	Newsome et al., 2021
	Sphaerochaeta	Newsome et al., 2021	*Veillonella*	Vestad et al., 2022; Zhang et al., 2022
	Synergistes	Newsome et al., 2021	*Flavonifractor*	Tian et al., 2021; Vestad et al., 2022
	Parabacteroides	Schult et al., 2022	*Streptococcus*	Zhang et al., 2022
	Veillonella	Zhang et al., 2022	*Rothia*	Tian et al., 2021; Zhang et al., 2022
	Coprobacillus	Vodnar et al., 2020; Zuo et al., 2020b	*Erysipelatoclostridium*	Tian et al., 2021
	Clostridium ramosum	Vodnar et al., 2020; Zuo et al., 2020b	*Acidimicrobiia*	Tian et al., 2021
	Clostridium hathewayi	Vodnar et al., 2020; Zuo et al., 2020b	*Micrococcaceae*	Tian et al., 2021
	Bacteroides massiliensis	Zuo et al., 2020b; Yeoh et al., 2021	*Microtrichaceae*	Tian et al., 2021
	Erysipelotrichaceae	Zuo et al., 2020b	*Actinomyces*	Zhang et al., 2022
	Streptococcus	Zhang et al., 2022	*Bifidobacterium dentium*	Yeoh et al., 2021
	Rothia	Zhang et al., 2022	*Candidatus Microthrix*	Tian et al., 2021
	Ruminococcus gnavus	Yeoh et al., 2021	*Ruminococcus gnavus*	Liu et al., 2022
	Ruminococcus torques	Yeoh et al., 2021	*Bacteroides vulgatus*	Liu et al., 2022

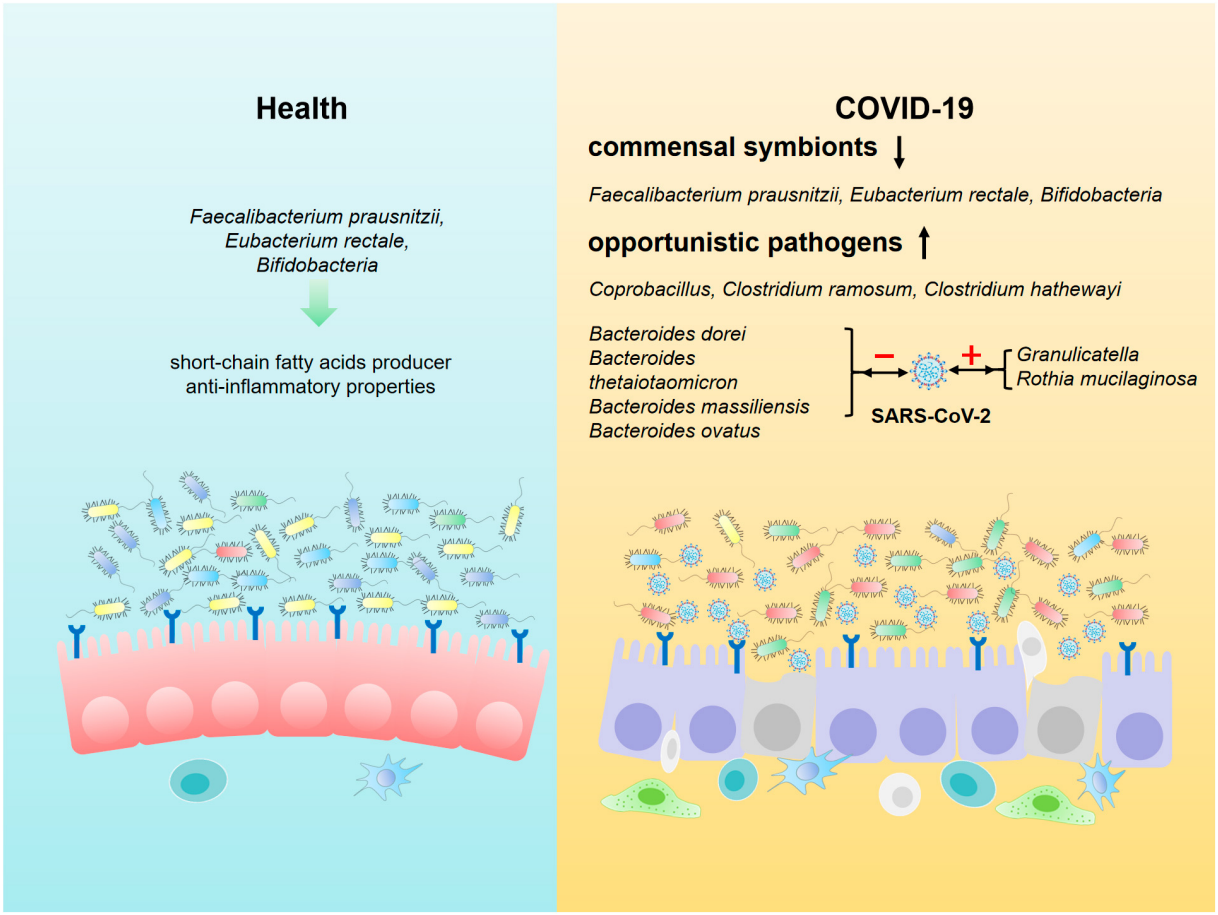

FIGURE 2
Composition of intestinal microbiota in healthy individuals and COVID-19 patients. Beneficial commensal bacteria such as *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Bifidobacteria* are dominant in the intestine of healthy population. However, the commensal symbionts are depleted, and opportunistic pathogens such as *Coprobacillus*, *Clostridium ramosum* and *Clostridium hathewayi* are markedly enriched in gut of COVID-19 patients. In addition, the composition of intestinal microbiota is closely associated with SARS-CoV-2 load in COVID-19 patients. *Bacteroides dorei*, *Bacteroides thetaiotaomicron*, *Bacteroides massiliensis* and *Bacteroides ovatus* are negatively correlated with the viral load, whereas the abundance of *Granulicatella* and *Rothia mucilaginosa* in oral and intestine of COVID-19 patients shows a positive correlation with SARS-CoV-2 load.

vulnerability to intestinal inflammation (**Figure 2**). There is evidence showing that gut microbiota imbalance is related to changes in gut-lung axis (Dang and Marsland, 2019), gut-liver axis (Tripathi et al., 2018; Manzoor et al., 2022), and gut-brain axis (Mitrea et al., 2022) homeostasis in COVID-19 patients. Transmission electron microscopy analysis showed that the existence of SARS-CoV-2 particles is on the surface and inside of intestinal bacteria, suggesting that SARS-CoV-2 infection with human gut bacteria may be another mechanism leading to ecological imbalance in COVID-19 patients (Clerbaux et al., 2022). Moreover, SARS-CoV-2 infection in IECs triggers an immune response leading to gut dysbiosis, characterized by reduced levels of short-chain fatty acid produced by anti-inflammatory bacteria and an increase in the abundance of opportunistic pathogens, such as *Enterobacteriaceae* (Gu et al., 2020; Gaibani et al., 2021; Ren et al., 2021; Xu et al., 2021). Hence it is crucial to thoroughly assess the gut dysbiosis in long-COVID patients with diarrhea. If necessary, fecal metagenomic sequencing should be employed to identify novel therapeutic targets for the treatment of long-term GI pathogenesis by analyzing the characteristics of gut microbiota changes in COVID-19 patients.

3.4 Drug toxicity

Numerous anti-COVID-19 drugs have been undergone clinical trials. A combination of two or three drugs were often used for the treatment of COVID-19, which increased the risk of GI injury. It was reported that acute diarrhea was associated with the use of drugs such as oseltamivir and arbidol in 55.2% of COVID-19 patients (Ren et al., 2021). Furthermore, a study revealed that remdesivir, while used to combat COVID-19, may have adverse effects such as nausea, elevated ALT, and constipation (Panda et al., 2020), indicating that drug toxicity plays a critical role in the occurrence of GI injury among COVID-19 patients.

Moreover, antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are frequently employed in clinical settings to treat tissue hypoxia and cells injury caused by respiratory infection, including COVID-19. It was observed that

TABLE 2 Composition of gut microbiota in COVID-19 patients.

Correlation type	Microbiota taxa	References
Positive correlation with COVID-19 severity	*Coprobacillus*	Zuo et al., 2020b; Najmi et al., 2022
	Clostridium ramosum	Zuo et al., 2020b; Najmi et al., 2022
	Clostridium hathewayi	Khan et al., 2021
	Erysipelotrichaceae	Li S. et al., 2021
	Actinomyces	Gu et al., 2020; Liu et al., 2022
	Enterobacteriaceae	Hughes et al., 2020; Amarsy et al., 2022; Marsico et al., 2022
	Parabacteroides	Al Bataineh et al., 2021; Farsi et al., 2022; Schult et al., 2022
	Alistipes_indistinctus	Zuo et al., 2020a
	Fusobacterium	Howell et al., 2021; Wolff et al., 2021
	Streptococcus	Amin-Chowdhury et al., 2021; Nair and Niederman, 2021; Parker et al., 2022
	Morganella	Zuo et al., 2021
	Neisseria	Rahaman et al., 2022; Romani et al., 2022
	Burkholderia	Zhong et al., 2021; Doubravská et al., 2022
	Desulfovibrionaceae	Sencio et al., 2022
	Granulicatella	Wu et al., 2021
	Rothia mucilaginosa	Marotz et al., 2021; Wu et al., 2021
Negative correlation with COVID-19 severity	*Bifidobacterium*	Zuo et al., 2020a; Ng S. C. et al., 2022; Romani et al., 2022
	Dorea	Zuo et al., 2020a
	Bacteroides	Prasad et al., 2022; Sun et al., 2022
	Anaerostipes	Romani et al., 2022
	Lachnospiraceae	Seibert et al., 2021; Sencio et al., 2022
	Roseburia	Ng S. C. et al., 2022
	Alistipes_onderdonkii	Zuo et al., 2021
	Faecalibacterium	Cortes et al., 2022; Sun et al., 2022
	Blautia	De Maio et al., 2021; Wang Y. et al., 2022
	Ruminococcus	Albrich et al., 2022
	Coprococcus	Romani et al., 2022; Xu et al., 2022
	Eggerthella	Romani et al., 2022
	Akkermansia	Cortes et al., 2022
	Eubacterium rectale	Tang et al., 2020; Yeoh et al., 2021

many patients experience symptoms including nausea, abdominal pain and acute diarrhea following such treatment (Tian et al., 2020). In a study involving 1,099 patients with COVID-19, intravenous antibiotics were used in 57.5% of the patients, and among these patients 3.8% experienced acute diarrhea as an adverse effect (Guan and Zhong, 2020). By analysis of stool samples from 96 COVID-19 patients, Bernard-Raichon et al. (2022) concluded that opportunistic pathogens with antibiotic resistance massively proliferate and contribute to the aggravation of gut dysbiosis. Without administration of antibiotics, COVID-19 patients showed an enrichment of abundance in *Ruminococcus gnavus*, *Ruminococcus torques* and *Bacteroides dorei* and a reduction of *Bifidobacterium adolescentis*, *Faecalibacterium prausnitzii* and *Eubacterium rectale*. However, COVID-19 with antibiotic treatment was primarily related to an increase of *Parabacteroides*, *Sutterella wadsworthensis* and *Bacteroides caccae* and a decrease in *Adlercreutzia equolifaciens*, *Dorea formicigenerans* and *Clostridium leptum*. Administration of antibiotics during hospitalization was associated with the severity of COVID-19 (Yeoh et al., 2021). Therefore, digestive symptoms in COVID-19 patients could also be caused by the administration of anti-viral drugs, antibiotics, NSAIDs and corticosteroids. To mitigate long-term GI injury these drugs should be accompanied by protective agents as a combinational therapy.

3.5 Psychological effects of long-COVID

COVID-19 is a rapidly evolving infectious disease characterized by swift transmission, high pathogenicity, elevated mortality and lacks specific drugs for treatment. Consequently, most patients experience negative emotions including panic and anxiety. Stress-induced brain interactions stimulate GI tract and lead to abnormalities in digestive, sensory, and immune functions,

resulting in GI symptoms such as abdominal pain and anorexia (Heinen et al., 2022; Taylor, 2022; Bassey et al., 2023). COVID-19 patients experience a wide range of physical and psychological symptoms, confirming that psychological factors can significantly slow down gastric emptying rates, that can ultimately lead to dysplasia (Aiyegbusi et al., 2021). A survey on the mental health and sleep status of COVID-19 patients found that compared with the control, the incidence of anxiety and sleep disorders are significantly higher in COVID-19 patients (Aiyegbusi et al., 2021), and the long-term GI symptoms in these patients has increased substantially (Anaya et al., 2021). Several studies suggest a causal impact of psychosocial stress on increased permeability of the intestine, possibly by corticotropin-releasing hormone-induced mast cell activation and reduced blood flow to the intestine under stimulation (Söderholm et al., 2002; Keita et al., 2010; Vicario et al., 2010). This suggests that psychological factors play a critical role in the development of GI symptoms in COVID-19 patients.

On the other hand, increased permeability of the intestine and abnormal influx of antigens from food and bacteria disrupts systemic immune homeostasis, which in turn harms brain function and structure (Genedi et al., 2019). Patients with structural and functional abnormalities in GI barrier, such as in colitis ulcerosa and Crohn's disease, display a more frequently psychiatric comorbidity (Faresjö et al., 2007; Nicholl et al., 2008). Thus, GI symptoms mutually affect the outcome of psychological disorders.

3.6 Exacerbation of primary diseases

Patients with underlying diseases, such as chronic obstructive pulmonary disease, hypertension, coronary heart disease, diabetes, cerebrovascular disease, viral hepatitis B, and cancer are more likely to develop severe pneumonia and have poor prognosis from COVID-19 (Chen N. et al., 2020; Guan and Zhong, 2020). Patients with inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD) are at high risk of developing COVID-19 complications, while long-term use of corticosteroids and immunosuppressants in IBD patients also elevates the risk of opportunistic SARS-CoV-2 infection (Bezzio et al., 2020). Moreover, IBD patients are more prone to stress and anxiety, which can lead to long-term GI symptoms (**Figure 3**).

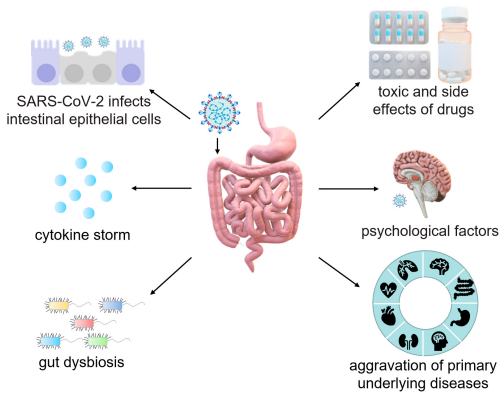

FIGURE 3
Schematic mechanisms of intestinal injury caused by SARS-CoV-2. The pathogenesis of intestinal injury in COVID-19 patients is mediated by the interaction between SARS-CoV-2 and ACE2, which leads to the destruction of epithelial cells and subsequent intestinal inflammation. In addition, of cytokine storm, gut dysbiosis, psychosocial factors, the aggravation of primary diseases, and potential side effects of antiviral drugs and antibiotics should also be considered during the treatment of GI injury in COVID-19 patients.

4 Preventive measures for long-term GI injury induced by SARS-CoV-2

4.1 Probiotics alleviate SARS-CoV-2-induced long-term GI injury

Probiotics are considered as an effective intervention for reducing morbidity and mortality in severe COVID-19 patients (Finlay et al., 2021). Despite of the continuous emergence of novel SARS-CoV-2 variant strains, the most potent prevention and control measure remains the widespread vaccination of the population with protective vaccines (Lynn et al., 2022). Studies have shown that gut microbiota of pre-vaccinated individuals can influence the immune balance within the host, consequently impacting the vaccine efficacy and immunogenicity (Pulendran, 2019; de Jong et al., 2020). It was reported that probiotics could improve the vaccine immunogenicity, and increase the ratio of seroprotection and seroconversion in adults vaccinated with influenza vaccine (Lei et al., 2017). Thus, the effectiveness and immune response of the vaccine in vulnerable populations can be improved by increasing the abundance of probiotics. *Lactobacilli* in the intestine of COVID-19 patients are beneficial to the maintenance of gastrointestinal homeostasis and alleviation of inflammatory illnesses (Sun et al., 2018; Huang et al., 2020). Wang M. et al. (2020) applied *Lactobacillus plantarum* to express SARS-CoV-2 S protein and showed that the protein could be expressed on the surface of *Lactobacillus plantarum* and bears a high antigenicity for stimulating the generation of specific monoclonal and polyclonal antibody against SARS-Cov-2-S-protein. Thus, *Lactobacillus plantarum* recombined with the gene expressing SARS-CoV-2 S protein could be developed as an ideal oral vaccine for SARS-CoV-2 infection (Wang M. et al., 2020). In addition, probiotics downregulate the proportion of pro-inflammatory and anti-inflammatory cytokines, which is of great benefit to the alleviation of cytokine storms in patients with COVID-19 (Aziz et al., 2020; Li Q. et al., 2021). In a short, due to the role of probiotics in the regulation of innate immunity, preventive intake of probiotics (formulations) might be helpful to promote protective antiviral response and inhibit harmful excessive inflammatory response of host (Tomkinson et al., 2023).

Judicious use of probiotics and prebiotics can safeguard the intestinal barrier and reduce intestinal permeability, and hence alleviate intestinal symptoms in COVID-19 patients by regulating immune homeostasis and inflammatory response (Trompette et al., 2018; Mak et al., 2020; Hu et al., 2021). Emerging studies have shown that probiotics can increase the number and activity of T cells, which directly contribute to the immune response induced by T cells and enhances immune functions

(Dhar and Mohanty, 2020). Furthermore, probiotics also enhance microbiome diversity and fortify the integrity of intestinal barrier functions, thereby effectively preventing microbial translocation (Mack et al., 2003; Reiff and Kelly, 2010; Wei et al., 2018; Suez et al., 2019; Badgeley et al., 2021). VSL#3, a mixture of 8 probiotics, has been safely and effectively used worldwide for decades. It is proven that VSL#3 alleviates enteritis and reinforces intestinal mucous barrier by improving the gut microecology (Mimura et al., 2004; Sartor, 2006; Tankou et al., 2018). Administration with VSL#3 is able help to improve the biodiversity of microbiota in patients, reduce fungal colonization, and increase the abundance of *Lactobacillus* and *Bifidobacterium*, thereby could diminish the severity of COVID-19-induced GI symptoms (Kühbacher et al., 2006; Chitapanarux et al., 2010). A comprehensive meta-analysis evaluated the clinical efficacy of probiotics in preventing treatment-related diarrhea (Wei et al., 2018), and showed that combined capsules containing diverse strains of *Bifidobacterium* and *Lactobacillus* were beneficial in reducing the incidence of acute diarrhea (Osterlund et al., 2007; Wada et al., 2010). Moreover, d'Ettorre et al. (2020) treated COVID-19 patients with a combination of *Streptococcus thermophilus DSM 32345*, *L. acidophilus DSM 32241*, *L. helveticus DSM 32242*, *Lacticaseibacillus paracasei DSM 32243*, *L. plantarum DSM 32244*, *LeviL. brevis DSM 27961*, *B. lactis DSM 32246,* and *B. lactis DSM 32247* for 7 days and found that patients given probiotics showed a reduction in GI symptoms as compared to those treated with the placebo. Gutiérrez-Castrellón et al. (2022) performed a randomized clinical trial involving 150 outpatients with COVID-19 treated with *L. plantarum KABP022*, *KABP023* and *KAPB033*, and *Pediococcus acidilactici KABP021* for 30 days and found that acute diarrhea remission rates are 53% and 28% in probiotic and placebo treatment groups, respectively. Similarly, Tang et al. (2021) applied probiotics to eliminate COVID-19 transmission in exposed household contacts involving 1,132 COVID-19 patients treated with *L. rhamnosus GG* or placebo for 28 days and beneficial effects were obtained. Collectively, current clinical evidence highlights that probiotics help to improve the immunity in COVID-19 patients by inhibiting pathogens colonization in the intestine, hence alleviating the disease severity (Olaimat et al., 2020).

Healthy dietary helps to maintain the homeostasis of gut microbiota in COVID-19 patients. Multiple studies showed that inflammatory response in COVID-19-positive patients is related to dietary styles (Ebrahimzadeh et al., 2022; Majidi et al., 2022; Moludi et al., 2022). Supplements of fruits, nuts, olive oil, vegetables, and whole grains, promote the abundance of probiotics and are negatively related to inflammation status in COVID-19 patients (Majidi et al., 2021; Hajipour et al., 2022). Moreover, vitamins D and A, selenium, flavonoids, zinc, and unsaturated fatty acids can alleviate inflammatory response in patients with COVID-19 via inhibiting the activation of nuclear factor kappa-B (NF-κB), hence reducing the production of pro-inflammatory cytokines, such as IL-6 and TNF-α. However, foods with higher glycemic load, carbohydrates, saturated fatty acids, and processed foods are found to disrupt the homeostasis of gut microbiota and are positively related to inflammation conditions in COVID-19 patients (Faghfouri et al., 2020, 2021; Zabetakis et al., 2020). In addition, appropriate intake of specific dietary fiber (Zhao et al., 2018), promote the growth of beneficial bacteria in the intestine, which may reduce the risk of SARS-CoV-2 infection (Kalantar-Zadeh et al., 2020; Merino et al., 2021). Fermentable dietary fiber (such as inulin) is fermented by gut microbiota to produce short-chain fatty acids (SCAFs), such as butyric acid, which alleviate the excessive inflammatory response caused by leukocytes in the lung, and enhance the immunoregulatory function of CD8 + T cells (Trompette et al., 2018). Antunes et al. (2019) administrated the acetate, butyrate, or propionate in the drinking water into mice infected with respiratory syncytial virus (RSV), and found that acetate exerts an anti-viral effect by binding with G protein coupled receptor 43 (GPR43). It suggests that increased abundance of probiotics could boost anti-viral ability and reduce long term GI symptoms in COVID-19 patients through preventive and therapeutic strategies.

4.2 FMT maintains intestinal flora homeostasis and relieves long-term GI symptoms caused by SARS-CoV-2

Fecal microbiota transplantation (FMT) is a therapeutic intervention to restructure the intestinal microbes of patients by transplanting the bacteria of healthy subjects into patient's intestine (Xu et al., 2016; Cheng et al., 2018; Juul et al., 2018). Eiseman et al. (1958) treated pseudomembranous enteritis induced by antibiotics through FMT therapy, and obtained a notable improvement in patient condition, demonstrating the efficacy of FMT in treating Clostridium difficile infection and in the alleviation of intestinal inflammation (Hui et al., 2019). A significant proportion of COVID-19 patients bear gut dysbiosis, which indicates a close relationship between intestinal flora imbalance and SARS-CoV-2 (Cao et al., 2021). FMT treatment in severe COVID-19 patients, rapidly alleviates COVID-19 symptoms and cures Clostridium difficile infection (CDI) (Ianiro et al., 2020a; Biliński et al., 2022). Furthermore, Liu et al. (2021) observed that FMT improves the recovery of COVID-19 patients, alleviates residual gastrointestinal symptoms, and promotes the recovery of normal intestinal microbiota. Lastly, a retrospective study of 86 patients with CDI and COVID-19 showed that combination of antibiotics and FMT promotes the alleviation of abdominal pain and reduces relapse of CDI, and decreases levels of inflammation cytokines as compared to those treated with antibiotics alone (Boicean et al., 2022). Taken together, these studies suggest that FMT facilitates the relief of gastrointestinal symptoms, reduces intestinal inflammation, and promotes the recovery of COVID-19 patients.

Study in the germ-free SD rat model showed that the colonization of intestinal flora affects the expression of intestinal Ace2, Lcn2, and Nlrc5, and regulates systemic inflammatory responses, which impacts the susceptibility of IECs to SARS-CoV-2, indicating that normal gut microbiota may play a role in mitigating SARS-CoV-2 infectivity and resultant injury in gastrointestine (Yang et al., 2020). Therefore, selection of healthy subjects for FMT donor may prevent potential side effects transmitted from donor's stool in gastrointestinal therapy (Ianiro et al., 2020b).

4.3 Rational medication for Long-COVID-19

COVID-19 patients may experience long term digestive symptoms due to the higher intake of antiviral drugs, antibiotics,

NSAIDs and corticosteroids, with antibiotics being particularly associated with antibiotic-induced acute diarrhea (Ng T. M. et al., 2022). Despite the international community emphasis on reasonable antibacterial intervention for COVID-19 patients, clinical practice often deviates from this principle, resulting in an upsurge risk of developing antibiotic-induced acute diarrhea. Antibacterial treatment should be reserved for cases with clear evidence of bacterial infection (phlegm, procalcitonin, leukopenia and neutropenia) (Shchikota et al., 2021). Traditional Chinese medicine (TCM) has been reported to reduce the risk of mild and severe cases of COVID-19 developing to critical stages and significantly shorten the course of disease and improve overall clinical effectiveness (Liu et al., 2020). A large number of clinical trials tested the administration of TCM to treat COVID-19 and showed that some herbs could regulate the immune response, limit SARS-CoV-2 infection, and protect organs from viral infection-induced damage (Li et al., 2022). For example, Qiao et al. (2021) found that application of Qingfei Paidu decoction, Lianhua Qingwen Capsule, and Xuanfei Baidu formula could relieve the symptoms and reduce the production of pro-inflammatory cytokines in COVID-19 patients. Moreover, Li et al. reported that Lianhua Qingwen Capsule and Pudilan Xiaoyan Oral Liquid inhibit the expression of inflammatory factors, such as TNF-α, IL-6, CCL2/MCP-1, and CCL10/IP-10 (Deng et al., 2020; Runfeng et al., 2020). Polysaccharides are a kind of substances with active pharmacological activity and widely found in TCM. Polysaccharides play a crucial role in immunomodulatory, anti-fibrotic and antiviral functions. It was reported that polysaccharides alleviate the symptoms of COVID-19 by targeting the regulatory axis of transforming growth factor-β/Smad2/3 and DANCER/AUF-1/FOXO3 (Chen R. R. et al., 2020; Chen N. et al., 2020). In addition, polysaccharides that regulate homeostasis of intestinal flora for a long time by promoting the growth of probiotics (Xu et al., 2017), These findings suggest that TCM herbs and their active substances may be used in the prevention and treatment of COVID-19. Rational use of antibiotic or combination with TCM may be an effective way to reduce antibiotic related acute diarrhea in COVID-19.

4.4 Psychotherapy for overcoming post COVID-19 symptoms

COVID-19 patients often experienced concomitant psychological disorders like anxiety. Unfortunately, clinical focus tends to minimize physical symptoms, neglecting the accompanying mental and psychological problems that can contribute to the exacerbation of COVID-19 condition. The psychological state of COVID-19 patients has a significant impact on the development of the disease as an excessive psychological stress can lead to increased activation of GI tract, which increases the risk of dyspepsia and intestinal inflammation (Everitt et al., 2019). Psychotherapy served as a significant approach to enhance the quality of life and alleviate stress could shorten the course of COVID-19. In the realm of clinical care, medical staff should prioritize psychological care to ensure comprehensive wellbeing of patient.

5 Challenges and opportunities for long-COVID-19 treatment

Currently, there are multiple types of vaccine against SARS-CoV-2 for the prevention of the virus. However, there is no specific vaccination or authorized drug developed to directly treat COVID-19 (Majumder and Minko, 2021; Gasmi et al., 2023). The pathogenesis of long-term intestinal injury in COVID-19 patients is well-understood. Interaction between SARS-CoV-2 and ACE2 destroys epithelial cells leading to intestinal inflammation. Furthermore, during the treatment of COVID-19 patients it is critically needed to pay more attention toward underlying symptoms such as cytokine storm, gut dysbiosis, psychosocial factors, the aggravation of primary diseases, side effects of antiviral drugs and antibiotics (**Figure 3**). Therefore, clinicians must maintain heightened vigilance regarding digestive symptoms in COVID-19 patients. The expectation is for medical professionals to delve further into the comprehension of long-term symptoms in COVID-19 patients and better comprehend the pathogenesis of the disease, so as to provide more targeted treatment while effectively reducing the spread of the virus.

6 Conclusion

The pathogenesis of long-COVID is undoubtedly multifactorial. Even after the acute effects of COVID-19 subside, vigilant attention is required for an extended period to monitor the poor prognosis and sequelae of long-COVID. This review aims to comprehensively summarize and enhance the understanding of long-term GI injury caused by long-COVID, thereby improving the efficacy of clinical diagnosis and treatment, and paves a way for new therapeutic strategies and targets for GI injury.

Author contributions

K-YH: Writing—original draft, Writing—review & editing. X-YL: Writing—original draft, Writing—review & editing. LZ: Writing—original draft. D-HW: Writing—original draft. J-QL: Writing—original draft. L-YL: Writing—original draft. UEL: Writing—original draft. C-YC: Writing—original draft. Y-PJ: Conceptualization, Funding acquisition, Writing—original draft, Writing—review & editing. Z-XX: Conceptualization, Funding acquisition, Writing—review & editing.

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Conflict of interest

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Impact of the COVID-19 pandemic on the prevalence of respiratory viral pathogens in patients with acute respiratory infection in Shanghai, China

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Objective: This study aimed to evaluate the impact of nonpharmaceutical interventions (NPIs) taken to combat COVID-19 on the prevalence of respiratory viruses (RVs) of acute respiratory infections (ARIs) in Shanghai.

Methods: Samples from ARI patients were collected and screened for 17 respiratory viral pathogens using TagMan low density microfluidic chip technology in Shanghai from January 2019 to December 2020. Pathogen data were analyzed to assess changes in acute respiratory infections between 2019 and 2020.

Results: A total of 2,744 patients were enrolled, including 1,710 and 1,034 in 2019 and 2020, respectively. The total detection rate of RVs decreased by 149.74% in 2020. However, detection rates for human respiratory syncytial virus B (RSVB), human coronavirus 229E (HCoV229E), human coronavirus NL63 (HCoVNL63), and human parainfluenza virus 3 (HPIV3) increased by 91.89, 58.33, 44.68 and 24.29%, in 2020. The increased positive rates of RSVB, HPIV3, resulted in more outpatients in 2020 than in 2019. IFV detection rates declined dramatically across gender, age groups, and seasons in 2020.

Conclusion: NPIs taken to eliminate COVID-19 had an impact on the prevalence of respiratory viral pathogens, especially the IFVs in the early phases of the pandemic. Partial respiratory viruses resurged with the lifting of NPIs, leading to an increase in ARIs infection.

KEYWORDS

COVID-19, acute respiratory infection, nonpharmaceutical interventions, viral pathogen, Shanghai

Introduction

Acute respiratory infections (ARIs) are common with significant morbidity and mortality worldwide, causing more than 2.5 million deaths in 2017 (1). Respiratory viruses (RVs), particularly influenza virus, coronavirus, parainfluenzavirus, adenovirus, respiratory syncytial virus, human metapneumovirus, bocavirus, rhinovirus, and enterovirus are known as common

viral pathogens causing ARIs (2–10). In late 2019, a new respiratory virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged and caused more than 2.4 million deaths within a year (11).

Nonpharmaceutical interventions (NPIs) (12) were used to prevent the spread of SARS-CoV-2 nationwide at the beginning of COVID-19 in China, including travel restrictions, physical distancing measures and wearing masks. However, socialdistancing, masking, school closures, shelter-in-place, travel restrictions, etc (13), also changed the prevalent characteristics of ARIs caused by RVs.

Since 2011, surveillance of RVs has begun with influenza-like illness (ILI) in outpatients (14) and ARI cases (15) in Shanghai. In China, the outbreak of COVID-19 has been quickly contained, but the impact of NPIs on ARIs is still unclear. The research was conducted to investigate and evaluate whether NPI could change the etiological characteristics of ARIs in patients before and during the COVID-19 epidemic in Shanghai between 2019 and 2020.

Materials and methods

Ethics statement

Data on participants in this study were collected from the respiratory surveillance system of Pudong New Area Center for Disease Control and Prevention. Participants gave their verbal informed consent before collecting their information and samples. Samples and information were not collected if refused. No written consent was obtained and no measures were taken to document the process as the data would be analyzed anonymously. All information was input in the respiratory surveillance system after collection. Pursuant to the Helsinki Declaration of 1975, the study protocol and consent procedure were approved by Pudong Centre for Disease Control and Prevention Ethics Review Committee.

Patients and specimen collection

Patients, judged by clinicians, had at least one of the following symptoms: cough, sore throat, runny nose or shortness of breath, with or without fever, were considered eligible for ARI (15). Respiratory samples (nasopharyngeal swab or sputum) and clinical data were collected and recorded by attending physicians of patients with ARI in 9 hospitals in Shanghai between January 2019 and December 2020 according to the ARI surveillance plan. The specimen was stored in 3.5-mL Viral Transport Media (VTM™, Yocon, Cat No: MT0301-1, Beijing, China) and transported to Shanghai Pudong New Area Center for Disease Control and Prevention for pathogen screening.

Screening for pathogens

Suspension of VTM was used directly for nucleic acid extraction. MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics, Cat. NO: 06543588001, Mannheim, Germany) were used to extract the nucleic acid on MagNA Pure 96 System (Roche Diagnostics, Switzerland) according to the manufacturer's instructions. The input volume was 200 μL and the elution volume was set at 100 μL.

Pathogens, including Adenovirus (HAdV), Human Coronavirus 229E (HCoV229E), Human Coronavirus HKU1 (HCoVHKU1), Human Coronavirus NL63 (HCoVNL63), Human Coronavirus OC43 (HCoVOC43), Human Enterovirus (EV), Influenza A/H1-2009 (IFVA-H1), Influenza A/H3 (IFVA-H3), Influenza A (IFVA), Influenza B (IFVB), Human Bocavirus (HBoV), Human Metapneumovirus (HMPV), Human Parainfluenza virus 1 (HPIV1), Human Parainfluenza virus 2 (HPIV2), Human Parainfluenza virus 3 (HPIV3), Human Parainfluenza virus 4 (HPIV4), Human Respiratory Syncytial Virus A (RSVA), Human Respiratory Syncytial Virus B (RSVB), Human Rhinovirus (HRV) were screened by the TaqMan low density array (TLDA) method (16) with TagMan low-density microfluidic chip technology by TaqMan™ Fast Advanced Master Mix (Applied Biosystems, Cat. NO: 4398986, USA) according to the manufacturer's instructions.

Epidemiological description and statistical analysis

On March 13, 2020, COVID-19 was declared a pandemic by WHO[1] and on May 8, 2020, The Joint Prevention and Control Mechanism of the State Council issued a guideline on regular prevention and control of COVID-19.[2] Accordingly, surveillance data were divided into three periods: Phase I (Jan. 1 to Mar. 1), Phase II (Mar. 1, to May 31), and Phase III (May 31 to Dec. 31). Positive rates and mean percent change for each RV were compared between phases with those of 2019.

The statistical analysis was performed using R 3.2.3 (R Core Team, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). The Mantel–Haenszel Chi square test or Fisher's exact two-tailed test was used to examine differences in discrete variable levels, with the Bonferroni corrected value of p of <(0.05/number of groups) indicating statistical significance.

Results

Characteristics of the study cases

During the two-year study period, a total of 2,744 patients were admitted the study, with 46.3% being female. The patients' median age was 14.0 years (IQR: 4.00–43.0). The cases were divided into five groups, with 700 (25.50%), 674 (24.60%), 137 (4.99%), 768 (28.00%), and 465 (16.90%) cases, respectively. The most common symptoms of any virus positive patient observed were fever (12.21%), cough (10.39%), runny nose (6.89%), and sore throat (3.21%). Notably, the

1 https://www.who.int/director-general/speeches/detail/who-director-general-s-opening-remarks-at-the-mission-briefing-on-covid-19---13-march-2020

2 https://english.www.gov.cn/policies/latestreleases/202005/08/content_WS5eb54d41c6d0b3f0e9497377.html

TABLE 1 Characteristics of ARI-patients in Shanghai, China, 2019–2020.

Characteristics	Total*	2019*	2020 *	*p* value
	N = 2,744 (100)	*N* = 1710 (62.32)	*N* = 1,034 (37.68)	
Demography characteristics				
Gender				0.076↓
Female	1,271 (46.3)	815 (47.7)	456 (44.1)	
Male	1,473 (53.7)	895 (52.3)	578 (55.9)	
Age median (IQR#, years)	14.0 [4.00; 43.0]	17.0 [4.00; 44.0]	12.5 [4.00; 42.0]	0.908↓
Age group(years)				0.268↓
~5	700 (25.5)	439 (25.7)	261 (25.2)	
~15	674 (24.6)	402 (23.5)	272 (26.3)	
~24	137 (4.99)	90 (5.26)	47 (4.55)	
~60	768 (28.0)	497 (29.1)	271 (26.2)	
≥60	465 (16.9)	282 (16.5)	183 (17.7)	
Clinical characteristics				
Fever	2,125 (12.21ᵀ)	1,428 (17.19ᵀ)	697 (3.97ᵀ)	**<0.001**↓
Cough	2000 (10.39)	1,380 (15.09)	620 (2.61)	**<0.001**↓
Runny nose	836 (6.89)	543 (9.82)	293 (2.03)	**<0.001**↓
Sore throat	1,120 (3.21)	686 (4.39)	434 (1.26)	**<0.001**↓
Expectoration	777 (2.33)	488 (3.63)	289 (0.19)	**<0.001**§
Weakness	367 (0.15)	271 (0.23)	96 (0)	**<0.001**§
Headache	307 (0.44)	261 (0.7)	46 (0)	**<0.001**§
Asthma	119 (0.07)	94 (0.12)	25 (0)	**<0.001**§
Difficulty breathing	20 (2.48)	16 (3.86)	4 (0.19)	**<0.001**§
Chest pain	48 (2.92)	30 (4.5)	18 (0.29)	0.2255§
Abdominal pain	39 (0.07)	21 (0.12)	18 (0)	0.656§
Diarrhea	31 (0.04)	14 (0.06)	17 (0)	1§
Hospital admission				**<0.001**↓
Inpatient	889 (32.4)	667 (39.0)	222 (21.5)	
Outpatient	1855 (67.6)	1,043 (61.0)	812 (78.5)	
Underlying disease	189 (6.89)	96 (5.61)	93 (8.99)	**<0.001**↓
Type of specimen				0.801↓
Nasopharyngeal swab	2016 (73.5)	1,253 (73.3)	763 (73.8)	
Sputum	728 (26.5)	457 (26.7)	271 (26.2)	

The characteristics contain demography characteristics, Clinical characteristics, hospital admission, underlying disease, and specimen. *p* values with statistical differences are shown in bold. #IQR, interquartile rang. ↓, Mentel-Haenszel $\chi 2$ test. §, Fisher exact test. ₸, Positive rate of any virus infection. *Data is presented as no. (%) of patients unless otherwise indicated.

number of inpatient and outpatient visits in 2020 was significantly lower than the number in 2019. Additionally, there was a significant difference in underlying diseases observed at the time of hospitalization (Table 1).

Viral etiology detected in ARI cases

During the study period, eight different pathogens and their subtypes were detected, however, HBoV was not detected in 2020. The overall detection rate of viral infections was lower in 2020 (15.2%) compared to the same period in 2019 (34.5%), and this difference was found to be statistically significant ($p < 0.05$). Among the viruses detected, the positive rates of IFV-H1, IFV-H3, IFV-B, RSVA, HPIV1, HPIV2, and HCoVOC43 were decreased in 2020 compared to 2019. However, the detection rates of RSVB, HPIV3, HCoVNL63, and HCoV229E were significantly increased by 91.89, 24.29, 44.68 and 58.33% in 2020 (Table 2).

Changes of viral pathogens in hospital admission

The detection rates of IFVA-H1 (5.1 to 0.45%, $p < 0.05$), HAdV (8.4 to 2.7%, $p < 0.05$), RSVA (3.45% to.1454%, $p < 0.05$) and HMPV (3.15 to 0.45%, $p < 0.05$) in inpatients and IFVA-H1 (9.49

TABLE 2 Characteristics of ARI-patients in Shanghai, China, 2019–2020.

	Total				*P* value	Inpatient				*P* value	Outpatient				*p* value
	2019		2020			2019		2020			2019		2020		
	n = 1710*	%#	*n* = 1,034	%		*n* = 667	%#	*n* = 222	%		*n* = 1,043	%#	*n* = 812	%	
IFVA-H1	133	7.78	5	0.48	**<0.001**↓	34	5.1	1	0.45	**0.002**§	99	9.49	4	0.49	**<0.001**§
IFVA-H3	105	6.14	15	1.45	**<0.001**↓	18	2.7	2	0.9	0.192§	87	8.34	13	1.6	**<0.001**↓
IFVB	77	4.5	23	2.22	**0.002**↓	13	1.95	5	2.25	0.998↓	64	6.14	18	2.22	**<0.001**↓
HAdV	86	5.03	28	2.71	**0.003**↓	56	8.4	6	2.7	**0.004**↓	30	2.88	22	2.71	0.829↓
HPIV1	12	0.7	2	0.19	0.070§	6	0.9	0	0	0.345§	6	0.58	2	0.25	0.474§
HPIV2	8	0.47	1	0.1	0.193§	5	0.75	0	0	0.438§	3	0.29	1	0.12	0.800§
HPIV3	24	1.4	18	1.74	0.486↓	14	2.1	0	0	0.062§	10	0.96	18	2.22	0.027↓
HPIV4	8	0.47	3	0.29	0.688§	3	0.45	0	0	0.577§	5	0.48	3	0.37	0.999§
RSVA	28	1.64	3	0.29	**0.001**§	23	3.45	1	0.45	**0.017**§	5	0.48	2	0.25	0.667§
RSVB	19	1.11	22	2.13	0.033↓	11	1.65	7	3.15	0.270↓	8	0.77	15	1.85	0.037↓
HCoV229e	8	0.47	7	0.68	0.472↓	6	0.9	3	1.35	0.845§	2	0.19	4	0.49	0.472§
HCoVHKU1	11	0.64	4	0.39	0.377§	4	0.6	0	0	0.577§	7	0.67	4	0.49	0.848§
HCoVNL63	2	0.12	2	0.19	1.000§	1	0.15	0	0	1.000§	1	0.1	2	0.25	0.828§
HCoVOC43	19	1.11	5	0.48	0.087↓	9	1.35	2	0.9	0.863§	10	0.96	3	0.37	0.131§
HRV	33	1.93	15	1.45	0.354↓	12	1.8	0	0	0.094§	21	2.01	15	1.85	0.797↓
HMPV	41	2.4	5	0.48	**<0.001**↓	21	3.15	1	0.45	0.025§	20	1.92	4	0.49	**0.007**§
HBoV	7	0.41	0	0	0.095§	4	0.6	0	0	0.577§	3	0.29	0	0	0.344§

P values with statistical differences are shown in bold. #, Percentage of detected rate: Detected number of positive specimens/Total number of specimens. ↓, Mentel-Haenszel χ^2 test. §, Fisher exact test. *Case numbers.

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to 0.49%, $p < 0.05$), IFVA-H3 (8.34 to 1.6%, $p < 0.05$), IFVB (6.14 to 2.22%, $p < 0.05$) and HMPV (1.92 to 0.49%, $p < 0.05$) in outpatients in 2019 were significantly higher than those in 2020. However, the detection rates of HPIV3 (0.96 to 2.22%, $p < 0.05$) and RSVB (0.77 to 1.85%) in outpatients were increased significantly in 2020 (Table 2).

Change of viral pathogens in gender

During the study period, significant differences were observed in the detection rate of IFVA-H1, IFVA-H3, IFVB, and HMPV for female ARI patients (8.22 to 0.44%, $p < 0.05$; 5.38 to 1.75%, $p < 0.05$; 5.15 to 2.63%, $p < 0.05$; and 2.21 to 0.66%, $p < 0.05$, respectively) and male patients (7.37 to 0.63%, $p < 0.05$; 5.92 to 1.46%, $p < 0.05$; and 2.57 to 0.42%, $p < 0.05$, respectively; Table 3).

Changes of viral pathogens in age groups

During the study period, the prevalence of IFVA-H1, IFVA-H3, HAdV, and RSVA was higher in the 0–4 year age group in 2019 compared to 2020 by 100, 90.19, 68.45% and 91.67, respectively. Additionally, the 5–14 year age group had a higher prevalence of IFVA-H3, HAdV, and HMPV in 2019 than in 2020 by 74.3, 78.13 and 100%, respectively. For the 15–24 year age group, IFVA-H1was more prevalent in 2019 compared to 2020 while HPIV3 was exactly the opposite, the decrease or increase rate was 100%. Similarly, IFVA-H1, IFVA-H3, IFV-B, and HMPV were significantly more prevalent in the 25–59 year age group in 2019 by 94.25, 71.11, 63.35, and 85.88%, respectively. The older 60 years age group also saw higher prevalence of IFVA-H1, IFVA-H3, in 2019 by 91.84 and 78.02%, respectively. The differences between years were statistically significant ($p < 0.05$; Table 3).

Changes of viral pathogens in different seasons

In October of 2019, only 5.77% (6/104) of respiratory viral pathogens were screened, compared to 70.14% (155/221) at the beginning of the year. It is evident that during the winter months of 2019 and 2020, there was a significant increase in respiratory virus prevalence. From July 2020, the detection rate gradually rose until reaching its peak in December of that year. Notably, there were no respiratory viruses detected in March, April, May, or July of 2020 (see Figure 1).

Changes of viral pathogens in different career

All 8 surveillance respiratory pathogens were detected in nursery children and students in 2019 and no RVs were detected in teachers in 2020. ARI-patients of medical personnel and farmers were infected with RSV and HCoV in 2020, respectively (Figure 2).

Change pattern of positive rate

The detection rates of the RVs decreased from 2019 to 2020 by 22.53% (from 63.05% to 40.52), 37.89% (from 37.89 to 0%), and 9.94% (from 22.71 to 12.77%) in three phases, respectively (Table 4).

Significant changes were identified in test-positive rates for IFV-H1, IFV-H3, IFVB, HAdV, HPIV1, HPIV2, HPIV3, RSVA, RSVB, HBoV and HMPV from 2019 to 2020. The largest decline in annual cumulative positive rates was observed by HBoV (100, 0.41 to 0%), followed by IFVA-H1 (93.83, 7.78 to 0.48%), RSVB (82.32, 1.64 to 0.29%) and HMPV (80, 2.4 to 0.48%). However, the annual cumulative positive rates for RSVB, HCoVNL63, and HCoV229e increased by 91.89% (1.11 to 2.13%), 58.33% (0.12 to 0.19%), and 44.68% (0.47 to 0.68%), respectively (Table 4).

Change pattern in RV detection rates varied significantly in three phases. In Phase I, the results showed that positive rates of HAdV (100%), HPIV1 (100%), HPIV2 (100%), HPIV3 (100%), RSVA (100%), HCoVHKU1 (100%), HCoVNL63 (100%), HCoVOC43 (100%), and IFVA-H1 (85.30%) decreased by more than 85%. The relative change in IFVB's positive rate was the only increase in this phase and the change ratio was 7,832%. In Phase II, dramatic reductions in RV detection rates were observed and no RV detected in this phase in 2020. In Phase III, the positive rates for IFVA-H3 (100%), IFVB (100%), HBoV (100%) still decreased dramatically. However, positive rates for HCoVHKU1, RSVB and HPIV3 continued to increase, with percentage changes of 91.67, 71.63 and 10.11%, respectively (Table 4).

The detection rates of respiratory viruses decreased across three phases, representing a 22.53, 37.89, and 9.94% reduction. Notably, the annual cumulative positive rates of HBoV, IFVA-H1, RSVB, and HMPV all underwent substantial changes. Some viruses demonstrated an increase in the detection-positive rate, including RSVB, HCoVNL63, and HCoV229e.

Discussion

To combat the COVID-19 pandemic, the Wuhan government implemented measures such as closing entertainment venues, suspending indoor public transport, and banning public gatherings (17). Subsequently, non-pharmaceutical interventions (NPIs) such as limiting social gatherings, wearing masks, practicing hand hygiene, and postponing the spring 2020 semester in primary and secondary schools were put in place to prevent the spread of SARS-CoV-2. There were no effective vaccines available globally at the time, so these NPIs were implemented to mitigate the spread of the virus (13). These interventions also had an impact on other respiratory viruses, including influenza, which was the most significant viral pathogen in ARI cases in Shanghai (14, 15).

This study analyzed the prevalence of respiratory viruses causing acute respiratory infections (ARIs) in Shanghai from 2019 to 2020. Results showed no significant differences in demographic characteristics among ARI patients in 2019 and 2020 (Table 1). Samples of ARI patients were detected by the TaqMan Low Density Array (TLDA) method from January 2019 to December 2020, despite the fact that ARI surveillance in Shanghai began in 2012 (15). This reflects the real impact of non-pharmacological interventions (NPIs) on the prevalence of respiratory viral pathogens in ARI patients.

TABLE 3 Distribution of viral etiology of the ARI-patients by age and gender, Shanghai, China, 2019–2020.

	0-4y		*p* value	5-14y		*p* value	15-24y		*p* value	25-59y		*p* value	≥60y		*p* value	female		*p* value	male		*p* value
	2019	2020		2019	2020		2019	2020		2019	2020		2019	2020		2019	2020		2019	2020	
	*N**=439(%) #	*n* = 261 (%)		*n* = 402 (%)	*n* = 272(%)		*n* = 90 (%)	*n* = 47(%)		*n* = 497(%)	*n* = 271(%)		*n* = 282(%)	*n* = 183(%)		*n* = 815(%)	*n* = 456(%)		*n* = 895(%)	*n* = 478(%)	
IFVA-H1	23 (5.24)	0 (0)	**<0.001**§	11 (2.74)	2 (0.74)	0.064§	16 (17.78)	0 (0)	**0.002**§	64 (12.88)	2 (0.74)	**<0.001**§	19 (6.74)	1 (0.55)	**0.001**§	67 (8.22)	2 (0.44)	**<0.001**§	66 (7.37)	3 (0.63)	**<0.001**§
IFVA-H3	17 (3.87)	1 (0.38)	**0.005**§	23 (5.72)	4 (1.47)	**0.006**§	13 (14.44)	2 (4.26)	0.07§	38 (7.65)	6 (2.21)	**0.002**↓	14 (4.96)	2 (1.09)	0.025§	52 (6.38)	8 (1.75)	**<0.001**§	53 (5.92)	7 (1.46)	**<0.001**↓
IFVB	8 (1.82)	2 (0.77)	0.418§	17 (4.23)	12 (4.41)	0.909↓	11 (12.22)	2 (4.26)	0.229§	35 (7.04)	7 (2.58)	**0.009**↓	6 (2.13)	0 (0)	0.117§	42 (5.15)	12 (2.63)	0.033↓	35 (3.91)	11 (2.3)	0.031↓
HAdV	32 (7.29)	6 (2.3)	**0.005**↓	27 (6.72)	4 (1.47)	**0.001**§	1 (1.11)	2 (4.26)	0.563§	17 (3.42)	9 (3.32)	0.942↓	9 (3.19)	7 (3.83)	0.714↓	28 (3.44)	8 (1.75)	0.083↓	58 (6.48)	20 (4.18)	0.011↓
HPIV1	4 (0.91)	1 (0.38)	0.735§	3 (0.75)	0 (0)	0.402§	1 (1.11)	0 (0)	1.000§	2 (0.4)	1 (0.37)	1.000§	2 (0.71)	0 (0)	0.522§	5 (0.61)	1 (0.22)	0.578§	7 (0.78)	1 (0.21)	0.234§
HPIV2	4 (0.91)	0 (0)	0.304§	2 (0.5)	0 (0)	0.518§	0 (0)	0 (0)	1.000§	0 (0)	1 (0.37)	0.353§	2 (0.71)	0 (0)	0.522§	3 (0.37)	1 (0.22)	1.000§	5 (0.56)	0 (0)	0.180§
HPIV3	12 (2.73)	4 (1.53)	0.304§	2 (0.5)	0 (0)	0.518§	0 (0)	2 (4.26)	0.116§	4 (0.8)	9 (3.32)	0.022§	6 (2.13)	3 (1.64)	0.977§	14 (1.72)	10 (2.19)	0.962↓	10 (1.12)	8 (1.67)	0.321↓
HPIV4	3 (0.68)	0 (0)	0.459§	1 (0.25)	1 (0.37)	1.000§	0 (0)	0 (0)	1.000§	3 (0.6)	0 (0)	0.499§	1 (0.35)	2 (1.09)	0.705§	2 (0.25)	2 (0.44)	0.946§	6 (0.67)	1 (0.21)	0.333§
RSVA	20 (4.56)	1 (0.38)	**0.002**§	1 (0.25)	2 (0.74)	0.733§	0 (0)	0 (0)	1.000§	2 (0.4)	0 (0)	0.543§	5 (1.77)	0 (0)	0.177§	11 (1.35)	1 (0.22)	0.090§	17 (1.9)	2 (0.42)	**0.010**§
RSVB	15 (3.42)	8 (3.07)	0.801↓	4 (1)	3 (1.1)	1.000§	0 (0)	1 (2.13)	0.343§	0 (0)	6 (2.21)	**0.004**§	0 (0)	4 (2.19)	0.048§	9 (1.1)	10 (2.19)	0.125↓	10 (1.12)	12 (2.51)	0.139↓
HCoV229e	3 (0.68)	1 (0.38)	1.000§	0 (0)	1 (0.37)	0.404§	0 (0)	0 (0)	1.000§	0 (0)	2 (0.74)	0.124§	5 (1.77)	3 (1.64)	1.000§	3 (0.37)	2 (0.44)	1.000§	5 (0.56)	5 (1.05)	0.708↓
HCoVHKU1	5 (1.14)	3 (1.15)	1.000§	1 (0.25)	0 (0)	1.000§	0 (0)	0 (0)	1.000§	3 (0.6)	0 (0)	0.499§	2 (0.71)	1 (0.55)	1.000§	3 (0.37)	1 (0.22)	1.000§	8 (0.89)	3 (0.63)	0.613§
HCoVNL63	1 (0.23)	1 (0.38)	1.000§	1 (0.25)	0 (0)	1.000§	0 (0)	0 (0)	1.000§	0 (0)	1 (0.37)	0.353§	0 (0)	0 (0)	1.000§	2 (0.25)	1 (0.22)	1.000§	0 (0)	1 (0.21)	0.392§
HCoVOC43	9 (2.05)	2 (0.77)	0314§	4 (1)	1 (0.37)	0.636§	0 (0)	0 (0)	1.000§	4 (0.8)	1 (0.37)	0.804§	2 (0.71)	1 (0.55)	1.000§	10 (1.23)	1 (0.22)	0.122§	9 (1.01)	4 (0.84)	0.530§
HRV	8 (1.82)	2 (0.77)	0.418§	6 (1.49)	4 (1.47)	1.000§	1 (1.11)	0 (0)	1.000§	13 (2.62)	9 (3.32)	0.576↓	5 (1.77)	0 (0)	0.177§	15 (1.84)	8 (1.75)	0.912↓	18 (2.01)	7 (1.46)	0.246↓
HMPV	11 (2.51)	3 (1.15)	0.215§	6 (1.49)	0 (0)	0.108§	1 (1.11)	0 (0)	1.000§	13 (2.62)	1 (0.37)	0.052§	10 (3.55)	1 (0.55)	0.077§	18 (2.21)	3 (0.66)	0.038§	23 (2.57)	2 (0.42)	**0.001**§
HBoV	4 (0.91)	0 (0)	0.304§	2 (0.5)	0 (0)	0.518§	0 (0)	0 (0)	1.000§	1 (0.2)	0 (0)	1.000§	0 (0)	0 (0)	1.000§	3 (0.37)	0 (0)	0.487§	4 (0.45)	0 (0)	0.273§

P values with statistical differences are shown in bold. #: Percentage of detected rate: Detected number of positive specimens/Total number of specimens. ↓, Mentel-Haenszel χ^2 test, §, Fisher exact test. *Case numbers.

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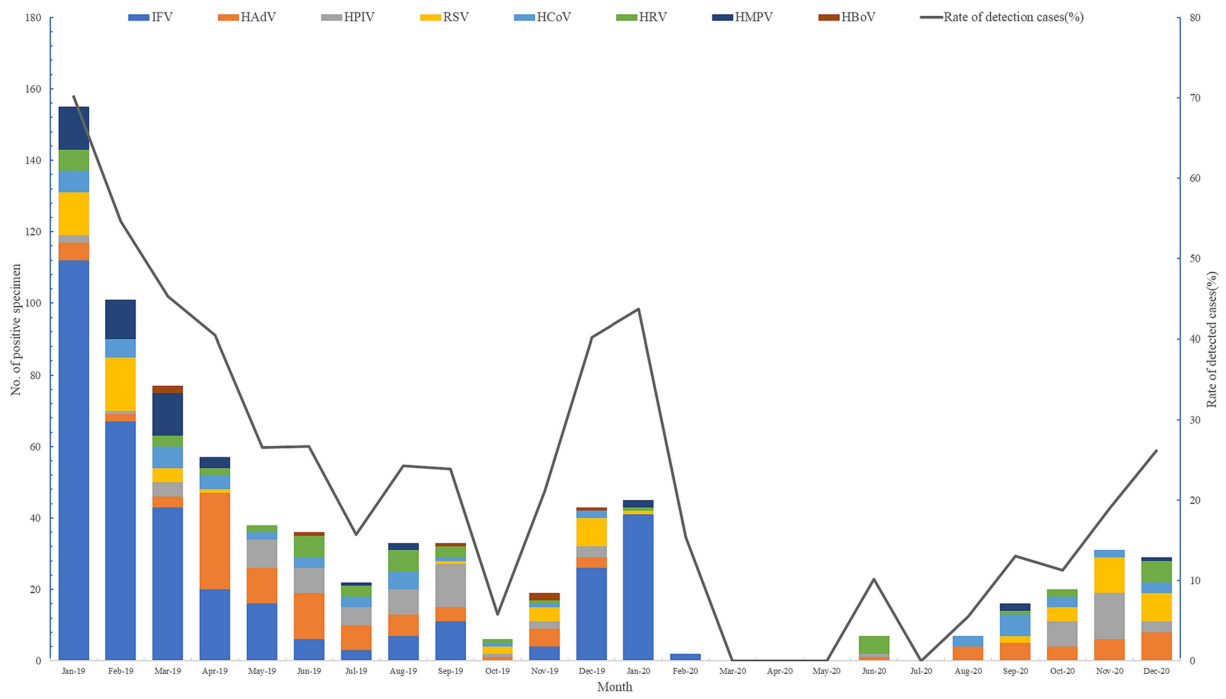

FIGURE 1
Monthly distribution of ARI-patients positive for viral etiology in Shanghai, 2019–2020.

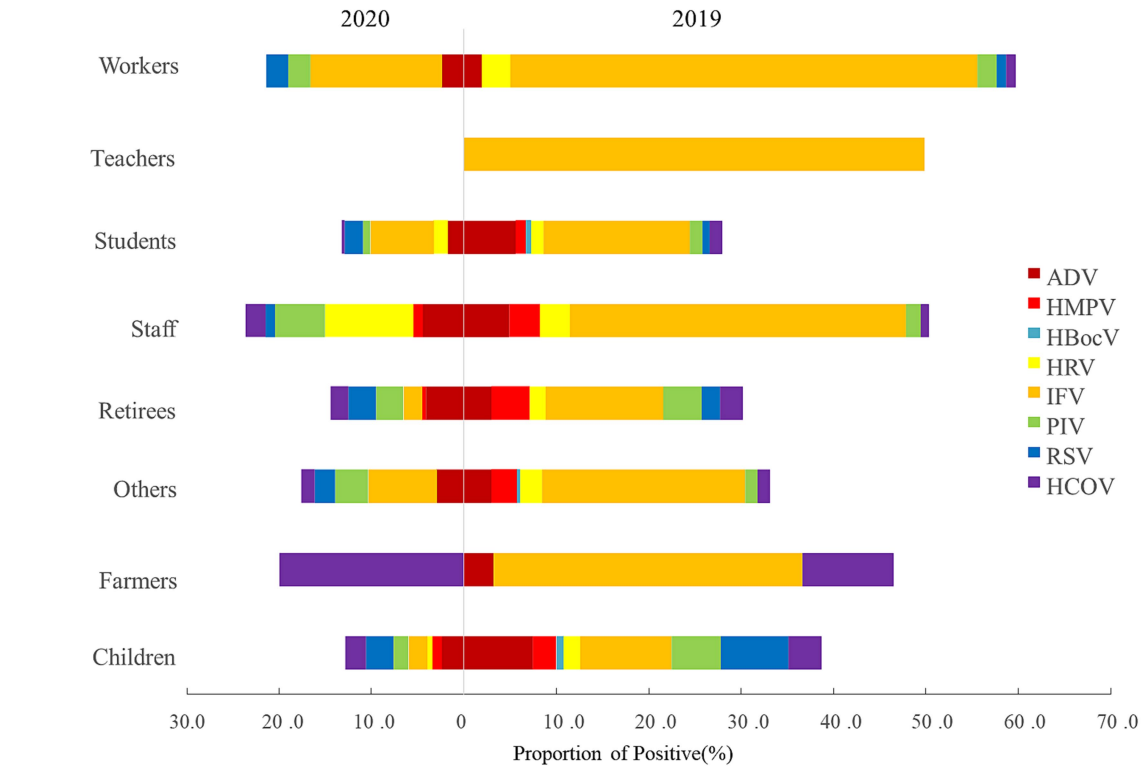

FIGURE 2
Career distribution of viral etiology in ARI-patients from 2019 to 2020, in Shanghai, China.

TABLE 4 Comparison of test positive rate (%) of respiratory viruses between 2019 and 2020 in Shanghai.

	Overall			Phase I*			Phase II*			Phase III*		
	2019	2020	Relative change₸	2019	2020	Relative change₸	2019	2020	Relative change₸	2019	2020	Relative change₸
IFVA-H1	7.78	0.48	−93.83%#	29.31	4.31	−85.30%#	3.08	0	−100%#	/		
IFVA-H3	6.14	1.45	−76.38%#	14.53	12.93	−11.01%	5.07	0	−100%#	2.71	0	−100%#
IFVB	4.5	2.22	−50.67%#	0.25	19.83	+7,832%#	9.25	0	−100%#	4	0	−100%#
HAdV	5.03	2.71	−46.12%#	1.72	0	−100%#	8.81	0	−100%#	4.59	3.22	−29.85%
HPIV1	0.7	0.19	−72.86%#	0.25	0	−100%#	0.44	0	−100%#	1.06	0.23	−78.30%
HPIV2	0.47	0.1	−78.72%#	/			0.88	0	−100%#	0.47	0.12	−74.47%
HPIV3	1.4	1.74	24.29%#	0.49	0	−100%#	1.32	0	−100%#	1.88	2.07	+10.11%
HPIV4	0.47	0.29	−38.30%	/			/			0.94	0.35	−62.77%
RSVA	1.64	0.29	−82.32%#	5.67	0	−100%#	0.44	0	−100%#	0.35	0.35	0%
RSVB	1.11	2.13	+1.89%#	0.99	0.86	−13.13%	0.66	0	−100%#	1.41	2.42	+71.63%
HCoV229e	0.47	0.68	+44.68%	/			0.22	0	−100%#	0.82	0.81	−1.22%
HCoVHKU1	0.64	0.39	−39.06%	0.74	0	−100%#	1.32	0	−100%#	0.24	0.46	+91.67%
HCoVNL63	0.12	0.19	+58.33%	0.49	0	−100%#	/			0	0.23	0
HCoVOC43	1.11	0.48	−56.76%	1.48	0	−100%#	1.1	0	−100%#	0.94	0.58	−38.30%
HRV	1.93	1.45	−24.87%	1.48	0.86	−41.89%	1.54	0	−100%#	2.35	1.61	−31.49%
HMPV	2.4	0.48	−80%#	5.67	1.72	−69.66%	3.3	0	−100%#	0.35	0.35	0%
HBoV	0.41	0	−100%#	/			0.44	0	−100%#	0.59	0	−100%#

*Phase I: from 1st Jan. to 28th Feb.; Phase II: from 1st Mar. to 31st May.; Phase III: from 1st Jan. to 31st Dec. #Statistically significant changes (value of $p<0.05$, Mentel-Haenszel chi-square test or Fisher's exact with two-tailed). ₸, Calculated by $\frac{(PR2020 - PR2019)}{PR2019} * 100\%$, PR 2019: positive rate in 2019, PR2020: positive rate in 2020.

In light of the directive to remain confined from March 1st to May 30th, 2020, the quantity of specimen collection for ARI cases decreased, which caused the decrease of the respiratory viral pathogens. Although it remains possible that ARIs were present in Shanghai during this period, this measure successfully suppressed the occurrence of respiratory viral pathogens. Correspondingly, a reduction in the number of ARI cases recorded through the surveillance system occurred, decreasing from 1,710 to 1,034, alongside a decrease in the percentage of detection of all viral pathogens, from 34.5 to 15.2%. Notably, the prevalence of ARI cases recorded was comparable to that of 2011–2015 in Shanghai (14, 15), 2016–2019 in Rome (17), and 2019–2020 in Canada (18).

Our study indicates that there are no significant differences in the demographics of ARI patients monitored over 2 years, as shown in Table 1. However, the implementation of NPIs has led to a decline in both outpatient and inpatient visits due to ARI, with the modes of transmission, including droplets, aerosols, and physical contact, being the same as those observed in COVID-19 cases, which were first detected in Shanghai after March 1, 2020. The above pattern has also been observed in respiratory tract infections in other countries, including Japan (19), Germany (20), and the United States (21).

Patients infected with IFVA had clinical symptoms such as fever and chills (22). Generally, the clinical symptoms of RVs' infection with ARI are mild and should always be ignored. It was surprising to find that the incidence of most clinical features of ARI decreased significantly in our study. Future studies should investigate whether NPIs could relieve infection symptoms. Numerous previous studies (23–27) have shown a decrease in seasonal influenza cases in Europe, Canada, New Zealand, Japan, and the United States in late 2020. Our study indicates that IFVA incidence declined significantly during phase I and phase II, indicating that the prevalence of IFVA in early 2020 may be ending. The relative surge in IFVB cases was noteworthy, with a 7,832% increase observed in phase I, suggesting that IFVB prevalence in Shanghai began in late 2020. However, with the implementation of NPIs, the incidence and positive rates of IFVB became zero in phase II and phase III, effectively preventing the spread of the virus.

Studies in children about ADV infection showed that ADV positive rates in 2020 were significantly lower than the same period in 2019 in Hangzhou (28), Shenzhen (29), China. Our results also showed that ADV was the main RV that infected children under 14 years of age other than IFV, and ADV detection rates were much lower than in 2020.

At the beginning of 2020, a significant decline in the detection rate of RSVA was observed among children below the age of four, which is consistent with findings reported by Australian researchers (30). Furthermore, there was no notable increase in the winter season of 2020, as RSVA has consistently been the leading cause of viral pneumonia in children (23). We found that the increased positive rate of RSVB in the medical personal group in the 25-59y age group, which was also a viral pathogen in our study, increased the detection rate in this age group in Phase III in 2020.

All pathogens were affected by NPI because the test positive rates for Phase II in 2020 were zero. IFVA-H1, HAdV, HPIV1, HPIV3, RSVA, HCoVHKU1, HCoVNL63, and HCoVOC43 decreased significantly and IFVB increased dramatically in phase I. IFVA-H1 and IFVB decreased significantly in phase III. However, the detection rates of IPIV3, RSVB, and HCOVHKU1 increased (Table 4).

The biological characteristics of HCoV are similar to those of SARS-CoV-2. Interestingly, the existing four HCoVs have not changed significantly, which means that the NPIs implemented to prevent the spread of SARS-CoV-2 did not prevent existing human CoVs, especially for HCoVHKU1.

Our study used the same detection methods and patient distribution did not change significantly. There are still a number of limitations and shortcomings. Firstly, the viral activity did not know as the method was based on TLDA, one kind of polymerase chain reaction (PCR), which could not distinguish whether the virus was alive or not, although Ct values can provide information on contagiousness and NPI measures could not be inferred with the detection results. Second, the COVID-19 pandemic could change the behavior of patients with respiratory infections by viral pathogens and their health-seeking behavior could change in the future. Third, the study period is only 2 years, and the small number of Phase II and Phase III samples may lead to some errors and lack of analysis. Fourth, NPIs lead to a harsh environment for respiratory viruses, which may increase the rate of viral mutation in the near future.

Understanding the circulation pattern and prevalence of respiratory viruses is crucial for designing strategies to combat infections and outbreaks. The utilization of NPIs to combat COVID-19 has resulted in behavioral and habitual changes among individuals, consequently impacting the transmission of other respiratory viruses.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Pudong Centre for Disease Control and Prevention Ethics Review Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from The acute respiratory surveillance in Pudong New Area, Shanghai, which began in 2010. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

LP: Writing – original draft, Conceptualization, Funding acquisition, Data curation, Software. QC: Writing – original draft, Data curation and Software. LH: Conceptualization and Funding acquisition. YY: Investigation. YH: Investigation. QL: Investigation. WZ: Investigation. BZ: Investigation. XZ: Data curation and Software. 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

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Gut microbiota and metabolites in patients with COVID-19 are altered by the type of SARS-CoV-2 variant

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Introduction: Patients with COVID-19 have dysbiosis of the intestinal microbiota with altered metabolites in the stool. However, it remains unclear whether the differences among SARS-CoV-2 variants lead to differences in intestinal microbiota and metabolites. Thus, we compared the microbiome and metabolome changes for each SARS-CoV-2 variant in patients with COVID-19.

Materials and methods: We conducted a multicenter observational study of patients with COVID-19 and performed fecal microbiome, metabolome, and calprotectin analyses and compared the results among the different SARS-CoV-2 variants.

Results: Twenty-one patients with COVID-19 were enrolled and stratified according to the SARS-CoV-2 strain: six with the Alpha, 10 with the Delta, and five with the Omicron variant. Fecal microbiome analysis showed that α-diversity was reduced in the order of the Omicron, Delta, and Alpha variants ($p = 0.07$). Linear discriminant analysis revealed differences in the abundance of short-chain fatty acid-producing gut microbiota for each SARS-CoV-2 variant. Fecal metabolome analysis showed that the Omicron and Delta variants had markedly reduced propionic and lactic acid levels compared to the Alpha strain ($p < 0.05$).

Conclusion: The intestinal microbiota of patients with COVID-19 varies depending on the SARS-CoV-2 variant. Dysbiosis of the intestinal microbiota due to differences in SARS-CoV-2 variants causes a decrease in intestinal short-chain fatty acids.

KEYWORDS

fecal calprotectin, metabolome, microbiome, short-chain fatty acids, Omicron

1 Introduction

Coronavirus disease 2019 (COVID-19) is an infectious disease that was first reported in December 2019 and has since become a global pandemic. Patients with COVID-19 are known to have a high frequency of gastrointestinal symptoms such as abdominal pain,

diarrhea, nausea and vomiting, and anorexia (Gu et al., 2020). COVID-19 patients with gastrointestinal symptoms have a more severe phenotype and are associated with poor prognosis (Hayashi et al., 2021; Bishehsari et al., 2022). One mechanism by which severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infects human cells is via the angiotensin-converting enzyme 2 (ACE2)-mediated pathway (Penninger et al., 2021). ACE2 is highly expressed in intestinal epithelial cells, such as those in the small intestine and colon (Penninger et al., 2021), and previous studies have shown that SARS-CoV-2 is able to infect human cells of the gastrointestinal tract (Carvalho et al., 2020; Cheung et al., 2021; Jiao et al., 2021). ACE2 regulates human gut microbiota by regulating antimicrobial peptides in the small intestine (Hashimoto et al., 2012). Patients with COVID-19 are known to have altered gut microbiota depending on the severity of the illness, with dysbiosis being more significant in more severe cases (Chakraborty et al., 2022). Our institution has reported markedly decreased gene expression of ACE2 in the small intestine of patients with severe COVID-19, followed by impaired tryptophan metabolism (Yokoyama et al., 2022). Furthermore, studies analyzing the intestinal metabolites in patients with COVID-19 have shown a decrease in short-chain fatty acids such as butyric acid (Zhang et al., 2022). However, the differences between each SARS-CoV-2 variant in the gut microbiome and the metabolome of patients with COVID-19 remain unclear.

In this study, we compared the microbiome and metabolome changes for each SARS-CoV-2 variant in patients with COVID-19. We found that the intestinal microbiota was altered by SARS-CoV-2 variants, defined as Alpha, Delta, and Omicron strains and that the number of short-chain fatty acid-producing bacteria was particularly reduced. Furthermore, metabolomic analysis revealed that a decrease in short-chain fatty acids occurred with a decrease in the intestinal microbiota.

2 Materials and methods

2.1 Patients and sample collection

We included adult patients admitted at Sapporo Medical University and at Teine-Keijinkai hospital who showed positive results for SARS-CoV-2 infection by real-time PCR or antigen testing of nasopharynx or sputum samples. A positive real-time PCR result was defined as a cycle threshold value of 40 or lower, and a positive antigen test result was defined as a quantitative value of 100 pg./mL or higher. We divided the study subjects into critical and non-critical groups according to a previous report (Yokoyama et al., 2022). Patient backgrounds and hematological findings were obtained from their medical records.

Serum samples were leftover blood from routine blood tests and stored at −80°C. Stool samples were collected from patients and stored at 4°C for microbiome analysis and − 80°C for metabolomic analysis.

Abbreviations: ACE2, angiotensin-converting enzyme 2; COVID-19, coronavirus disease 2019; FCP, fecal calprotectin; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; PCoA, principal coordinate analysis; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

2.2 Fecal microbiome analysis

Genomic DNA from fecal samples was extracted using NucleoSpin® DNA Stool (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions and quantified using a Qubit 4 Fluorometer (Promega, Madison, WI, United States). 16S rRNA gene sequencing PCR and data processing were performed as previously described (Ariyoshi et al., 2021). Taxonomic classification for the microbiome was calculated by comparing all amplicon sequence variants with SILVA 138.1 using the q2-feature-classifier (Bokulich et al., 2018). α-diversity and β-diversity were calculated using QIIME2 (Hall and Beiko, 2018). Principal coordinate analysis (PCoA) was applied to distance matrices to create a two-dimensional plot. We used the linear discriminant analysis (LDA) effect size (LEfSe) approach to identify bacterial taxa that differed significantly between the critical and non-critical groups (Segata et al., 2011). Taxa groups with >3 log 10 LDA scores were considered significantly enriched at a p-value of <0.05.

2.3 Fecal metabolome analysis

Approximately 30–50 mg of feces were mixed with 500 μL of Milli-Q water containing internal standards (H3304-1002, HMT, Tsuruoka, Yamagata, Japan). The mixture was centrifuged at 2,300 × g and 4°C for 5 minutes, after which 80 μL of the supernatant was centrifugally filtered through a Millipore 5-kDa cutoff filter (ULTRAFREE MC PLHCC, HMT) at 9,100 × g and 4°C for 120 min to remove macromolecules. Subsequently, the filtrate was evaporated to dryness under vacuum and reconstituted in 20 μL of Milli-Q water for metabolome analysis. Metabolome analysis was conducted according to the HMT Basic Scan package using capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS) based on previously described methods (Ohashi et al., 2008; Ooga et al., 2011). Briefly, CE-TOF-MS analysis was performed using an Agilent CE capillary electrophoresis system equipped with an Agilent 6,210 TOF mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA, United States). The systems were controlled by Agilent G2201AA ChemStation software version B.03.01 (Agilent Technologies) and connected by a fused silica capillary (50 μm i.d. × 80 cm total length) with commercial electrophoresis buffer (H3301-1001 and I3302-1023 for cation and anion analyses, respectively, HMT) as the electrolyte. The spectrometer was scanned from m/z 50 to 1,000, and peaks were extracted using MasterHands automatic integration software (Keio University, Tsuroka, Yamagata, Japan) to obtain peak information, including m/z, peak area, and migration time (Sugimoto et al., 2010). Signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites were excluded, and the remaining peaks were annotated according to the HMT metabolite database based on their m/z values and migration times. The areas of the annotated peaks were normalized to the internal standards and sample amounts to obtain the relative levels of each metabolite. Hierarchical cluster analysis and principal component analysis (Yamamoto et al., 2014) were performed by HMT proprietary MATLAB and R programs, respectively.

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2.4 Fecal calprotectin analysis

Fecal calprotectin (FCP) levels were calculated using a sandwich enzyme immunoassay kit (Calprotectin ELISA Kit, Calprotectin Mochida®). In healthy control, the 95% confidence interval for FCP ranges from 0 to 94 μg/g (mean value = 43.0 μg/g, SD = ±26.0 μg/g) (Nancey et al., 2013). We defined a result as 30 μg/g if the measured FCP value was less than 30 μg/g, which is below the sensitivity limit of the assay.

2.5 Statistics

We analyzed normally distributed continuous variables using an unpaired Student's *t*-test, and non-normally distributed data using the Wilcoxon rank-sum test. For comparison of values among COVID-19 variants, one-way ANOVA with Tukey's test was applied. A Kruskal-Wallis test was applied for α-diversity and LEfSe and *p*-values for β-Diversity were calculated by using permutational analysis of variance.

We defined two-sided *p*-values less than 0.05 as indicate statistical significance. All analyses were performed using JMP, version 15 (SAS Institute, Cary, NC, United States).

2.6 Study approval

This multicenter, prospective, observational study was undertaken at Sapporo Medical University, Sapporo, Japan, and was approved by the ethical committee of Sapporo Medical University (IRB number: 332-111). This study is part of a comprehensive clinical trial registered with the University Hospital Medical Information Network Clinical Trial Registry (UMIN-CTR: 000046106). We obtained written informed consent for all the patients participating in the study.

To ensure the accuracy and completeness of the data and the fidelity of the protocol, all authors contributed to the collection and analysis of the data. This study was conducted in accordance with the principles of the Declaration of Helsinki.

3 Results

3.1 Study patients

We obtained informed consent from 53 patients with COVID-19 between July 2021 and January 2023. Of these, 21 were positive for COVID-19 variants: six for Alpha, 10 for Delta, and five for Omicron variants. The median age was 49.0 years (Alpha/Delta/Omicron: 44.5/50.0/45.0), body mass index was 26.7 (28.8/25.6/27.9) kg/m^2, and disease severity was classified as mild/moderate/severe/critical and 12/2/2/5 patients. The period between the SARS-CoV-2 test positive and collection of fecal samples was 4 days (2–12), for each variant: Alpha 5 (3–9), Delta 4.5 (2–12) and Omicron 4 (3–10), respectively. Gastrointestinal symptoms included diarrhea in four patients (19.0%), anorexia in four patients (19.0%), abdominal pain in three patients (14.3%), and nausea in three patients (14.3%). Patient characteristics are shown in Table 1.

TABLE 1 Clinical characteristics of the patients with COVID-19 included in the study.

	All (*n* = 21)	Alpha (*n* = 6)	Delta (*n* = 10)	Omicron (*n* = 5)
General characteristics				
Age, y	49.0 ± 15.4	44.5 ± 15.6	50.0 ± 14.8	45.0 ± 18.5
Gender, Male	14 (66.7)	5 (83.3)	6 (60.0)	3 (60.0)
Body mass index, kg/m^2	26.7 ± 5.4	28.8 ± 4.6	25.6 ± 5.9	27.9 ± 5.1
Current smoking	5 (23.8)	2 (33.3)	2 (20.0)	1 (20.0)
Covid-19 disease severity category				
Mild	12 (57.1)	3 (50.0)	6 (60.0)	3 (60.0)
Moderate	2 (9.5)	1 (16.7)	1 (10.0)	0 (0)
Severe	3 (14.3)	0 (0)	2 (20.0)	1 (20.0)
Critical	4 (19.0)	2 (33.3)	1 (10.0)	1 (20.0)
Vaccination	6 (28.6)	1 (16.7)	3 (30.0)	2 (40.0)
Period between SARS-CoV-2 test positive and sample collection, days	4 (2–12)	5 (3–9)	4.5 (2–12)	4 (3–10)
Comorbidity				
Hypertension	8 (38.1)	3 (50.0)	4 (40.0)	1 (20.0)
Diabetes mellitus	4 (19.0)	1 (16.7)	2 (20.0)	1 (20.0)
Hyperlipidemia	3 (14.3)	3 (50.0)	0 (0)	0 (0)
COPD	2 (9.5)	1 (16.7)	0 (0)	1 (20.0)
Symptoms at hospitalization				
High fever >37.5°C	10 (47.6)	2 (33.3)	6 (60.0)	2 (40.0)
General fatigue	10 (47.6)	3 (50.0)	6 (60.0)	1 (20.0)
Cough	13 (61.9)	4 (66.7)	7 (70.0)	2 (40.0)
Diarrhea	4 (19.0)	2 (33.3)	2 (20.0)	1 (20.0)
Appetite loss	4 (19.0)	0 (0)	3 (30.0)	1 (20.0)
Abdominal pain	3 (14.3)	1 (7.7)	1 (10.0)	1 (20.0)
Nausea	3 (14.3)	2 (15.4)	1 (10.0)	0 (0)
Therapeutics after admission				
Steroids	14 (66.7)	5 (83.3)	7 (70.0)	2 (40.0)
Antiviral antibodies	12 (57.1)	3 (50.0)	6 (60.0)	3 (60.0)
Anti-inflammatory drugs	14 (66.7)	5 (83.3)	8 (80.0)	1 (20.0)
Anti-SARS-CoV-2 antibody cocktail	7 (33.3)	3 (23.1)	3 (30.0)	1 (20.0)

Data are expressed as the mean ± SD or number (percentage). Percentages may not add up to 100% due to rounding or overlap. COPD, chronic obstructive pulmonary disease.

3.2 Fecal microbiome analysis

We analyzed the fecal microbiome of patients with COVID-19 and classified them according to SARS-CoV-2 variants (Figure 1).

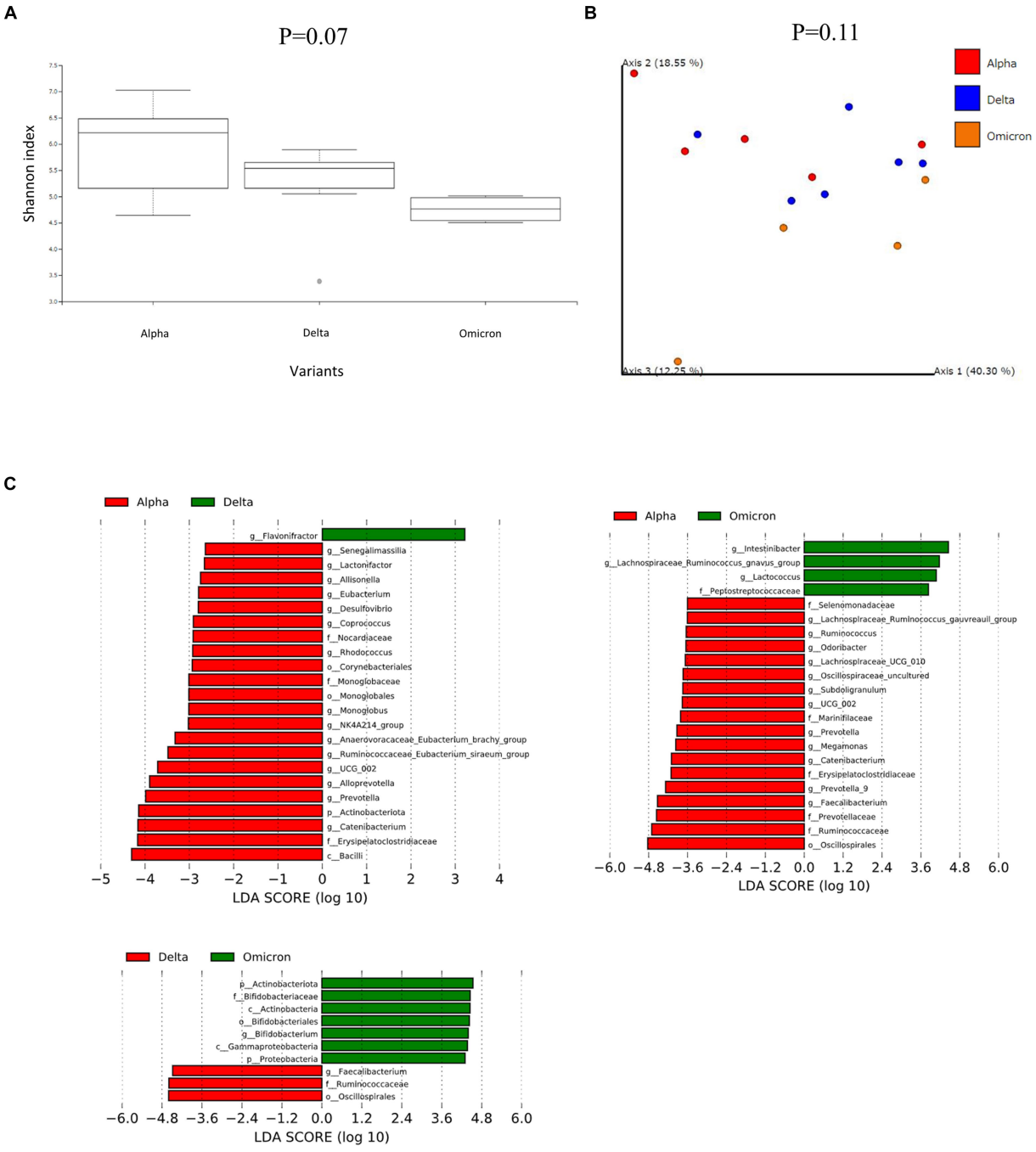

FIGURE 1
Fecal microbiome analysis comparing each infected variants in patients with COVID-19. **(A)** The α-diversity represented by the Shannon index. The vertical axis represents Shannon's index. Box-and-whisker plots show the median, 25th percentile, and 75th percentile, with whiskers extending to the minimum and maximum values. Data were analyzed using the Kruskal-Wallis test. Statistical significance was set at $p < 0.05$. **(B)** β-diversity evaluated by the unweighted UniFrac distances applied for principal coordinates analysis (PCoA). Statistical analysis was performed using permutational analysis of variance (PERMANOVA). Statistical significance was set at $p < 0.05$. **(C)** Enriched gut microbiota constituents were identified using the linear discriminant analysis (LDA) effect size (LEfSe). The histogram of the LDA scores with (log 10) values >3 and $p < 0.05$ revealed the most differentially abundant taxa among the different reproductive stages.

α-diversity, as represented by the Shannon index, decreased in the order of the microbiomes of patients infected with Alpha, Delta, and Omicron variants ($p = 0.07$, Figure 1A). β-diversity evaluated by the unweighted UniFrac distances applied for PCoA was analyzed for each SARS-CoV-2 variant, no significant differences were obtained despite a slight divergence between Omicron and other strains ($p = 0.11$, Figure 1B). Next, we used LEfSe analysis to identify bacterial taxa that were significantly different in abundance between each SARS-CoV-2 variant (Figure 1C). First, a comparison of the Alpha and Delta strains showed that short-chain fatty acid-producing bacteria, such as *Catenibacterium*, *Ruminococcus*, and *Eubacterium,* were more abundant in the microbiome of patients infected with the Alpha strain

than with the Delta strain. *Oscillospirales*, *Faecalibacterium*, *Catenibacterium*, and *Subdoligranulum*, which also produce short-chain fatty acids, mainly butyric acid, were more abundant in the microbiome of patients infected with the Alpha strain than Omicron. Finally, comparing the Delta and Omicron strains, *Oscillospirales*, *Ruminococcus*, and *Faecalibacterium* were abundant in the Delta strain, while *Bifidobacterium* was abundant in the Omicron strain, indicating that both strains produce short-chain fatty acids but differ in the composition. These data suggest that the gut microbiota in patients with COVID-19 varied depending on the SARS-CoV-2 variants and that the changes were mainly due to the different compositions of the bacteria producing short-chain fatty acids.

We then performed further analysis of the microbiome data for each SARS-CoV-2 variant, classifying them into critical and non-critical cases (Figure 2). Interestingly, in the non-critical group, the microbiome of patients with COVID-19 infected with omicron strains showed a significant reduction in α-diversity compared to Alpha strains (Figure 2A). However, no characteristic differences in β-diversity were found when classified as critical or non-critical (Figure 2B). LEfSe analysis of the non-critical group showed a pattern similar to that in Figure 1C (Figure 2C). These results indicate that patients with COVID-19 infected with the Omicron strain, at least in non-critical cases, have a clearly altered microbiota compared to patients infected with the Alpha strain.

3.3 Fecal metabolome analysis

Based on the results of the fecal microbiome analysis, we focused on short-chain fatty acids in the stool metabolome analysis. For comparison with COVID-19 patients, we used data from a group of healthy controls not affected by COVID-19 ($n = 5$). Compared to data from healthy controls, lactic acid and propionic acid were lower in COVID-19 patients, with only the Omicron strain showing statistically significant differences ($p < 0.05$; Figure 3). In a comparison among the SARS-CoV-2 strains, lactic acid and propionic acid were markedly decreased in the stool metabolites of patients infected with the Delta and Omicron strains compared to the Alpha strain ($p < 0.05$). Butyric acid was decreased in the stool of patients infected with the Delta and Omicron strains compared with the Alpha strain; however, the difference was not significant. Succinic and valeric acid levels did not vary among the strains. We further analyzed the metabolomic data for each SARS-CoV-2 variant and classified them into critical and non-critical cases (Figure 4). Lactic acid and propionic acid levels were decreased in the stool of patients with COVID-19 infected with the Delta and Omicron strains compared to those infected with the Alpha strain. These data provide direct evidence that SARS-CoV-2 variants contribute to the production of short-chain fatty acids in stool by influencing the gut microbiome.

3.4 Fecal calprotectin analysis

We measured the FCP levels in the stool of patients with COVID-19 and compared them for each SARS-CoV-2 variant (Figure 5A). Mean FCP levels in patients infected with the Alpha, Delta, and Omicron variants were 198.6 μg/g (SD ±231.2 μg/g), 103.7 μg/g (SD ±79.2 μg/g), and 272.3 μg/g (SD ±194.4 μg/g), respectively. There were no significant differences in FCP levels among these variants. We analyzed FCP levels in patients with and without gastrointestinal symptoms such as diarrhea or abdominal pain (Figure 5B). The FCP level was not affected by the presence or absence of abdominal symptoms. We further analyzed FCP levels according to the SARS-CoV-2 variant and severity of COVID-19, classifying them as critical or non-critical (Figure 5C). The data showed that the FCP levels were higher in critical cases than in non-critical cases, regardless of the SARS-CoV-2 variant. Interestingly, while FCP levels were not elevated in the non-critical group of the Alpha strain, markedly elevated FCP levels were observed in the non-critical group of the Delta and Omicron strains.

4 Discussion

This study showed that the gut microbiota of patients with COVID-19 differed according to the SARS-CoV-2 variant. α-diversity was decreased in the order of Omicron, Delta, and Alpha strains, with a significant difference between the Omicron and Alpha strains, especially in the non-critical group. We also confirmed a decrease in short-chain fatty acids in the stool owing to a decrease in short-chain fatty acid-producing bacteria. In addition, FCP was found to be more elevated in the fecal samples of patients with COVID-19 infected with the Omicron strain than in those infected with the Alpha and Delta strains in a comparison of the non-critical groups.

To our knowledge, this is the first comparative analysis of gut microbiota and metabolites among patients infected with three different SARS-CoV-2 variants. COVID-19 causes gastrointestinal symptoms during the early stages of the disease (Wiersinga et al., 2020). The mechanism of gastrointestinal symptoms in COVID-19 is speculated to involve the abundant expression of ACE2, the entry portal of SARS-CoV-2 into the gastrointestinal tract (Cheung et al., 2021; Jiao et al., 2021). Studies on intestinal bacteria in patients with COVID-19 showed that intestinal bacteria, including *Faecalibacterium prausnitzii*, *Eubacterium rectale*, and several species of bifidobacteria, decreased with disease severity (Yeoh et al., 2021). Metabolomic analysis of patients with COVID-19 showed decreased short-chain fatty acid and L-isoleucine biosynthesis in the gut microbiota, which was correlated with disease severity (Zhang et al., 2022). Furthermore, our laboratory reported a marked decrease in indole 3-propionate, a tryptophan metabolite, in patients with severe COVID-19 (Yokoyama et al., 2022). However, the effects of SARS-CoV-2 variants on intestinal microbiota and intestinal metabolites have not been elucidated (Zhang et al., 2023).

First, we analyzed the gut microbiota by classifying SARS-CoV-2 variants into Alpha, Delta, and Omicron strains and found that α-diversity decreased in patients infected with the Omicron strain. LEfSe analysis, which reflects the abundance of microbiota, showed that *Catenibacterium*, *Ruminococcus*, and *Eubacterium* were more abundant in the Alpha strain than in the Delta strain, and *Oscillospirales*, *Faecalibacterium*, *Catenibacterium*, and *Subdoligranulum* were more abundant in the Alpha strains than in Omicron strains. All these bacteria are known to produce short-chain fatty acids such as propionic acid and butyric acid (Ohira et al., 2017; Fernández-Veledo and Vendrell, 2019; Van Hul et al., 2020; Yang et al., 2021; Hazan et al., 2022; Xie et al., 2022; Huang et al., 2023). One hypothesis as to why the composition of the gut microbiota was

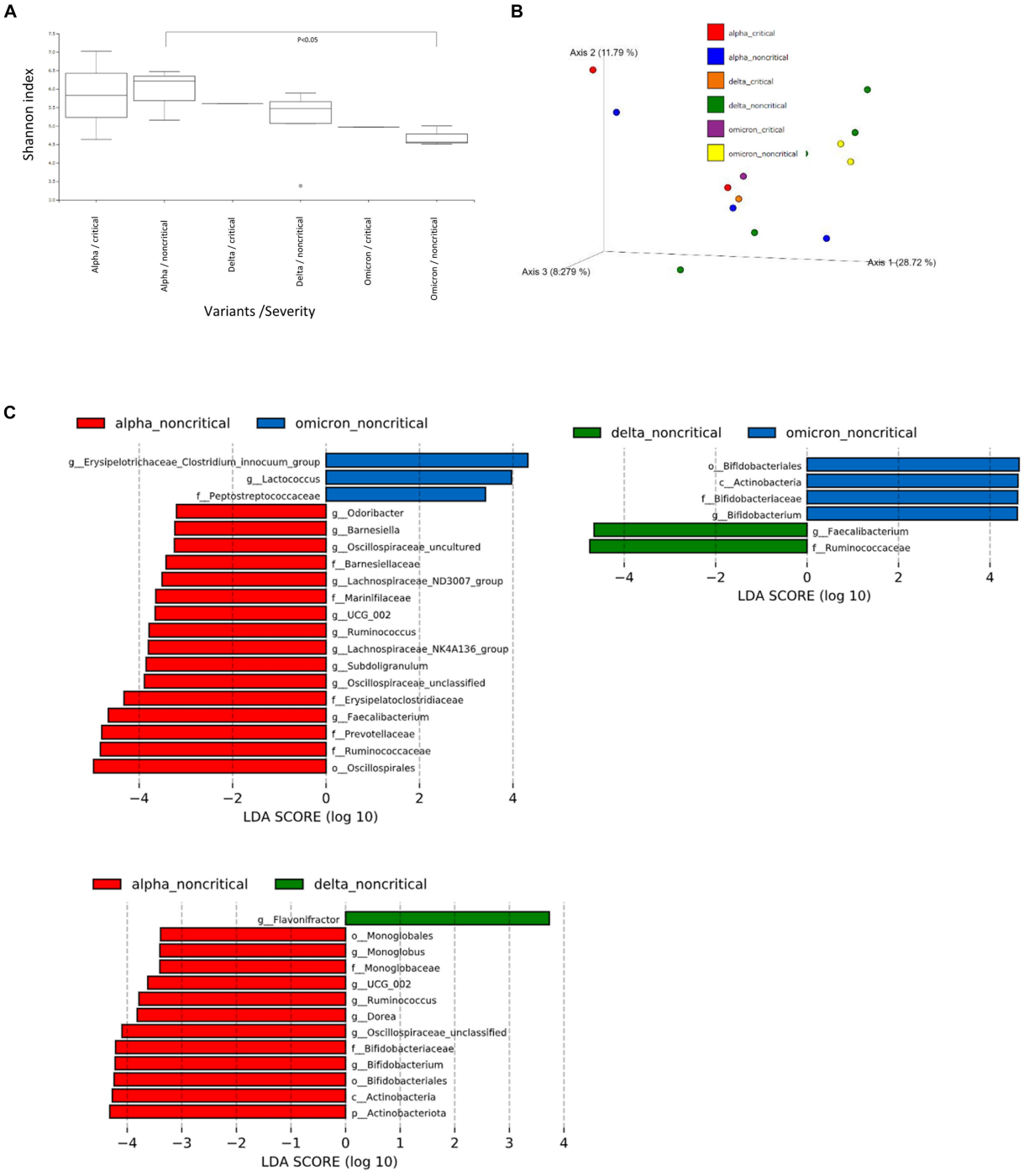

FIGURE 2
Fecal microbiome analysis of COVID-19 patients compared by infected variants and critical/noncritical group. **(A)** The α-diversity represented by the Shannon index classified by SARS-CoV-2 variant and severity. The vertical axis represents Shannon's index. Box-and-whisker plots show the median, 25th percentile, and 75th percentile, with whiskers extending to the minimum and maximum values. Data were analyzed using the Kruskal-Wallis test. Statistical significance was set at $p < 0.05$. **(B)** β-diversity evaluated by the unweighted UniFrac distances applied for principal coordinates analysis (PCoA) classified by SARS-CoV-2 variant and severity. **(C)** The enriched gut microbiota constituents were identified by linear discriminant analysis (LDA) effect size (LEfSe) and classified by the SARS-CoV-2 variant and severity. The histogram of the LDA scores with (log 10) values >3 and $p < 0.05$ revealed the most differentially abundant taxa among the different reproductive stages.

different for each SARS-CoV-2 variant may explain the response of SARS-CoV-2 to the gut epithelium and ACE2. Using intestinal organoids, Jang et al. reported that the infectivity of SARS-CoV-2 for the intestinal epithelium was strongest in the Omicron strain and that the infectivity correlated with ACE2 expression levels (Jang et al., 2022). Furthermore, *in vivo* experiments showed that the Omicron

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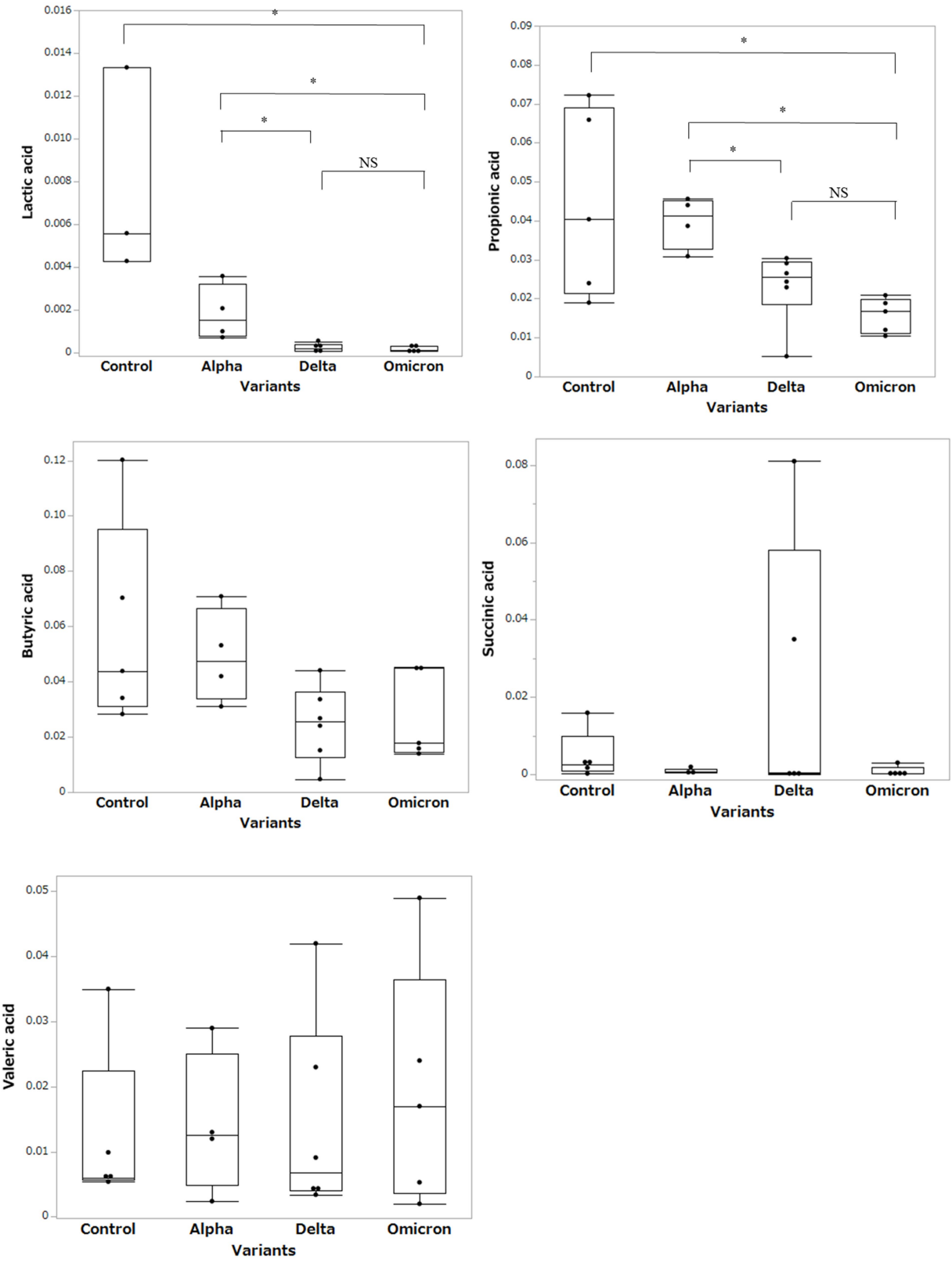

FIGURE 3
Comparison of short-chain fatty acids in stool metabolites classified by the SARS-CoV-2 variants. Statistical analysis was performed using a one-way ANOVA with Tukey's test. Statistical significance is indicated by asterisks at $P < 0.05$.

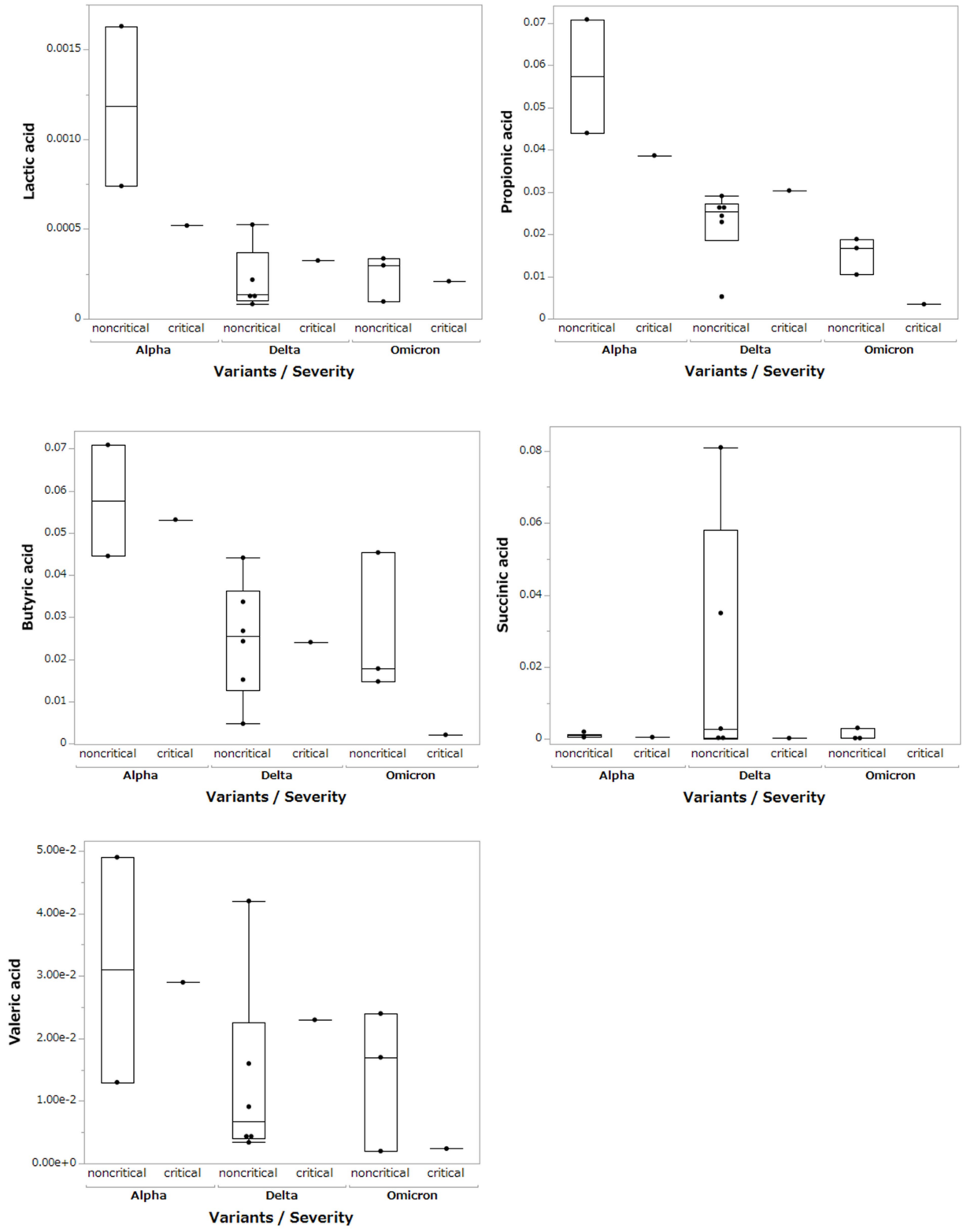

FIGURE 4
Comparison of short-chain fatty acids in stool metabolites classified by SARS-CoV-2 variant and severity.

variant is susceptible to binding to the human ACE2 receptor (Lupala et al., 2022). Based on these basic data, we considered that mutant strains of SARS-CoV-2 (Omicron and Delta) bind more strongly to the ACE2 receptor than Alpha strains, thereby reducing ACE2 expression in the intestinal tract and causing dysbiosis (Hashimoto et al., 2012). Experimental studies using animal and *in vivo* models are required to confirm this mechanism. Since short-chain fatty acids play a role in regulating the immune response in the intestinal tract

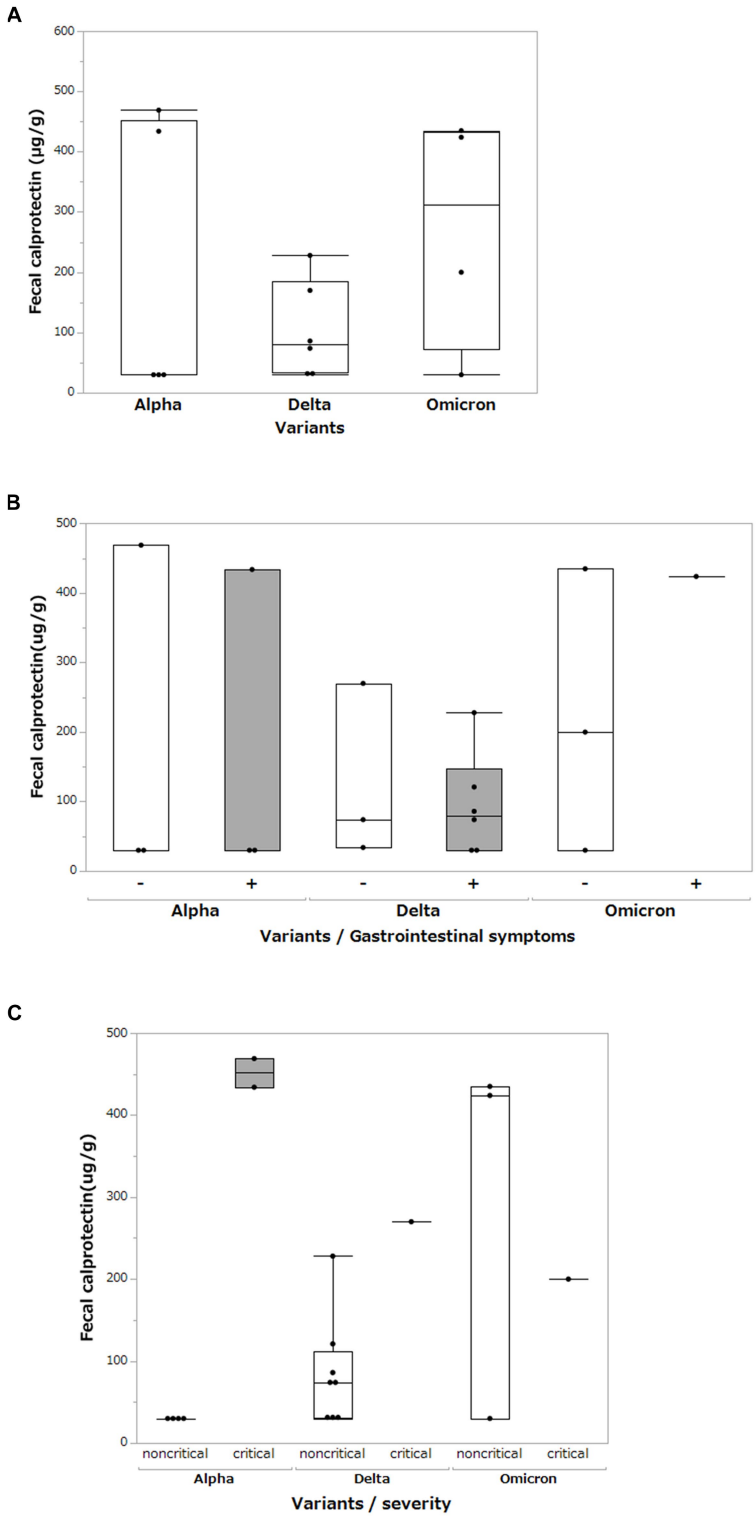

FIGURE 5
Fecal calprotectin levels between different SARS-CoV-2 variants. **(A)** Comparison of fecal calprotectin levels between the Alpha, Delta, and Omicron SARS-CoV-2 variants. Box-and-whisker plots show the median, 25th percentile, and 75th percentile, with whiskers extending to the minimum and maximum values. Each dot represents an individual value. Statistical analyses were performed using the Wilcoxon rank-sum test. Statistical significance was set at $P < 0.05$. If the measured FCP value was under 30 μg/g, which is less than the sensitivity limit of the assay, the result was defined as 30 μg/g. **(B)** Comparison of fecal calprotectin levels among the Alpha, Delta, and Omicron SARS-CoV-2 variants with and without gastrointestinal symptoms (diarrhea and abdominal pain). **(C)** Comparison of fecal calprotectin levels among the Alpha, Delta, and Omicron variants of SARS-CoV-2 and the severity of COVID-19.

(Akhtar et al., 2022), a decrease in short-chain fatty acids could induce intestinal inflammation.

The FCP level correlates with disease severity in patients with COVID-19 (Effenberger et al., 2020; Ojetti et al., 2020), and our previous data showed a marked increase in FCP in critical cases (Yokoyama et al., 2022). The present study also examined non-critical cases and found that FCP levels were higher in patients infected with the Omicron strain than in those infected with the Alpha strain. Furthermore, the α-diversity of the microbiome was lower in patients infected with the Omicron strain than in patients infected with the Alpha strain. These facts suggest that the Omicron strains may cause dysbiosis and inflammation of the gastrointestinal tract, regardless of disease severity. Further studies are needed to determine whether differences in SARS-CoV-2 variants, particularly in the reduction of short-chain fatty acids in the Delta and Omicron strains, induce intestinal inflammation and their clinical significance.

This study has several limitations. First, this study is the lack of the WHO classification of patients by disease severity. As previously reported, the microbiome and metabolome of patients with COVID-19 correlate with disease severity (Yeoh et al., 2021; Yokoyama et al., 2022). In this study, although we could classify non-critical cases by SARS-CoV-2 variant, our sample size was small, and we could not fully examine critical cases. Thus, statistical tests could not be performed and the interpretation of the results was difficult. Second, because this study was a multicenter study in Japan, only Japanese patients were enrolled in the study, so comparisons between races were not performed. Third, vaccination affects the severity and the intestinal microbiota in patients with COVID-19, but we could not examine in detail the timing of vaccination or the type of vaccine in this study. Finally, we could not compare the healthy control and COVID-19 groups in our microbiome analysis because there were no adequate fecal samples from healthy individuals during the COVID-19 pandemic.

In conclusion, we found that the type of SARS-CoV-2 variant causes changes in intestinal inflammation and bacterial diversity, as well as a decrease in fatty acids in the intestinal tract. SARS-CoV-2 continues to mutate; however, the mechanism by which it affects the gastrointestinal tract has not been elucidated. Further studies on the disruption of the intestinal immune system by SARS-CoV-2 and changes in the intestinal microbiota will play an important role in treating new viral infections that may occur.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJDB13936/.

Ethics statement

The studies involving humans were approved by the Ethical Committee of Sapporo Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YY: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. TI: Data curation, Formal analysis, Writing – review & editing. TY: Data curation, Writing – review & editing. YT: Supervision, Writing – review & editing. KK: Supervision, Writing – review & editing. ST: Supervision, Writing – review & editing. EN: Supervision, Writing – review & editing. AK: Supervision, Writing – review & editing. HN: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

HN reports receiving personal fees from Abbvie Inc., Kissei Pharmaceutical Co., Ltd., KYORIN Pharmaceutical Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Janssen Pharmaceutical K.K., Takeda Pharmaceutical Co., Ltd., Pfizer Japan Inc., Celgene K.K., EA Pharma Co., Ltd., Zeria Pharmaceutical Co., Ltd., Mochida Pharmaceutical Co., Ltd., Nippon Kayaku Co., Ltd., and Daiichi Sankyo Co., Ltd., JIMRO Co., Ltd., and grants for commissioned/joint research from Hoya Group Pentax Medical, Boehringer Ingelheim GmbH, Bristol-Myers Squibb Company. ST reports personal fees from MSD K.K., grants from Shino-Test Corporation, Roche Diagnostics K.K., Fuji Rebio Inc., Abbott Japan Co., Ltd. Author Contributions.

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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Resurgence of respiratory syncytial virus with dominance of RSV-B during the 2022–2023 season

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Background: Respiratory syncytial virus (RSV) is a common cause of upper and lower respiratory tract infections. This study aimed to explore the prevalence of respiratory syncytial virus (RSV) and other respiratory viruses in Bulgaria, characterize the genetic diversity of RSV strains, and perform amino acid sequence analyses of RSV surface and internal proteins.

Methods: Clinical and epidemiological data and nasopharyngeal swabs were prospectively collected from patients with acute respiratory infections between October 2020 and May 2023. Real-time PCR for 13 respiratory viruses, whole-genome sequencing, phylogenetic, and amino acid analyses were performed.

Results: This study included three epidemic seasons (2020–2021, 2021–2022, and 2022–2023) from week 40 of the previous year to week 20 of the following year. Of the 3,047 patients examined, 1,813 (59.5%) tested positive for at least one viral respiratory pathogen. RSV was the second most detected virus (10.9%) after SARS-CoV-2 (22%). Coinfections between RSV and other respiratory viruses were detected in 68 cases, including 14 with SARS-CoV-2. After two seasons of low circulation, RSV activity increased significantly during the 2022–2023 season. The detection rates of RSV were 3.2, 6.6, and 13.7% in the first, second, and third seasons, respectively. RSV was the most common virus found in children under 5 years old with bronchiolitis (40%) and pneumonia (24.5%). RSV-B drove the 2022–2023 epidemic. Phylogenetic analysis indicated that the sequenced RSV-B strains belonged to the GB5.0.5a and GB5.0.6a genotypes. Amino acid substitutions in the surface and internal proteins, including the F protein antigenic sites were identified compared to the BA prototype strain.

Conclusion: This study revealed a strong resurgence of RSV in the autumn of 2022 after the lifting of anti-COVID-19 measures, the leading role of RSV as a causative agent of serious respiratory illnesses in early childhood, and relatively low genetic diversity in circulating RSV strains.

KEYWORDS

respiratory syncytial virus, molecular epidemiology, genetic variability, genotype, evolution, whole-genome sequencing

1 Introduction

Viral acute respiratory infections (ARIs) are a leading cause of infectious morbidity and mortality and have serious health, economic, and social consequences. Among the known respiratory viruses (more than 200), respiratory syncytial virus (RSV) is one of the most common etiological agents of upper and lower respiratory tract infections (LRTIs), with the greatest disease burden in infants, young children, the elderly, and immunocompromised adults. It is also a leading cause of serious LRTI (bronchiolitis and pneumonia) in children aged < 5 years. According to epidemiological data from 2019, 33 million episodes of acute LRTI, 3.6 million hospital admissions, and 101,400 death cases in children aged 0–60 months have been attributed to RSV (Li et al., 2022). RSV belongs to the family *Pneumoviridae* and the genus *Orthopneumovirus* (Lefkowitz et al., 2018). The genome of this virus is a non-segmented, single-stranded, negative-sense RNA approximately 15.2 kb in length. It encodes 11 proteins, namely NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2, and L, each with specific functions (Collins and Karron, 2013). The surface glycoproteins G and F play crucial roles in the initial stage of viral infection. The G protein is accountable for attaching the virus to host cells. On the other hand, the F protein makes it possible for the viral ribonucleoprotein to enter the cell's cytoplasm as a result of the fusion of the viral envelope with the host cell membrane (McLellan et al., 2013). During the fusion process, RSV F undergoes dramatic pre-fusion-to-post-fusion conformational changes. The active pre-fusion conformation includes six major antigenic sites (Ø and I–V) and produces more potent neutralizing antibodies. Only four sites (I, II, III, and IV) are present in the inactive post-fusion conformation (Chang et al., 2022). The F protein is the major viral antigen and is relatively conserved, whereas the G protein contains two hypervariable regions (HVRs) in the superficial ectodomain and exhibits greater genetic diversity. The highly variable C-terminal region of the G gene (HVR2) is commonly used in molecular, epidemiological, and evolutionary studies (Gimferrer et al., 2019; Sáez-López et al., 2019; Kim et al., 2023). The G and F proteins are heavily glycosylated with N-linked and O-linked sugars, which limit antibody access to the antigenic epitopes, thereby facilitating RSV evasion from pre-existing immunity (Holmes, 2013).

RSV has a single serotype divided into two major subgroups, RSV-A and RSV-B, each comprising multiple genotypes (Mufson et al., 1985). Based on the genetic variability of the G gene, RSV-A is divided into 14 genotypes (GA1–GA7, SAA1, NA1–NA4, CB-A, and ON1), whereas RSV-B comprises 37 genotypes (GB1–GB6, GB12, GB13, SAB1–SAB4, URU1, URU2, CB1, THB, BA1–BA14, BA-C, BA-CCA, BA-CCB, JAB1, NZB1, and NZB2; Gaymard et al., 2018; Muñoz-Escalante et al., 2021). Goya et al. (2020) proposed a new classification in which the number of genotypes was reduced to three for RSV-A (GA1–GA3) and seven for RSV-B (GB1–GB7), with two additional levels of classification defined as sub-genotypes and lineages (Goya et al., 2020). Variations in the temporal and geographical distributions of individual RSV genotypes and the periodic replacement of predominant genotypes were observed (Esposito et al., 2015; Sáez-López et al., 2019; Kamau et al., 2020; Lee et al., 2021; Tabatabai et al., 2022; Kim et al., 2023). Viruses of different genotypes can co-circulate during a single epidemic within the same community. Genotyping of circulating RSV is essential for monitoring the emergence and clinical burden of new genotypes and for developing therapeutic and preventive strategies. Furthermore, analyzing the amino acid composition of RSV surface glycoproteins enables tracing of the evolutionary dynamics of the pathogen and understanding the molecular mechanisms underlying its genetic and antigenic variability.

During the COVID-19 pandemic, various public health and social distancing measures (e.g., mandatory mask-wearing, working from home, school closures, limited social gatherings, travel restrictions, quarantine, and patient isolation) were implemented to prevent the spread of SARS-CoV-2; these measures resulted in greatly reduced transmission of RSV and other seasonal respiratory viruses (Agca et al., 2021; Groves et al., 2021). After two seasons of low circulation, in the winter of 2022–2023, RSV activity returned to levels similar to those in the seasons preceding the pandemic (Jia et al., 2022; Guo et al., 2023; Pierangeli et al., 2023). In Bulgaria, RSV has been detected using real-time RT-PCR since 2010 within the national influenza surveillance system. This study aimed to explore the circulation patterns of RSV and other respiratory viruses during the COVID-19 pandemic, describe the epidemiological and clinical characteristics of RSV infection and the genetic diversity of RSV strains in the first RSV post-pandemic season, and perform an amino acid sequence analysis of the RSV surface and internal proteins.

2 Methods

2.1 Patients and specimen collection

The present study was conducted from October 2020 to May 2023, covering three epidemic seasons (2020–2021, 2021–2022, and 2022–2023) from week 40 of the previous year to week 20 of the following year. A total of 3,047 patients from different regions of the country who were ambulatory-treated or hospitalized for ARIs were enrolled in the National Influenza Surveillance Program. ARIs were defined according to the European Center for Disease Prevention and Control case definition.[1] The study population consisted of patients of all age ranges: 728 in the first season, 482 in the second, 1,597 in the third season, and 240 inter seasons. In the first two seasons, the number of patients examined was significantly smaller due to the disruption of the national surveillance system caused by the COVID-19 pandemic. Nasopharyngeal and oropharyngeal swab specimens were prospectively collected from enrolled patients and inserted into a

Abbreviations: ARIs, Acute respiratory infections; AdV, Adenovirus; BoV, Bocavirus; CTD, Carboxy-terminal domain; Cap, Capping domain; CD, Connector domain; FP, Fusion peptide; HR, Heptad repeats; hMPV, Human metapneumovirus; HVRs, Hypervariable regions; IRR, International Reagent Resource; L, Large protein; LRTIs, Lower respiratory tract infections; M, Matrix protein; MT, Methyltransferase domain; N, Nucleoprotein; PIV, Parainfluenza virus; p, Phosphoprotein; RSV, Respiratory syncytial virus; RV, Rhinovirus; SH, Small hydrophobic surface protein; SP, Signal peptide; TM/CT, Transmembrane/carboxy terminus.

1 https://ecdc.europa.eu/en/infectious-diseases-public-health/surveillance-and-disease-data/eu-case-definitions

container containing 2 mL of virus transport medium. Specimens were obtained during the visit to the doctor or within the first 24 h of admission, within 7 days of the onset of respiratory symptoms. After collection, specimens were stored at 2°C–8°C for up to 24 h at the health facilities and then sent in ice packs to the National Laboratory "Influenza and ARI" for the detection of viral respiratory pathogens. Specimens were processed immediately, and aliquots of the primary samples were stored at −80°C.

2.2 Molecular detection of respiratory viruses

Viral nucleic acids were extracted automatically from 400 µL of each respiratory specimen with an elution volume of 100 µL using a commercial ExiPrep Dx Viral DNA/RNA kit and ExiPrep 16DX instrument (Bioneer, Daejeon, Republic of Korea), according to the manufacturer's instructions. The SARS-CoV-2 and influenza A/B viruses were simultaneously screened using the FluSC2 Multiplex Real-Time RT-PCR Kit provided by the International Reagent Resource (IRR; United States) (CDC, 2022). Real-time RT-PCR was performed to subtype influenza A viruses and determine the influenza B genetic lineage using the SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), with specific primers and probes provided by IRR (USA). Amplification was performed using a CFX96 thermal cycler (Bio-Rad Laboratories, Inc., Singapore), according to the protocol recommended by CDC-Atlanta (United States; Shu et al., 2011).

The presence of eight common non-influenza respiratory viruses, namely RSV, human metapneumovirus (hMPV), parainfluenza virus (PIV) types 1/2/3, rhinovirus (RV), adenovirus (AdV), and bocavirus (BoV), was screened using multiplex real-time PCR assays with primers and probes, as previously described (Kodani et al., 2022). Three PCR mixtures were developed, including the SuperScript III Platinum One-Step qRT-PCR kit and combinations of primers and TaqMan probes labeled with different fluorescent dyes: Mixture 1: AdV + RSV + PIV1; Mixture 2: BoV + RV + PIV2; and Mixture 3: hMPV + PIV3. Positive and negative controls were included in each experiment. The RNAase-P gene was used as an internal positive control during specimen extraction. For influenza type A and B viruses, positive controls were provided by IRR (United States), while for other viruses, AmpliRun DNA/RNA Amplification Controls (Vircell, Spain) were used. Samples with a cycle threshold (Ct) value <38 were considered positive. To distinguish RSV-A and RSV-B, two sets of oligonucleotides targeting the RSV F and N genes were used for multiplex real-time RT-PCR, as described previously (Zlateva et al., 2007). The primer and probe sequences and thermocycling conditions are listed in Supplementary Table 1.

2.3 RSV whole-genome sequencing

Fifty-two randomly selected RSV-B-positive samples with a Ct value <30 were subjected to whole-genome sequencing. The Respiratory Virus Panel Illumina RNA Prep with Enrichment (L) Tag (Illumina, San Diego, CA, United States) and the Illumina MiSeq system with the reagent kit v.3150 cycles (Illumina, San Diego, CA, United States) were used to perform whole-genome sequencing of 40 common respiratory viruses.[2] Before sequencing, the quality of the DNA pool libraries was verified by QIAxcel Advanced capillary electrophoresis (QIAGEN, Switzerland). Normalization of libraries was performed with a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States) and Invitrogen™ Quant-iT™ 1X High Sensitivity (HS) Broad Range (BR) dsDNA Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, United States). For genome analysis, the Explify RPIP Data Analysis software (v2.0.0), available on the BaseSpace platform (Illumina, Cambridge, United Kingdom), was used. Complete or almost complete RSV genomes were obtained from 47 RSV-B-positive samples with coverage greater than 99%, mainly from samples with higher viral loads. The RSV-B sequences reported in this study have been deposited in the EpiRSV database of the GISAID under the accession numbers listed in Supplementary Table 2.

2.4 Phylogenetic analysis

For phylogenetic analysis, the RSV-B sequences obtained in this study were aligned with published sequences representing known genotypes and sequences of recently circulating RSV strains from different geographical regions retrieved from GenBank and the EpiRSV of GISAID. Geneious Prime® 2020.1.2. software[3] was used for alignment and phylogenetic tree construction using the following algorithms: Tamura–Nei genetic distance model, maximum likelihood build method, and 1,000 replicates bootstrap. The final tree was visualized and annotated using iTOL software.[4] Pairwise nucleotide distances (*p*-distances) were calculated using Geneious Prime software to compare the differences within and between genotypes. The phylogenetic tree consisted of 47 nucleotide sequences from this study, 46 reference sequences, and other RSV-B sequences available in GenBank and GISAID databases.

2.5 Deduced amino acid sequence analysis and glycosylation prediction

Translation of the nucleotide code into the amino acid code was performed using BioEdit software (version 7.2). To identify amino acid substitutions in G, F, and other proteins, the studied sequences were aligned and compared with the sequence of the NH118 strain (accession number MF185752), one of the first BA strains with a completely published genome.

NetNGlyc 1.0 web server[5] was used to predict putative N-glycosylation sites with a threshold value of 0.5. To identify potential O-glycosylation sites, we used the NetOGlyc 4.0 web server, which can be accessed at https://services.healthtech.dtu.dk/service.php?NetOGlyc-4.0. N-linked glycosylation occurs when the amino acid sequence has N–X–S/T (sequon), where X is any amino acid except proline. O-glycosylation occurs when the amino acid sequence has serine and threonine configuration.

2 https://support-docs.illumina.com/LP/Illumina_RNA_Prep_Checklist/Content/LP/Illumina_RNA/RNA-Prep/Checklist.htm

3 https://www.geneious.com/

4 https://itol.embl.de/

5 https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0

2.6 Statistics

GraphPad Prism version 6.0 was used for statistical analyses. Categorical data were presented as frequencies and percentages and compared using Chi-square (χ^2) and Fisher's exact tests. Continuous data were expressed as either means or medians, depending on the specific context. A p-value of less than 0.05 was considered statistically significant.

3 Results

3.1 Patient characteristics

Of the 3,047 patients examined, 71.2% (2170) were admitted to the hospital as inpatients, while 28.8% (877) were treated as outpatients. The patient's age range was from 3 days to 91 years, with a median age of 5. Out of all study participants, 505 (16.6%) were <11 months old, 260 (8.5%) were 11–23 months old, 265 (8.7%) were 24–35 months old, 378 (12.4%) were 36–59 months old, 883 (12.4%) were 5–14 years old, 136 (4.5%) were 15–29 years old, 98 (3.2%) were 30–64 years old, 455 (14.9%) were ≥ 65 years old, and 67 (2.2%) were of unknown age. Among the study subjects whose sex was known, there were 1,587 males and 1,428 females, resulting in a male-to-female ratio of 1.11.

3.2 Virus detection

Viral respiratory pathogens were identified in 1,813 (59.5%) of the 3,047 patients examined. At least one viral respiratory agent was detected in 433 (49.4%) outpatients and 1,380 (63.5%) inpatients ($p < 0.05$). Single infections were proven in 1,651 (54.2%) patients, 155 (5.1%) patients were co-infected with two viruses, and seven (0.2%) were co-infected with three viruses. A total of 669 (22%) patients were positive for SARS-CoV-2, and 469 (15.4%) were infected with influenza viruses: A(H1N1)pdm09 (223, 7.3%), A(H3N2; 155, 5.1%), and B/Victoria lineages (91, 3%). Non-influenza respiratory viruses, including RSV (331, 10.9%), hMPV (59, 1.9%), PIV-1 (8, 0.3%), PIV-2 (6, 0.2%), PIV-3 (43, 1.4%), RV (199, 6.5%), AdV (106, 3.5%), and BoV (107, 3.5%), were detected in 859 (28.2%) patients (Table 1). RSV was the most prevalent seasonal non-influenza respiratory virus, followed by RV ($p < 0.05$). PIV and hMPV were identified as having the lowest frequency. The detection rates of RSV were 3.2%, 6.6%, and 13.7% during the first, second, and third seasons, respectively. The incidence rates of RSV infection among outpatients and inpatients were 10.8 and 10.9%, respectively. Approximately 20.5% (68/331) of the RSV-positive patients were co-infected with other respiratory viruses. The most common co-infecting virus was RV (23 cases, 6.9%), followed by SARS-CoV-2 (14 cases, 4.2%), BoV (13 cases, 3.9%), AdV (9 cases, 2.7%), and influenza A(H1N1)pdm09 (8 cases, 2.4%). Single cases of mixed RSV infections were found with hMPV, PIV-1, PIV-3, influenza A(H3N2), and B/Victoria lineage. Among the study participants with RSV co-infections, 55 (80.9%) were inpatients.

During the 2022–2023 season, 217 (99%) RSVs identified were subgrouped as RSV-B, and only two (1%) were RSV-A. The RSV epidemic started in October 2022 and peaked in December 2022, a month earlier than the usual peak observed during the pre-pandemic

TABLE 1 Detected respiratory viruses among outpatients and inpatients.

	Total tested	Total positive, *n* (%)	Influenza viruses, *n* (%)			SARS-CoV-2, *n* (%)	Seasonal non-influenza respiratory viruses, *n* (%)							
			A(H1N1)pdm09	A(H3N2)	B/Vic		RSV	hMPV	PIV-1	PIV-2	PIV-3	RV	AdV	BoV
Outpatients	877	433 (49.4)	79 (9.0)	65 (7.4)	33 (3.8)	45 (5.1)	95 (10.8)	21 (2.4)	1 (0.1)	1 (0.1)	6 (0.7)	69 (7.9)	28 (3.2)	26 (3)
Inpatients	2,170	1,380 (63.6)	144 (6.6)	90 (4.1)	58 (2.7)	624 (28.8)	236 (10.9)	38 (1.8)	7 (0.3)	5 (0.2)	37 (1.7)	130 (6)	78 (3.6)	81 (3.7)
Total	3,047	1,813 (59.5)	223 (7.3)	155 (5.1)	91 (3)	669 (22)	331 (10.9)	59 (1.9)	8 (0.3)	6 (0.2)	43 (1.4)	199 (6.5)	106 (3.5)	107 (3.5)

seasons (Korsun et al., 2021). RSV was mainly identified in specimens collected between November 2022 and January 2023. Throughout the study period, the highest detection rate for RSV occurred in July 2021 (75.8%, 25 cases), followed by December 2022 (33.9%, 103 cases), when the incidence rates of SARS-CoV-2 were low (3% and 1.3%, respectively; Figure 1).

3.3 Age and gender distribution

Viral respiratory infections were observed in all age groups. The proportion of positive cases in the age groups < 11 months, 11–23 months, 24–35 months 36–59 months, 5–14 years, 15–29 years, 30–64 years, and ≥ 65 years was 49.5% (250/505), 58.5% (152/260), 61.1% (162/265), 57.9% (219/378), 48% (424/883), 49.3% (67/136), 60.2% (59/98), and 96.7% (440/455), respectively. Co-infections were detected more often in patients aged 0–4 years (7.6%, 107/1,408). The highest incidence rate for SARS-CoV-2 was found in patients aged ≥ 65 years (95.6%), and for influenza viruses, the highest rate occurred in 5–14-year-olds (27.2%). Seasonal non-influenza viruses were most prevalent in the 24–35-month age group, accounting for 52.8%. Patients infected with RSV ranged in age from 1 month to 67 years, with a mean age of 4.3 ± 11.3 years and a median age of 2 years. The incidence of RSV infection was highest among the youngest age group (0–11 months, 17.7%; Figure 2) and decreased with increasing age to 1.3% in the oldest age group (≥ 65 years). A relatively high rate (6%)

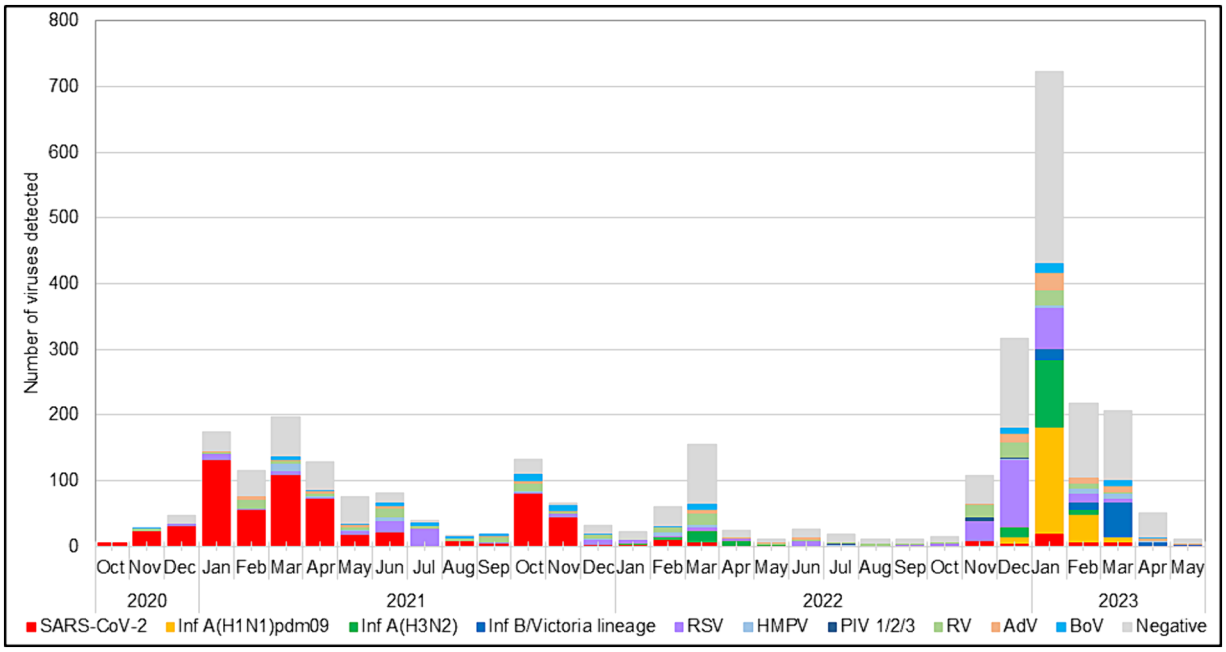

FIGURE 1
Monthly distribution of respiratory viruses detected among patients with ARI.

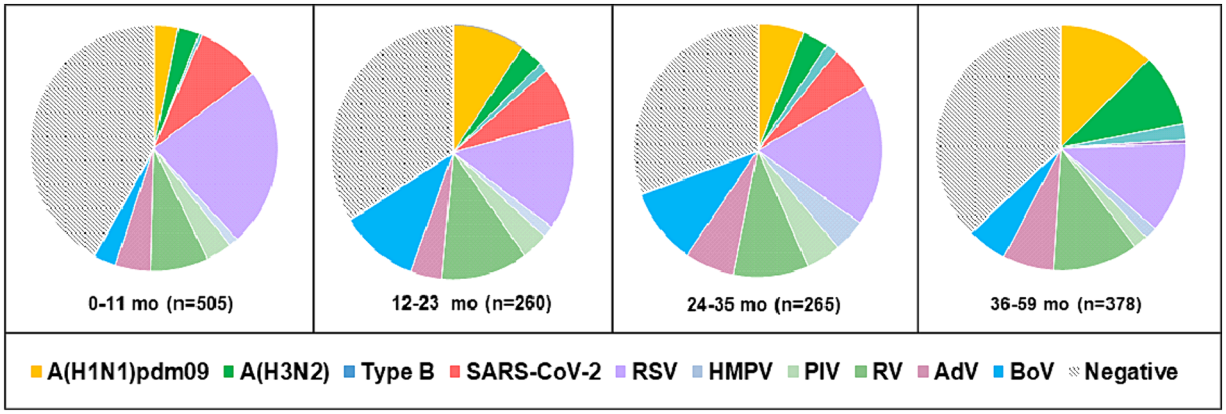

FIGURE 2
Age distribution of patients aged 0–59 months with detected respiratory viruses. Positive cases represent the sum of single infections and co-infections for each virus.

of RSV infection was observed in older children and adolescents (5–14 years). Children aged 0–4 years accounted for 75.5% (250/331) of the patients with confirmed RSV infection and 84.3% (198/236) of those hospitalized because of RSV infection; the highest rate of RSV co-infection (75%, 51/68) was also found in this age group (Table 2). Among the RSV-positive patients of known sex, the male-to-female ratio was 1.22 (180 males and 147 females; $p = 0.3791$), and among the RSV-positive hospitalized patients, the ratio was 1.24 (129 males and 104 females).

3.4 Clinical characteristics

In addition to upper respiratory tract symptoms, respiratory viruses can cause complications in the lower respiratory tract, heart, and central nervous system. We explored the participation of SARS-CoV-2, influenza viruses, and seasonal non-influenza respiratory viruses in the development of the most common complications—tracheobronchitis, bronchiolitis, pneumonia, and central nervous system involvement (febrile seizures, cerebral edema, viral meningitis, and encephalopathy). Data from the 2022–2023 season were analyzed, as they contained more complete clinical information. The proportions of influenza viruses detected in patients with tracheobronchitis, bronchiolitis, pneumonia, and neurological complications were 21.6% (8/37), 7.6% (13/170), 22.2% (53/239), and 24% (6/25), respectively; regarding seasonal non-influenza viruses, the proportions were 35.1% (13/37), 53.5% (91/170), 31% (74/239), and 12% (3/25), respectively. In SARS-CoV-2-infected patients, the proportions were 2.7%, 0.6%, 1.3%, and 4%, respectively (Figure 3). RSV was the most common virus identified in patients with bronchiolitis, accounting for 40% (68/170) of cases, and was the second most common cause of pneumonia after influenza viruses, responsible for 19.7% (47/239) of cases in the entire study population ($p < 0.05$). A total of 142 children aged <5 years were diagnosed with pneumonia, of whom 36 (25.4%) and 28 (19.7%) had confirmed RSV and influenza infections, respectively. Among the patients with lower respiratory tract complications due to RSV, 80% (72/90) were children under 2 years of age.

3.5 Phylogenetic analysis of RSV

A phylogenetic tree based on G gene nucleotide sequences of RSV-B strains was created, including RSV-B strains detected in this study, recently circulating viruses in other countries, and

TABLE 2 Age distribution of patients with confirmed RSV mono-infections and co-infections.

Age group (years)	RSV positive (n = 331)		Mono-infections	Dual infections	Triple infections
	n	%			
0–4 years (n = 1,408)	249	17.7	199	49	2
5–14 years (n = 883)	53	6.0	45	8	-
15–29 years (n = 136)	6	4.4	5	1	-
30–64 years (n = 98)	3	3.1	3	0	-
≥ 65 years (n = 455)	6	1.3	1	5	-
Without data (67)	13	19.4	10	3	-
Total (n = 3,047)	331	10.9	263	66	2

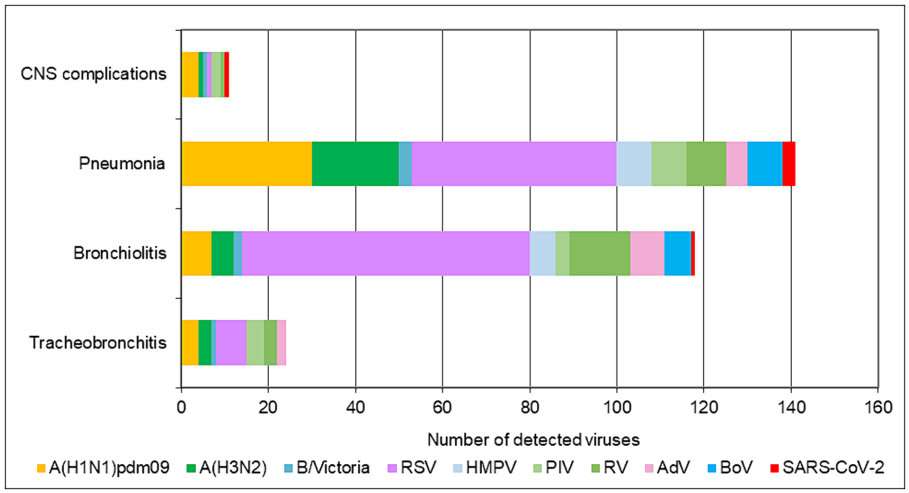

FIGURE 3
Number of respiratory viruses detected in patients with tracheobronchitis, bronchiolitis, pneumonia, and CNS complications during the 2022–2023 season.

representative strains of all known genotypes from GenBank. The phylogenetic analysis illustrated that the 34 sequenced Bulgarian RSV-B strains clustered together with the consensus sequence of GB5.0.5a, and the remaining 13 strains were classified into a new lineage, GB5.0.6a, within the GB5 genotype (Figure 4). The Bulgarian sequences were phylogenetically close to the sequences of RSV circulating during the same period in other European countries (the United Kingdom, Germany, Austria, Spain, Italy, and Macedonia) and the United States.

At the nucleotide level, the average patristic distance between the study GB5.0.5a strains and another GB5.0.5a strain (KY249660) was 0.0282 (±0.0021). In the remaining 13 sequences, the patristic distance with the KY249660 strain was 0.0328 (SD 0.0032), indicating that these sequences represent a new lineage of RSV-B, assigned as GB5.0.6a (Goya et al., 2020).

Next-generation sequencing identified 17 strains in which, in addition to the RSV sequence, high-quality sequence of another respiratory virus was found: SARS-CoV-2 (8 strains), influenza A(H1N1)pdm09 (3), A(H3N2) (2), B/Victoria lineage (1), and AdV (1). In strain RSV/B/Bulgaria/1305/2023, two additional sequences (SARS-CoV-2 and BoV) were identified, and in strain RSV/B/Bulgaria/2559/2022, three sequences (SARS-CoV-2, influenza virus, and BoV) were identified.

3.6 Amino acid polymorphisms in G and F surface proteins

Amino acid polymorphisms of the G and F surface glycoproteins reflect the evolutionary dynamics of RSV. Therefore, the deduced G

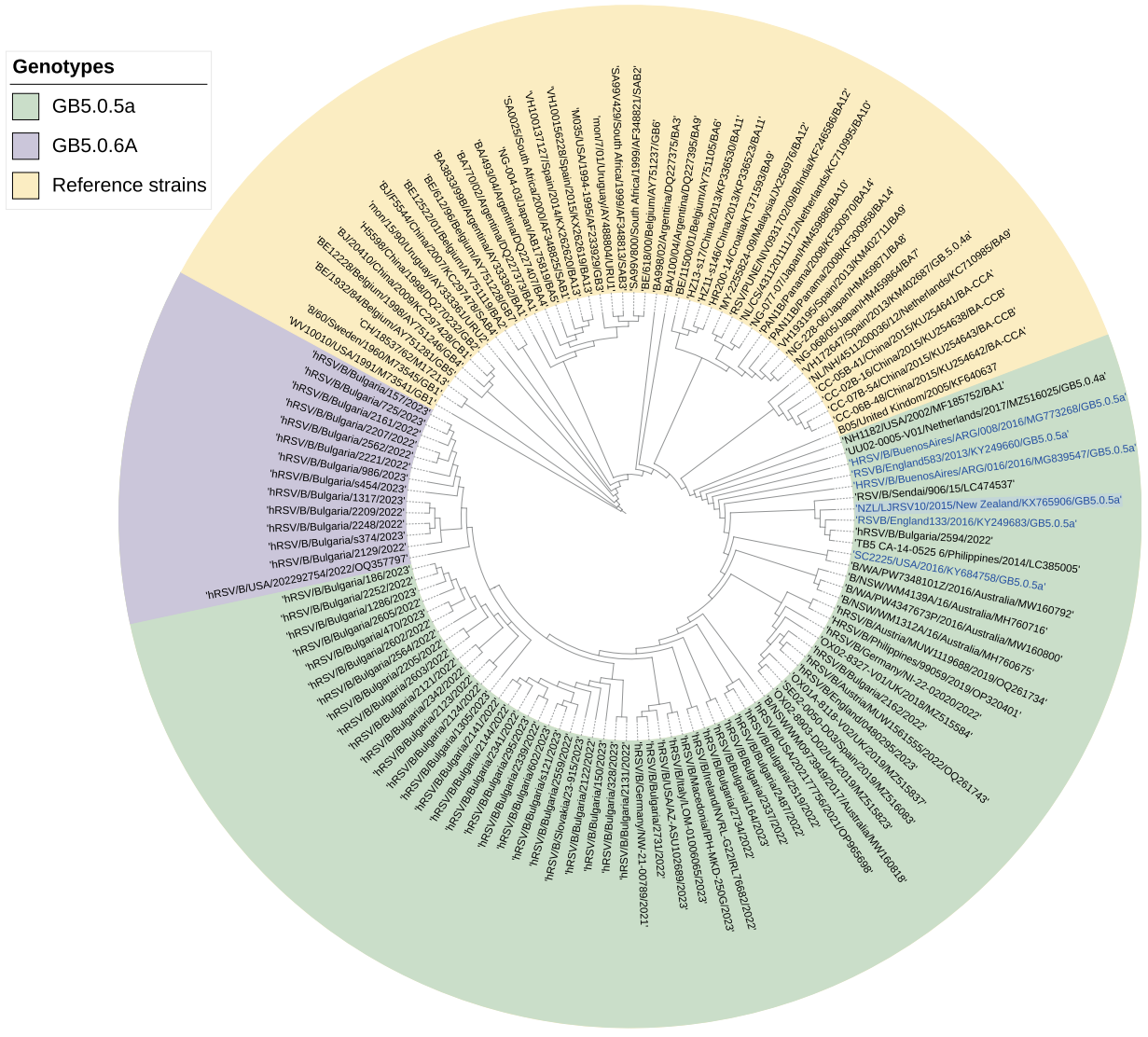

FIGURE 4
Phylogenetic analysis based on G gene nucleotide sequences of RSV-B strains. A phylogenetic tree was constructed using the maximum-likelihood method with 1,000 bootstrap iterations running within Geneious Prime software. Reference sequences were labeled with GenBank names and accession numbers, followed by country, year of isolation, and genotype. Reference RSV-B strains are indicated in yellow background. GB5.0.5a and GB5.0.6a strains detected during 2022–2023 are indicated in green and purple background, respectively. The names of the reference GB5.0.5a strains are highlighted in blue.

and F protein amino acid sequences of the 47 Bulgarian RSV-B strains were aligned and compared with the sequence of the NH1182 strain (accession number MF185752), which is one of the first BA strains to have a complete published genome. Reference strain hRSV/B/Australia/VIC-RCH056/2019 (EPI_ISL_1653999) and eight database-derived sequences of RSV-B strains with complete genomes, detected in other countries during 2021–2023, were also included in the analysis. The study identified amino acid substitutions in the G protein at 18 positions. Among these substitutions, HVR1 (aa 67–163) contained eight, while the heparin-binding domain (aa 186–223) contained two, and HVR2 (aa 224–311) contained eight. However, no changes were observed in the cytoplasmic (aa 1–35), transmembrane (aa 36–66), or centrally conserved regions (aa 164–185; Table 3; Jiang et al., 2023).

One of the unique characteristics of the BA genotypes is the presence of a 60-nucleotide duplication, leading to a duplication of 20 amino acids (TERDTSTSQSTVLDTTTSKH; aa 240–259 and 260–279) in HVR2. Consequently, protein G is extended to 312 amino acids (the stop codon in BA viruses is set to 313 amino acids). Five of the substitutions in HVR2 were observed in the Bulgarian strains detected in the 2017–2018 season (Korsun et al., 2021), and three (D253N, K258N, and S277P) were new and not present in the sequence of the reference strain hRSV/B/Australia/VIC-RCH056/2019. The substitutions V271A and S277P were located inside the duplicated region (Figure 5).

Fifteen amino acid substitutions in the G protein were conserved and represented a high frequency exceeding 90%. All identified substitutions were also found in the database-derived RSV sequences, except for the substitutions S100G, P216S, and K258N, which were present in some strains. Six N-linked glycosylation motifs were predicted in the G protein (at positions 81, 86, 230, 253, 258, and 296), three located in HVR2. The amino acid substitutions D253N and K258N led to the acquisition of novel N-linked glycosylation motifs in 28 and 96% of the strains, respectively. In contrast, substitution T312NI resulted in a loss of potential N-linked glycosylation sites in all strains. In HVR2, O-linked glycosylation was predicted in 42 serine and threonine residues with a G score $\geq$ 0.5, and 10 of these sites were found in the duplication region.

The F protein showed variations at 13 amino acid positions: two in F2 (aa 24–109), one in the p27 segment (aa 110–136), and 10 in the F1 subunit (aa 137–524; Lu et al., 2019). No amino acid substitutions were identified in the fusion peptide (aa 27–36; Figure 6). Amino acid variations were examined in the antigenic sites Ø and I–V (Tabor et al., 2020).

No amino acid changes were identified in the major neutralizing epitopes, specifically antigenic sites II, III, and IV, found on both the pre-and post-fusion conformations of the RSV F protein. Eleven amino acid changes were detected at the remaining three antigenic sites. Nine of these had a high level of sequence conservation and were present at a frequency above 96%. Polymorphisms (N201S, I206M, Q209R, and S211N) were observed in the immunodominant Ø epitope, which was unique to the pre-fusion conformation of the F protein (McLellan, 2015). The substitutions L172Q, S173L, K191R, and K191R were located at site V, F45L, and S389P at site I, and

TABLE 3 Amino acid variations identified in G and F proteins of RSV-B strains (n = 47) circulating in Bulgaria during the 2022–2023 season.

G protein (aa 1–315)		F protein (aa 1–574)			
Amino acid changes	Frequency (%)	Antigenic sites	Amino acid positions of antigenic sites	Amino acid changes	Frequency (%)
S100G	77	Ø	62–96; 195–227	N201S	28
S101P	100			I206M	98
T107A	98			Q209R	98
Y112H	96			S211N	96
P120L	28	I	27–45; 312–318; 378–389	F45L	100
R136T	100			S389P	96
T138S	100	II	254–277	-	
A141T	100	III	46–54; 301–311; 345–352; 367–378	-	
I200T	100	IV	422–471	-	
P216S	96	V	55–61; 146–194; 287–300	L172Q	100
S247P	100			S173L	100
D253N + CHO	28			S190N	96
K258N + CHO	96			K191R	98
V271A	98	P27	110–136	M115T	23
S277P	96			A103V	100
I281T	100			H250Y	100
T290I	98				
T312I -CHO	100				

Gain/Loss of glycosylation is represented by + CHO/-CHO.

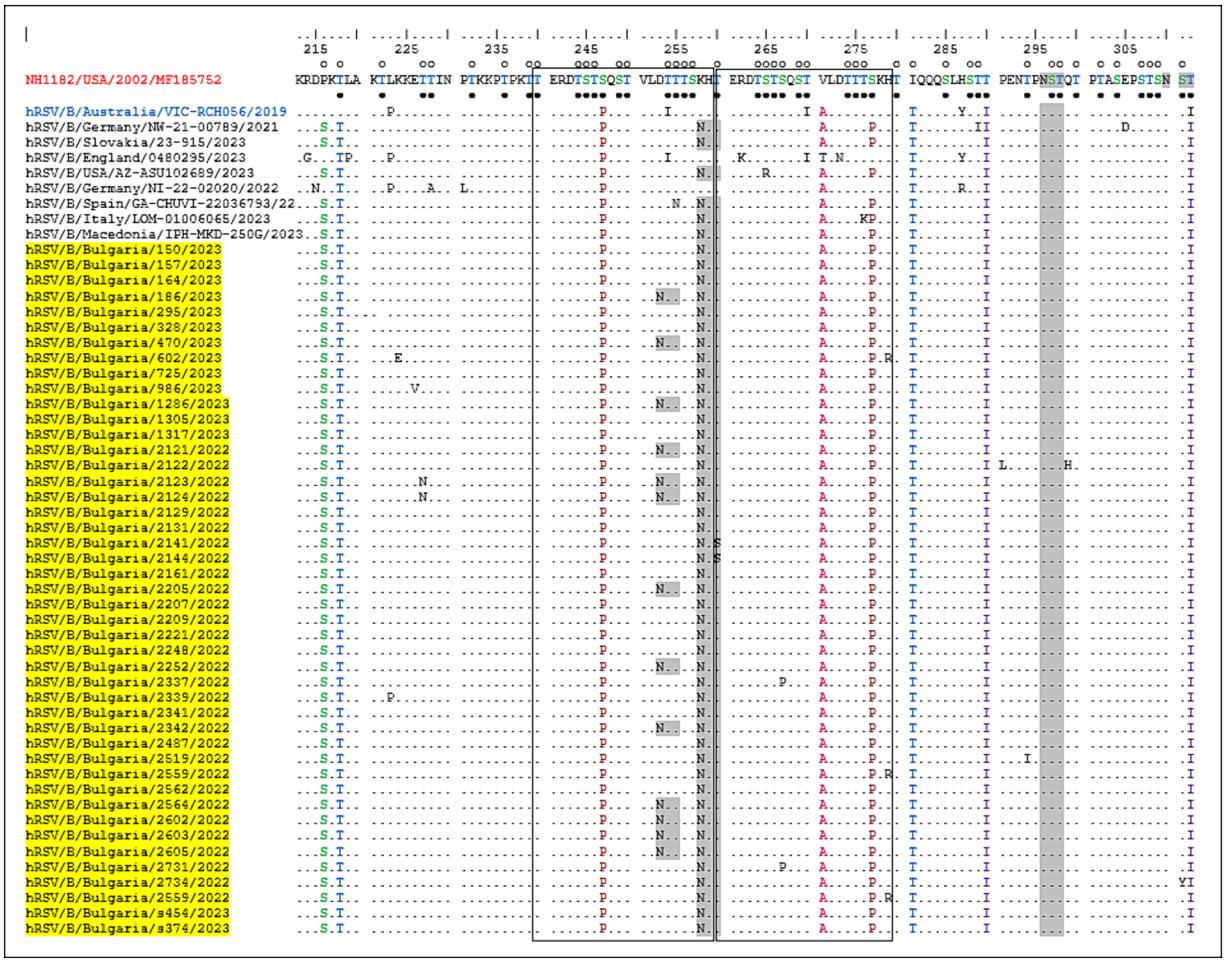

FIGURE 5
Deduced amino acid alignment of the G protein HVR2 in the RSV-B strains. Alignment is shown relative to the sequence of the prototype BA strain NH1182 (GenBank accession number MF185752). The amino acid numbers correspond to G protein positions 213–312 of strain MF185752. Identical residues are indicated by dots. Outlined rectangles represent two copies of the duplicated 20-amino acid region in the RSV-B strains. Light gray shading highlights the predicted N-glycosylation sites. Black circles indicate the predicted O-glycosylation sites of the BA prototype strain MF185752, and unfilled circles indicate the predicted O-glycosylation sites of the Bulgarian strains.

M115T at the p27 segment—an internal peptide released after cleavage of the two subunits F1 and F2. Only two substitutions (A103V and H250Y) were located outside the antigenic regions (Table 3). Except for S190N, S211N, and S389P, all analyzed database-derived RSV sequences contained the remaining substitutions. Six N-linked glycosylation motifs were predicted in the F protein (at aa positions 27, 70, 116, 120, 126, and 500). Three glycosylation sites (N116, N120, and N126) were located within the p27 segment.

3.7 Complete SH, N, P, M, M2-1, M2-2, and L protein analysis

Single amino acid substitutions were identified in other structural proteins: T49I and N64D in the small hydrophobic surface protein (SH; 65 aa in length); H216Y and A372T in the nucleoprotein (N) (391 aa); G229D in the phosphoprotein (P) (241 aa); V181I in the M2-1 (195 aa) and R41H in the M2-2 (90 aa) transcriptional regulators. No amino acid variations were identified in the matrix (M) protein (256 aa). The large (L) protein, consisting of 2,165 amino acids organized into five domains: RdRp domain, capping domain (Cap), connector domain (CD), methyltransferase domain (MT), and carboxy-terminal domain (CTD), harbored 11 amino acid variations at positions 56, 570, 715, 1,479, 1,712, 1,716, 1,723, 1,736, 1,759, 1,787, and 2,108.

4 Discussion

This study investigated the circulation patterns of RSV and other respiratory viruses among patients presenting with ARI symptoms in Bulgaria during the COVID-19 pandemic, with an emphasis on the first season after COVID-19 restrictions were lifted. The genetic characteristics of the RSV responsible for the 2022–2023 outbreak were also explored. Extensive non-pharmaceutical preventive interventions targeting SARS-CoV-2 have had a tremendous impact on viral respiratory infection epidemiology, such as the low prevalence and diminished genetic diversity of circulating viruses and the monthly and age distribution of cases (Garg et al., 2022; Chow et al., 2023). Viral interference, the behavior of individuals, and societal and

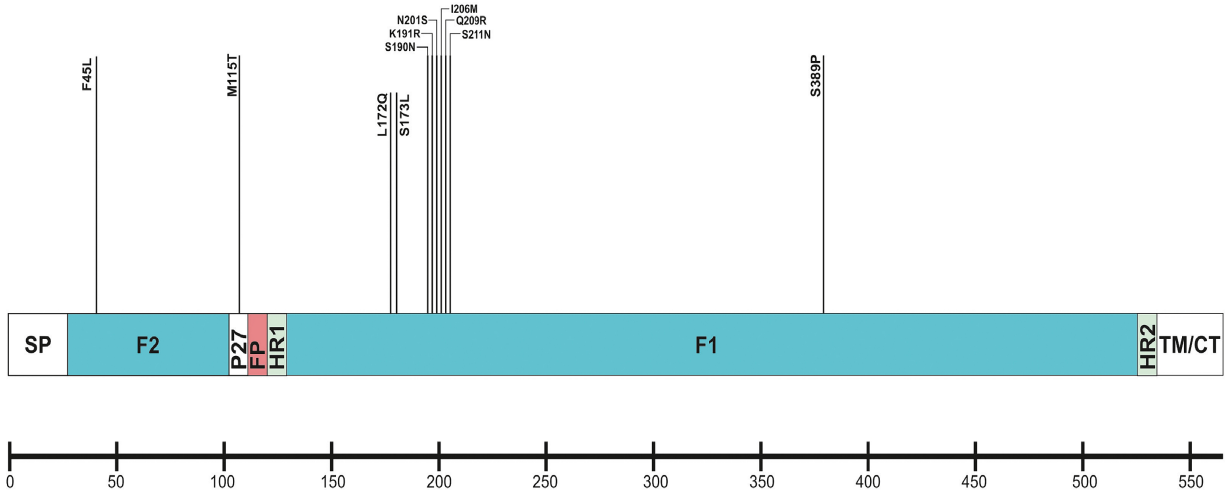

FIGURE 6
Location of amino acid substitutions identified in RSV-B F protein. Structural sites of the F protein sequence are shown: SP (signal peptide), F2 subunit (aa 24–109); p27 peptide; FP (fusion peptide); F1 subunit (aa 137–524); HR1 and HR2 (heptad repeats); and TM/CT (transmembrane/carboxy terminus).

health system factors may have also contributed to the significant reduction in respiratory virus circulation during the pandemic (Abu-Raya et al., 2023). During the first two winters, the activity of RSV and other seasonal respiratory viruses plummeted abruptly worldwide, including in Bulgaria (Hönemann et al., 2023). An unusual summer spike in RSV-associated activity was observed in 2021 during the first relaxation of COVID-19-related public health measures. The low RSV exposure during the winter of 2020–2021 probably resulted in a significant decline in population RSV-specific immunity and an atypical inter-seasonal resurgence of RSV infections (Reicherz et al., 2022; Bardsley et al., 2023). In the fall of 2022, with the complete withdrawal of containment measures, RSV returned to the seasonally high levels of activity observed in the preceding years. The sudden upsurge in RSV cases in the summer of 2021 and autumn of 2022, as well as the earlier start of the 2022–2023 epidemic, has been registered in many countries (Redlberger-Fritz et al., 2021; Goya et al., 2023; Hönemann et al., 2023; Munkstrup et al., 2023). The detection rate of RSV in the 2022–2023 outbreak was 13.7% vs. 3.2% and 6.6% during the previous two seasons, respectively ($p < 0.05$). Among all the studied respiratory viruses, RSV was the most frequently identified etiologic agent of ARIs during the 2022–2023 season and the second most commonly detected respiratory virus after SARS-CoV-2 during the previous two seasons. Temporal and geographic variations exist in the incidence of RSV infection, depending on the study population, detection methods, climate, and other factors (Gimferrer et al., 2019; Valley-Omar et al., 2022; Bimouhen et al., 2023; Kim et al., 2023). During the COVID-19 pandemic, the intensity of the applied anti-epidemic measures has played a crucial role in determining the spread of seasonal respiratory viruses. In this study, RSV was co-detected with other respiratory viruses in 68 (20.5%) patients, including SARS-CoV-2 in 14 patients. A high rate (23.3%, 55/236) of co-infections was found among hospitalized RSV-positive patients. The proportion of RSV co-infections varied between studies (Trento et al., 2010; Holmes, 2013; Gimferrer et al., 2019), and no co-infections with other respiratory viruses were detected in a study conducted in Washington, United Kingdom (Goya et al., 2023). Currently, there is no conclusive evidence linking the presence of co-infections with disease severity (Goka et al., 2014).

The strong dominance of RSV-B during the 2022–2023 epidemic can be explained by the low transmission of RSV in the previous two seasons, which could have caused a genetic bottleneck, resulting in reduced viral diversity (Chow et al., 2023). Significant declines in RSV genetic diversity during the implementation of COVID-19-related restrictions have also been observed in other countries (Eden et al., 2022). Our findings were consistent with reports from other European countries, where the 2022–2023 outbreak was also driven by RSV-B (Redlberger-Fritz et al., 2021; Munkstrup et al., 2023; Pierangeli et al., 2023). During the period preceding the pandemic, year-to-year fluctuations in the incidences of RSV-A and RSV-B were observed in Bulgaria (Korsun et al., 2021). Herd immunity against the RSV subgroup, which was dominant in the country in the preceding year, is likely the reason for the dominance of other subgroups in the following season. A consistent shift in the predominance of RSV-A to RSV-B at different time intervals (1–3 years) has been reported worldwide (Hall, 2001; Gimferrer et al., 2019).

During the study period, RSV affected all age groups across the population, with much more frequent involvement in children aged < 5 years, especially those under 2 years. In line with many reports, the rate of RSV infection was highest in the youngest age group (up to 1 year) and decreased with increasing age due to the development of immunity after repeated infections (Jepsen et al., 2018; Jallow et al., 2023). Administration of the recently approved Abrysvo vaccine during pregnancy could protect infants against LRTI from birth to 6 months of age (EMA; Saso and Kampmann, 2016). According to the literature, the burden of RSV infection is high in older adults who are at risk of severe infection (Savic et al., 2023). In contrast to a previous study (Nguyen-Van-Tam et al., 2022), the study older population aged ≥65 years was minimally affected by RSV infection (4.66% vs. 1.3%, respectively). For this age group, licensed vaccines (Arexvy and Abrysvo) have already been designed to protect against LRTI (such as

bronchitis and pneumonia) caused by RSV [European Medicine Agency (EMA), 2023a,b]. Our previous study showed that RSV infections follow a seasonal pattern, occurring predominantly in winter and early spring, with very few cases occurring during the summer (Korsun et al., 2021). During the 2022–2023 season, RSV was in circulation from November to late March, with peak activity in December 2022, which is consistent with data from other European countries (Jepsen et al., 2018; Gimferrer et al., 2019; Pierangeli et al., 2023). From 2020 to 2021, the typical seasonality of RSV was disrupted because of the applied anti-COVID-19 measures. Information on the monthly distribution of RSV infections is important for the implementation of prophylactic measures, including vaccines and monoclonal antibodies, among high-risk populations and for strengthening infection control to prevent nosocomial infections.

Similar to the pre-pandemic seasons, respiratory viruses were more frequently detected among inpatients (63.5%) than outpatients (49.4%; $p < 0.05$; Korsun et al., 2021). RSV was the major pathogen associated with hospitalization in children, and 59.8% (198/331) of all RSV cases occurred in hospitalized children aged < 5 years. RSV is well known as the leading etiological agent of LRTI in the pediatric population and the predominant cause of bronchiolitis and pneumonia (Hall, 2001). During the 2022–2023 season, RSV was the most common cause of bronchiolitis (40%) and pneumonia (25.4%) in children aged < 5 years. RSV-associated cases of LRTI were observed more frequently in children younger than 2 years of age, indicating a higher susceptibility of this age group to RSV infection. According to a previous report, boys are more likely to develop severe disease, and male infants are twice as likely to be hospitalized than female infants (Hall, 2004). In our study, no statistically significant differences were found in the incidence and hospitalization rates among males and females < 5 years old with confirmed RSV infection.

Whole-genome sequencing and phylogenetic analysis of 47 RSV-B sequences indicated that the GB5.0.5a and GB5.0.6a genotypes (both equivalent to the BA strains) were responsible for the 2022–2023 outbreak. The 2022–2023 RSV-B surge was also driven by GB5.0.5a in the United States (Adams et al., 2023), Austria (Redlberger-Fritz et al., 2021), Italy (Tramuto et al., 2023), and other countries (Jallow et al., 2023). Molecular analysis of RSV G genes revealed the presence of a 60-nucleotide duplication in HVR2, which is a landmark characteristic of BA strains initially described in Buenos Aires, Argentina, in 1999. Following its appearance, the BA genotype rapidly evolved and displayed significant diversification into at least 14 new genotypes (Ábrego et al., 2017; Lee et al., 2021; Kim et al., 2023). Since 2006, RSV-B strains harboring this partial duplication have become globally predominant and have completely replaced all previously circulating RSV-B genotypes (Trento et al., 2010). The worldwide spread of RSV-B strains carrying this 60-nucleotide duplication indicates that this unique insertion may enhance viral attachment to host cells and improve fitness, thereby facilitating transmission (Hotard et al., 2015).

Surface glycoproteins G and F are under selective immune pressure and undergo constant evolution. Therefore, they were subjected to molecular analysis. In Bulgarian RSV-B, amino acid substitutions were identified at 18 G protein positions, of which 16 were located in HVR1/2 and six N-linked glycosylation motifs, including two new ones, compared to the NH1182 strain discovered 20 years ago in the early years after the appearance of the BA genotype. Duplication of 20 amino acids in the RSV-B strain resulted in additional amino acid substitutions in HVR2. Some substitutions identified in the G protein (S247P, V271A, I281T, T290I, and T312I) have been described in other European countries and elsewhere (Kim et al., 2023; Tramuto et al., 2023). The RSV F protein is a vaccine antigen and a target of the monoclonal antibody product palivizumab (Synagis), which is administered as a passive immunoprophylaxis to high-risk infants, as well as several other monoclonal antibodies and small molecules that are under clinical development (Kopera et al., 2023). Key variations in the F protein antigenic sites could affect the antigenicity and susceptibility of RSV to prophylactic and therapeutic agents targeting these regions. In our study, the F protein showed 13 substitutions and six N-linked glycosylation sites, confirming its lower genetic variability than the G protein (Sun et al., 2022). No amino acid variations were found at antigenic sites II (target of palivizumab and motavizumab) or IV (target of 101F and MAb19; Lu et al., 2019). More variation was found in the antigenic sites Ø (target of nirsevimab) and V (target of suptavumab). This variability is likely a result of neutralization escape, given that these regions prompt the production of antibodies with high neutralizing activity (Ruckwardt et al., 2019; Tabor et al., 2020; Adhikari et al., 2022; Sun et al., 2022). The antigenic site Ø is located at the apex of the pre-fusion trimer and forms much of the variability of the F protein (~25%; McLellan et al., 2013). The variations L172Q, S173L, S190N, and K191R were located at site V (aa 148–194; target of the monoclonal antibody AM14), and the substitution F45L was located at site I (aa 27–45; target of human antibody MPE8; Hause et al., 2017). The substitutions A103V, L172Q, S173L, K191R, I206M, and Q209R have also been reported in other recent studies (Lu et al., 2019; Adhikari et al., 2022; Chen et al., 2022; Sun et al., 2022). The N-linked glycosylation sites of the F protein were relatively conserved, whereas those of the G protein were more variable. The acquisition or removal of N-linked glycosylation can affect viral antigenicity and facilitate immune evasion (Feng et al., 2022). The continuous accumulation of amino acid changes and extensive glycosylation of RSV G and F proteins with N-and O-linked sugars allow viruses to escape neutralization by pre-existing antibodies. Consequently, the continued emergence of genetically altered RSV has enabled this pathogen to cause repeated infections in the same individual and annual epidemics. Full-length sequencing of other structural proteins showed a high degree of similarity with the reference strain. In agreement with a previous study, a small number of amino acid changes were identified in the internal proteins (Jallow et al., 2023). The L56I, K570R, I715V, R1759K, and A1787E substitutions were located in the conserved enzymatic regions RdRp, Cap, and MT of L protein, which are potential targets for inhibitor development (Gilman et al., 2019). A recent study in Austria reported the L protein variations K570R, V1479A, and R1759K, which were also observed in Bulgarian strains (Redlberger-Fritz et al., 2021).

It is worth noting that this study had a few limitations. In the first two seasons of the COVID-19 pandemic, the number of clinical samples examined was notably smaller than that in the 2022–2023 season. This is because the national ARI surveillance system was disrupted during this period, as resources and specialists were redirected toward COVID-19 diagnosis, treatment, and contact tracing. A significant portion of the National Laboratory's "Influenza and ARI" work involved testing for SARS-CoV-2. The reporting forms for clinical samples were often incomplete and lacked the necessary

clinical information. This made it impossible to perform an extensive epidemiological and clinical analysis of respiratory infections other than SARS-CoV-2 during the acute phase of the pandemic. In addition, due to the limited detection of RSV-A, we did not sequence viruses in this subgroup and analyze their genetic characteristics. Finally, as the objectives of the study focused on RSV infection, other respiratory infections caused by RVs, AdVs, PIVs, and BoVs were not analyzed in detail. Despite these limitations, our study provides a comprehensive overview of RSV circulation patterns during the COVID-19 pandemic. Policymakers can utilize this information to devise effective strategies for controlling the transmission of RSV and preventing future epidemics. We identified complete genome sequences of one or more respiratory viruses in addition to the RSV sequence in some strains, a unique finding not present in other publications.

This study found a high incidence of viral respiratory infections, particularly RSV, during the first season after anti-COVID-19 restrictions were lifted. The high activity of RSV is likely a result of diminished population immunity and the accumulation of vulnerable individuals, particularly children, due to prolonged low exposure to natural infections. We identified RSV as the primary cause of severe respiratory illnesses in young children. This study emphasizes the need for ongoing local and global surveillance of this pathogen. Phylogenetic and molecular analyses of RSV play crucial roles in identifying new epidemic strains, tracking the evolutionary and epidemiological patterns of viruses, and evaluating the impact of genetic variation on the transmissibility, virulence, and effectiveness of preventative vaccines and medications.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving humans were approved by Institutional Review Board and Ethics Committee of the NCIPD (IRB Number 00006384). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

NK: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Methodology. IT: Data curation, Formal analysis, Investigation, Methodology, Visualization, Validation, Writing – review & editing. IM: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. IA: Methodology, Resources, Writing – review & editing. IU: Data curation, Formal analysis, Writing – review & editing. II: Data curation, Formal analysis, Writing – review & editing. PV: Data curation, Formal analysis, Writing – review & editing. TT: Data curation, Formal analysis, Writing – review & editing. IC: Formal analysis, Supervision, Writing – review & editing, Data curation.

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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/fmicb.2024.1376389/full#supplementary-material

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Implications of COVID-19 prevention on the occurrence of childhood diarrhea in the Semen Bench district, Bench Sheko zone, southwestern Ethiopia

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Background: Coronavirus (COVID-19) is a virus that occurred in Wuhan, China, in December 2019 and has spread to several countries. Although interventions in water, sanitation, and hygiene (WASH) for COVID-19 are likely a pre-existing response to childhood diarrhea, evidence of the effects of COVID-19 preventative strategies on childhood diarrhea has been lacking. This study aimed to assess the implications of COVID-19 prevention for the occurrence of childhood diarrhea in rural communities of Ethiopia.

Methods: A community-based cross-sectional study was conducted from 10 May 2020 to 30 July 2020 involving selected households in the Semen Bench district, Bench Sheko zone, southwestern Ethiopia. A single population proportion formula was used to obtain a total of 768 sample sizes. Data were collected from selected households using a simple random sampling technique. Epidata 3.1 was used to enter the data and then exported to Stata 14 for analysis. Descriptive statistics along with binary and multivariable logistic regression analyses were used to identify factors of COVID-19 knowledge and practices related to childhood diarrhea. The chi-squared test was used to check the association between COVID-19 prevention and childhood diarrhea reduction.

Results: A total of 720 (93.75%) households participated in the study to achieve the study objectives. Approximately 55% of the participants had a good understanding of COVID-19 prevention, while only 48.5% had good COVID-19 prevention practices. The prevalence of childhood diarrhea was 19.3% which was more common among households with poor practices of COVID-19 prevention. The respondents with poor COVID-19 prevention knowledge were 42% (AOR = 0.58, 95% CI: 0.398, 0.847, $P = 0.005$) less likely to develop childhood diarrhea than those who had good COVID-19 prevention knowledge. Households with poor practices for COVID-19 prevention were 75.1% more likely to develop childhood diarrhea than those who had good preventive practices for COVID-19 prevention (AOR = 1.751, 95% CI: 1.193, 2.571, $P = 0.004$). The lower risk of childhood diarrhea is significantly related to good COVID-19 prevention practices. However, households with no formal education and a lack of WASH facilities have a higher likelihood of having childhood diarrhea in the household.

Conclusion: COVID-19 preventative strategies help reduce the prevalence of diarrhea in children. More research using prospective study designs and

advanced statistical models is needed to better understand the implication of COVID-19 preventative efforts in reducing childhood diarrhea.

KEYWORDS

association, COVID-19, childhood, diarrhea, knowledge, practice, prevention, implications

Introduction

Coronavirus 2019 (COVID-19) is a globally burdensome virus transmitted through unhygienic handshakes, direct breathing, coughing, or sneezing from a COVID-19-infected person. The first case was identified on 13 March 2020 in Addis Ababa, Ethiopia. The WHO suggests handwashing with soap or using alcohol-based hand sanitizers, wearing a face mask, maintaining physical distancing, and staying at home if possible to prevent the spread of the pandemic (1).

The government of Ethiopia has implemented various non-pharmaceutical interventions (NPIs) to control the transmission of the virus. These measures include case identification, contact tracing, isolation, and quarantine, as well as promoting physical distancing and sanitary measures. In addition, the government has temporarily closed many social institutions, including all academic institutions and religious organizations. Additionally it has imposed restrictions on cross-country and inter-city public transport systems, and postponed the national election (2, 3) to curb the pandemic.

Handwashing with soap is a proven intervention method for reducing 45–55% of childhood diarrhea episodes, and it also serves as a major COVID-19 intervention (4). However, the findings revealed that handwashing with soap was not widely practiced in rural areas before the COVID-19 pandemic (5). This understanding of the pandemic's response has a substantial impact on reducing childhood diarrhea. Authors from eastern Ethiopia have reported that there is a reduction in childhood diarrhea among families which implement COVID-19 prevention knowledge and practices (6). Due to limited evidence regarding the implication of COVID-19 prevention practices and the occurrence of childhood diarrhea in rural communities of Ethiopia, it is crucial to conduct this study. Therefore, this study aimed at investigating the implication of COVID-19 preventative practices on the occurrence of childhood diarrhea. This research plays a crucial role in reducing the prevalence of childhood diarrhea and providing valuable information to decision-makers and researchers for generating hypotheses.

Materials and methods

Study area and period

This study was conducted in the Semen Bench district, Bench Sheko zone, southwestern Ethiopia from 10 May 2020 to 30 July 2020. It is located 550 km from Addis Ababa, Ethiopia. The total population in the study area was 148,285, including 71,177 men, 77,108 women, and 23,147 children under the age of 5 years. The study area has 24 kebeles with a total of 29,610 households with an average family size of 4.14 (Bench Sheko zone health department). The study area map is designed on ArcGIS 10.5 to depict the sampled kebeles such as Kasha, Serti, Endakel, and Yali (Figure 1).

Study design and sample population

A community-based cross-sectional study design was employed using a two-stage design to select the study participants who fulfilled the inclusion criteria.

Inclusion criteria

Households or caregivers who express the willingness to participate in this study will be included to provide the required information. Conversely, participants or caregivers who are unable to participate due to their busy time shall be excluded from this study.

Sample size calculation

The following assumptions were used to compute the sample size: due to a lack of prior similar research, a 50% proportion of the association of COVID-19 prevention practices in a rural community was used, along with a 95% confidence level and a 5% margin of error, including 10% for non-responses. A design effect of 1.82 since the study participants were recruited using a two-stage design, due to the constraint and limitations of the relevant resources such as budget and time, which must be considered for the study (7).

$$n = \frac{(z\alpha/2)^2\left(1-p\right) * 0.5}{d^2},$$

where, n = sample size, q = 1 – p, d = margin of error = 0.05(5%)

$$n = \frac{\left((1.96)^2 * (1.96)\right) * 0.5}{(0.5)^2}) * 1.82 * 10\% = 768$$

Therefore, 768 total sample sizes were obtained for the study and uneven distributions of sample size were applied to select the respondents from sampled kebeles.

Sampling technique

A simple random sampling technique was used to choose the study participants based on eligibility criteria, ensuring that every household in a population has an equal chance of being chosen for the sample. Simple random sampling is a type of probability

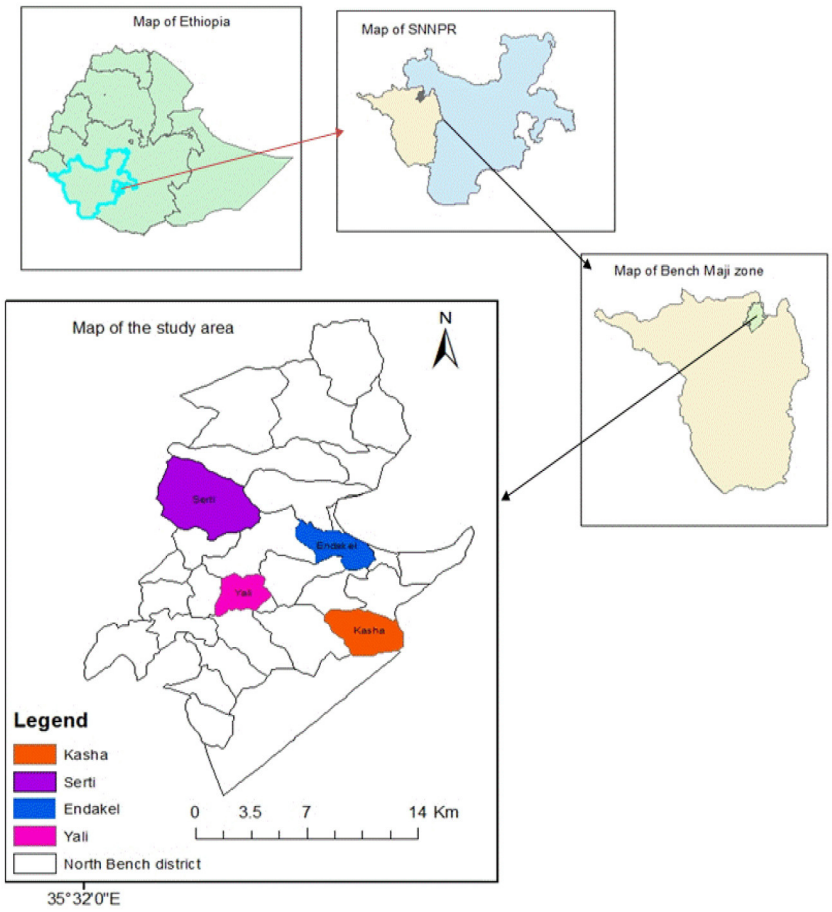

FIGURE 1
Map of the study areas. Esri reserves the right to grant permission for any other use of the image.

sampling technique in which the researcher randomly selects study participants from all households in the kebeles. Each member of the household has an equal chance of being selected for the study. The data are then collected from as large a percentage as possible of this random subset. Only one person from each family was selected to control the risk of contact with COVID-19 and to reduce the clustering effect and potential bias, specifically, the need to control for confounding variables related to family clustering.

Data collection process and tools

The survey questionnaires were adapted from previously published COVID-19 surveys that included demographic information, 11 knowledge items, and five practice items (8–10). A pretest was conducted on 5% of the samples in Sheko kebeles (Sheko district) and necessary changes were made to tools.

To assess the knowledge about COVID-19, 11 items were included in the participant knowledge questionnaires. Items 1–5 covered clinical presentations, items 6–8 focused on transmission routes, and items 9–11 were related to prevention and control of COVID-19. A correct response to an item earned one point, while an incorrect or uncertain response earned zero points. The total score ranged from 0 to 11 with higher scores indicating good COVID-19 knowledge. Similarly, the COVID-19 preventative practice questionnaire consisted of five items with a total score ranging from 0 to 5. The classification of knowledge and practice was adapted from previous research and applied to evaluate the knowledge and practice levels of the respondents. Additionally, knowledge and practices were operationalized as the awareness levels of the respondents exceeded the mean score on questions related to knowledge and practices regarding COVID-19 (9).

Study variables of interest

Outcome variable

The prevalence of childhood diarrhea within 2 weeks before the data collection is considered a dependent variable.

Independent variables

The independent variables included knowledge and practices of COVID-19 prevention, sociodemographic variables, and WASH facilities (critical handwashing times were identified as before preparing food, eating, providing supplementary feeding for children, breastfeeding, after defecation, and cleaning a child after defecation) (11). Per capita water consumption (l/c/d) is measured

as at least 20 l of water required for a person per day (an indication of the adequacy of water), and time to fetch water is defined as the total time taken to fetch water from sources within 30 min (an indication of the accessibility of water) (12).

Data quality assurances

The questionnaires were written in English before being translated into Amharic. Six data collectors and two supervisors were trained to ensure the quality of data. The principal investigator kept track of the data collection process daily.

Statistical analysis

Epidata3.1 was used to enter the data, and the data were exported to Stata 14 for analysis. Frequencies and percentages were used to summarize the studied variables. The variance inflation factor (VIF) less than 10 as a measure of the collinearity of each variable was used to examine the multicollinearity between independent variables (13, 14). A chi-squared test was performed to understand the association of COVID-19 prevention practices with the occurrence of childhood diarrhea. A binary logistic regression model was used to find the potential variables related to poor COVID-19 preventative practices and the occurrence of childhood diarrhea with a $p < 0.25$ obtained from univariate analysis. The results of the final model were interpreted using an adjusted odds ratio (AOR) with a 95 percent confidence interval at a statistical significance threshold of 0.05.

Ethical consideration

The Institutional Review Board (IRB) of Jimma University approved the study documents (IRB000197/20) and provided an ethical letter to conduct this study. In addition, consent was obtained from each participant to proceed with the data collection process.

Results

Sociodemographic characteristics of participants

The sociodemographic characteristics of participants were analyzed to assess the implication of COVID-19 prevention practices on the occurrence of childhood diarrhea. A total of 720 respondents completed the survey, yielding a response rate of 93.8%. More than three-fourths (73.8%) of the study participants were female individuals. The mean age of the respondents was 29.4 years with a standard deviation (SD) of 5.19. More than half, (54.6%), of the respondents, were over 40 years old, with 550 (76.4%) being married. Less than half (48.10%) of the respondents did not attend formal education. Less than half (46.9%) of respondents were farmers, while 554 (76.9%) of the respondents had more than five family members (Table 1).

TABLE 1 Sociodemographic characteristics of study participants.

Variable	Categories	Number	%
Age (in year)	18–29	120	16.7
	30–39	207	28.8
	≥40	393	54.6
	Mean (± SD)	29.4 ± 5.54	
Sex	Male	189	26.3
	Female	531	73.8
Marital status	Single	170	23.6
	Married	550	76.4
The educational level of the caregiver	No formal education	346	48.1
	Primary education	250	34.7
	More than secondary education	124	17.2
Occupation	Farmer	338	46.9
	Merchant	80	11.1
	Student	145	20.1
	Government employee	38	5.3
	Housewife	119	16.5
Family size	≥5	166	23.1
	<5	554	76.9

TABLE 2 WASH facilities of the study participants.

WASH facilities		Number	%
Handwashing facilities	Yes	177	24.6
	No	543	75.4
Handwashing at critical times	Yes	259	36
	No	461	64
Total time to fetch water from sources, in min	<30	245	34
	>30	475	66
Per capita water consumption per day (l/c/d)	>20	276	38.3
	<20	444	61.7

The WASH facilities available to the respondents in a rural community were evaluated in relation to COVID-19 prevention practices in the Semen Bench district, southwestern Ethiopia. Almost a quarter (24.6%) of the respondents owned hand-washing facilities. Approximately 259 (36%) respondents practiced hand washing at critical times. Only 245 (34%) households obtained water from sources within 30 min of walking distance, while 276 (38.3%) respondents consumed more than 20 l per capita per day (38.3%) (Table 2).

TABLE 3 Knowledge of respondents toward COVID-19 prevention practices.

Characteristics		Response	
		Yes (%)	No (%)
Mode of transmission	Breathing, sneezing, cough	337 (47)	383 (53)
	Physical contacts	298 (41)	422 (59)
	Handshake	344 (48)	376 (52)
Symptoms of COVID-19	Fever	669 (93)	50 (7)
	Dry cough	378 (52.5)	342 (47.5)
	Breathing difficulty	259 (36)	461 (64)
	Fatigue	89 (12.4)	631 (87.6)

Knowledge of respondents toward COVID-19

Less than half, 344 (48%), of the respondents know the mode of transmission of the pandemic. A majority of the participants, 669 (93%), know a symptom of COVID-19. In addition, dry cough and breathing difficulties were mentioned as symptoms of COVID-19 (Table 3).

COVID-19 prevention practices

The result revealed that less than half, 349 (48.5%), of the participants had good practices for preventing the COVID-19 pandemic. The participants with no formal education were 36% less likely to implement COVID-19 prevention practices than those with secondary education (AOR = 0.638, 95% CI: 0.421, 0.967, $P = 0.012$).

Similarly, the respondents with poor knowledge were 27.2% less likely to practice COVID-19 prevention methods than those with better knowledge levels of COVID-19 (AOR = 0.728, 95% CI: 0.542, 0.978, $P = 0.035$).

The respondents who traveled more than 30 min to fetch water from sources were 35.2% less likely to practice prevention than those who obtained water <30 min from sources (AOR = 0.675, 95% CI: 0.465, 0.980, $P = 0.039$) (Table 4).

Implication of COVID-19 prevention practices on occurrences of childhood diarrhea

This study explores the potential impacts of COVID-19 prevention measures, such as hand hygiene and increased sanitation practices, on the incidence and severity of childhood diarrhea. We can gain insights into how these prevention practices can contribute to reducing the burden of childhood diarrhea. Understanding the implications of COVID-19 prevention practices on occurrences of childhood diarrhea is crucial for developing effective strategies for disease prevention and management. By examining the reported associations between health-seeking behaviors, such as the adoption of a healthier lifestyle and good personal hygiene practices, we can assess the potential impact of these practices on reducing the transmission of diarrheal diseases among children during the COVID-19 pandemic.

The highest incidence of childhood diarrhea was observed among respondents with poor knowledge and practices regarding COVID-19 prevention. However, respondents with good knowledge of COVID-19 had a lower prevalence (7.92%) of childhood diarrhea than those with poor COVID-19 prevention knowledge (11.4%). Similarly, lower (7.22%) risks of developing childhood diarrhea were significantly associated with good preventive practices for COVID-19.

Moreover, the results from the multivariable analysis revealed that respondents with poor prevention knowledge of COVID-19 were 42% (AOR = 0.580, 95% CI: 0.398, 0.847, $P = 0.005$) less likely to develop childhood diarrhea than those who had good knowledge of COVID-19 prevention. Similarly, households with poor preventive practices for COVID-19 were 75.1% more likely to develop childhood diarrhea than those who had good preventive practices for COVID-19 (AOR = 1.751, 95% CI: 1.193, 2.571, $P = 0.004$). There is an observed significant positive association between COVID-19 prevention practices and a lower risk of childhood diarrhea (p-value less than 5) (Table 5).

Discussion

This study showed that good COVID-19 prevention practices reduced the prevalence of childhood diarrhea compared to groups with poorly practiced respondents. The previous findings suggested that COVID-19 intervention, particularly proper handwashing practices, could reduce episodes of diarrhea (15, 16). Moreover, the COVID-19 prevention practices of frequent hand washing with soap and sanitizers were also the existing key NPIs for preventing acute childhood diarrhea globally. These practices, promoted for COVID-19 prevention, provide a valuable opportunity to explore the implication of COVID-19 prevention practices on the occurrence of childhood diarrhea.

The prevalence of childhood diarrhea during the research period was 19.3%, which is lower than the previously reported prevalence from similar contexts before the pandemic (17). The lower prevalence of childhood diarrhea in the current study might suggest some behaviors, such as handwashing with soap, may have improved as a result of promotions of frequent and proper handwashing practices during the pandemic (17). Proper hand hygiene reduces the chances of ingesting or coming into contact with pathogens, leading to a decrease in diarrhea cases. However, the COVID-19 pandemic has increased the demand for water and sanitation worldwide, highlighting the need for basic WASH in both households and public places (18).

This study indicated that more than half of the respondents (55.3%) were aware of COVID-19 prevention methods, a finding that is similar to a study conducted in Bangladesh (54.87%) (19) but inconsistent with findings from Malaysia (80.5%) (20) and India (80.64%) (21). The limited COVID-19 prevention awareness in this

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TABLE 4 Factors of COVID-19 prevention practices.

Variable	COVID-19 prevention practice					
	Poor	Good	COR (95%CI)	*P*-value	AOR (95%CI)	*P*-value
Sex						
Male	92	97	1			
Female	262	269	0.832 (0.597, 1.161)	0.279		
Marital status						
Single	99	71	1.012 (0.718,1.428)	0.994		
Married	255	295	1			
Educational status						
No formal education	189	157	0.638 (0.421, 0.967)	0.034	0.640 (0.421,0.974)	0.037*
Primary education	131	119	0.665 (0.430, 1.028)	0.065	0.678 (0.437,1.051)	0.082
More than secondary education	51	73	1			
Occupational status						
Farmer	155	183	0.581 (0.392, 0.862)	0.007	0.569 (0.383,0.847)	0.005*
Merchant	42	38	1.176 (0.562, 2.459)	0.667	1.107 (0.527,2.327)	0.787
Student	86	59	0.798 (0.489, 1.302)	0.367	0.762 (0.465, 1.248)	0.281
Housewife	64	55	0.758 (0.438, 1.314)	0.607	0.753 (0.433, 1.309)	0.314
Government employee	24	14	1			
Family size						
≥5	85	81	0.983 (0.695, 1.391)	0.695		
<5	286	268	1			
Knowledge score						
Poor	198	181	0.579 (0.431, 0.778)	0.033	0.728 (0.542,0.978)	0.035*
Good	190	151	1			
Handwashing facilities						
Yes	60	117	1			
No	311	232	0.614 (0.436, 0.864)	0.005	0.621 (0.440, 0.875)	0.007*
Per capita water consumption						
Greater than 20	160	224	1			
Less 20	211	125	0.423 (0.313, 0.571)	<0.001	0.446 (0.329, 0.605)	0.001*
Time travel to fetch water from sources						
Greater than 30	251	143	0.516 (0.381, 0.698)	<0.001	0.675 (0.465, 0.980)	0.039*
Less 30	120	206	1			
Handwashing at critical times						
Yes	107	153	1			
No	264	196	0.699 (0.514, 0.949)	0.022	0.705 (0.519, 0.958)	0.025*

*Significant at $p < 0.05$; COR, Crude odds ratio; AOR, Adjusted odds ratio; CI, confidence. Interval.

study was influenced by the respondent's educational status and rural residency. These two factors affected their access to various social media platforms through which health information was disseminated. Several findings revealed that educated respondents had more access to various information sources on COVID-19 (21–23) to prevent the pandemic. This access may increase the likelihood of being more aware of the spread of the pandemic, enabling better implementation of recommended

TABLE 5 Implication of COVID-19 prevention practices on occurrences of childhood diarrhea.

COVID-19 prevention		Childhood diarrhea within two weeks					
		Yes (%)	No (%)	COR (95% CI)	*p*-value	AOR (95% CI)	*p*-value
Knowledge	Poor	82 (11.40)	259 (36)	0.559 (0.384, 0.814)	0.002	0.580 (0.398, 0.847)	0.005*
	Good	57 (7.92)	322 (44.72)	1			
Practices	Poor	87 (12.10)	267 (37.10)	1.817 (1.241, 2.661)	0.002	1.751 (1.193, 2.571)	0.004*
	Good	52 (7.22)	314 (43.61)	1			

*Significant at $p < 0.05$; COR, Crude odds ratio; AOR, Adjusted odds ratio; CI, confidence interval.

measures and potentially reducing the incidence of childhood diarrhea (24).

In this study, the COVID-19 prevention practice was less than half, 48.5%, which is inconsistent with the studies conducted in India (83.8%) (25), southern Ethiopia (80%) (26), and Saudi Arabia (81%) (27). The poor practices of COVID-19 prevention in this study area were affected by the poor knowledge level, absence of formal education, and WASH facilities (Table 5), which is similar to the earlier suggested factors (1). In developing countries, pre-existing WASH facilities were key intervention strategies against various infections. Currently, there are NPIs that control the spread of the COVID-19 pandemic (28); however, the coverage of WASH facilities in this study area needs due attention from the health sectors. The findings on prevention practices might be dissimilar because this study was conducted in rural communities with limited access to health information and a lower level of educational status compared to the participants in other studies. In addition, the mean score taken as a reference to distinguish between good and poor prevention practices of COVID-19 and the sample size might also contribute to the difference.

This study has provided evidence of the implications of COVID-19 prevention practices in relation to occurrences of childhood diarrhea that could aid health implementers, planners, and policymakers in integrating strategies and generating hypotheses.

Limitations of the study

Since this study used simple random sampling techniques to select a sample, sampling errors may have occurred. The sample may not accurately reflect the general population or the appropriate population of interest which may cause "sample bias" or "selection bias". This occurs when a study systematically differs from the population of interest resulting in a systematic error in the association or outcome. However, the chi-squared test was used to measure associations or implications that may not address the cause–effect relationship. Further study using prospective study designs with advanced statistical approaches is needed.

In this study, biases were managed by carefully utilizing simple random sample techniques, pretesting and translating data collection tools. The adjusted odds ratio analysis was also used to control for any confounding variables.

Conclusion

COVID-19 prevention practices were significantly associated with a lower risk of developing childhood diarrhea. The increasing level of knowledge is crucial for preventing the COVID-19 pandemic, which has consequently helped reduce the risk of childhood diarrhea.

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 humans were approved by the Institutional Review Board (IRB) of Jimma University (IRB000197/20). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

BA: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing. SM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing—review & editing. AA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing—review & editing.

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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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An outbreak of *Mycoplasma pneumoniae* in children after the COVID-19 pandemic, Shanghai, China, 2023

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Background: During the coronavirus disease 2019 (COVID-19) pandemic, the infection of *Mycoplasma pneumoniae* (MP) decreased significantly. At the beginning of the summer of 2023, there was an increasing trend of MP infection in China and the MP pneumonia (MPP) is surging when it comes to the school season and lasts for several months which has attracted widespread attention.

Objective: This study aims to investigate the prevalent characteristics of the MP and the difference between the COVID-19 pandemic and the post in Shanghai, China.

Methods: The demographic information and the results of laboratory pathogen detection from July 2021 to May 2024 were collected and analyzed to find out the prevalent characteristics of MP. Two periods, during the COVID-19 pandemic and the post-pandemic, were divided and compared. The P1 genotyping and macrolide resistance-associated gene of 23 s rRNA were detected using the remaining MP-positive samples.

Results: During the COVID-19 pandemic, the prevalence of the MP has significantly decreased. Female children are more susceptible to MP infection than the male. The school-aged group (>6 years) had the highest infection rate. The rate of MP P1 genotype during post panel is higher than that during COVID-19 pandemic, which is dominant from July 2021 to May 2024, while the macrolide-resistant associated mutations (A2063G) keep high percentage during or post pandemic.

Conclusion: After the COVID-19 pandemic, an outbreak of MP infection occurred from summer onwards in 2023 with children in Shanghai, China. Immunity debt and high rate of macrolide-resistance may take effects in this MP epidemic. Continuous surveillance of MP is necessary to help to alert the prevalence of MPP.

KEYWORDS

***Mycoplasma pneumoniae*, COVID-19, P1 genotyping, macrolide resistance, non-pharmaceutical intervention, epidemiology**

1 Introduction

Mycoplasma pneumoniae (*M. pneumoniae*, MP) was first isolated from the sputum sample of a patient having primary atypical pneumonia by Eaton et al. (1944). It is a crucial pathogen of community-acquired pneumonia (CAP) in children and is responsible for about 4%–8% of CAP cases in endemic periods and 40% in epidemics (Jain et al., 2015; Brown et al., 2016; Waites et al., 2017). It is transmitted by droplets from infected patients through the respiratory tract and can lead to respiratory infection, which is mild and self-limited generally but can still result in severe pneumonia and extrapulmonary manifestations particularly in the pandemic (Poddighe, 2020; Biagi et al., 2021).

MP is a kind of prokaryote without a rigid cell wall (Waites and Talkington, 2004). Thus, treatment of MP infection with beta-lactam antibiotics is ineffective. Macrolides, such as azithromycin, are recommended as the first-line antibiotics for treating children with MPP in many countries including China (Bradley et al., 2011; Tsai et al., 2021). However, with the wide application of macrolides, the resistance rate of MP to macrolides is increasing around the world, especially in China which is over 90% (Chen et al., 2020; Kim et al., 2022; Wang et al., 2022). The mechanism of macrolide resistance is the point mutations of the domain V in the 23 s ribosomal RNA with the frequent sites of A2063G/C/T, C2617G, and A2064G/C (Lucier et al., 1995). As for genotypes of MP, there are two major subtypes (type 1 and type 2) which aimed at the p1 adhesin gene.

During the epidemic of COVID-19, the prevalence of other respiratory pathogens like influenza A virus (IAV), respiratory syncytial virus (RSV), adenoviruses (ADV), and MP has significantly decreased (Liu et al., 2021; Chow et al., 2022; Ye and Liu, 2022; Meyer Sauteur and Beeton, 2023). The probable reason may be related to the adoption of strict non-pharmaceutical interventions (NPIs) like wearing masks, working at home, and closing schools, which blocked the spread of respiratory pathogens (Chow et al., 2022). Hence, experts predicted that an exceptionally large wave of MP infections could occur as a result of reduced exposure (Meyer Sauteur et al., 2022; Meyer Sauteur and Beeton, 2023). There have been reports of outbreaks of MP infection from Denmark, Netherlands, and France (Bolluyt et al., 2024; Nordholm et al., 2024; Zayet et al., 2024). Likewise, since the summer of 2023, outbreaks of MP pneumonia have emerged in various parts of China, characterized by the high rate of macrolide resistance and difficulty in the treatment of severe cases (Zhang et al., 2024).

Here, we conducted a retrospective epidemiologic analysis of MP between July 2021 and May 2024 spanning the COVID-19 pandemic (July 2021–December 2022) and the post (January 2023–May 2024) based on the "10 measures" policy announced by the China government at the onset of December 2022. The epidemiological characteristics, molecular type, and macrolide resistance were investigated in this study aiming to understand the MP prevalence better and reduce the morbidity and mortality caused by MP infection.

2 Methods

2.1 Study population

Patients with acute respiratory tract infection (ARTI) were enrolled at the Children's Hospital of Fudan University in Shanghai, from July 2021 to May 2024. The respiratory specimens (bronchoalveolar lavage fluid/sputum/ nasopharyngeal swab) were collected by trained professional staff and then sent to the microbiology laboratory timely for a multi-pathogen detection using a commercial multiplex capillary electrophoresis PCR-based panel (Health Gene Technologies, Ningbo, China). It can detect 11 common respiratory pathogens simultaneously including MP, IAV, influenza B virus (IBV), human parainfluenza virus (HPIV), RSV, ADV, human metapneumovirus (HMPV), human rhinovirus (HRV), human bocavirus (HBOV), human coronavirus (HCOV), and chlamydia (CH). The medical records and the detection results were used to analyze the prevalence of MP and the remaining samples positive for MP were stored at −70°C for the subsequent macrolide-resistant mutations sequencing and P1 typing. Two periods, the COVID-19 pandemic and the post-pandemic, were set up. The patients were divided into five age groups: under 28 days of age (≤28 d), under 12 months of age (~12 m), 1–3 years of age (~3 y), 4–6 years of age (~6 y) and more than 6 years of age (>6 y).

2.2 Detection of macrolide resistance-associated mutations

The nucleic acid of the stored MP-positive samples was extracted and purified with a kit on an automated extraction machine from Tianlong, China. The nucleotide position 1,758–2,684 of the 23S rRNA gene was amplified by the primer pair prescribed previously (Matsuoka et al., 2004). Each amplification was performed on a T100 thermal cycler (Bio-Rad, United States) with a mixture of 25 μL containing 12.5 μL of TaKaRa premix Taq™, 0.4 μM primers, 5 μL of template DNA, 5.5 μL of nuclease-free water. The reaction condition was as follows: initial pre-denaturation for 10 min at 95°C; followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 10 min. The amplification products were sequenced by Sanger sequencing (Sangon Biotech, Shanghai, China). All the sequences were aligned with the nucleotide sequence of *M. pneumoniae* M129 according to GenBank accession no. X68422 using MEGA11 software to seek out any point mutations.

2.3 P1 genotyping

A duplex real-time PCR assay described by Zhao et al. (2011) was used to identify the P1 genotypes of MP. The reaction mixture contained 12.5 μL of TaKaRa premix Ex TaqTM for probe, 0.5 μM of primers, 0.2 μM of probes, 5 μL of template DNA, and nuclease-free water to a final volume of 25 μL in total. The amplification conditions were as follows: 95°C for 2 min; followed by 45 cycles at 95°C for 15 s and 56°C for 15 s.

2.4 Statistical analysis

Continuous variables were presented as the mean or median, while the categorical variables were described by the number and percentage. Comparisons between different groups were conducted by Chi-square test using SPSS 23 (SPSS Inc., Chicago, IL, United States). A two-tailed p-value < 0.05 was considered statistically significant.

3 Results

3.1 Study population

After removing duplicate records, a total of 17,247 specimens of patients with ARTI were enrolled in this study, among which 4,461 (25.87%) were from the pandemic period and 12,786 (74.13%) from the post. The mean of the specimens during the pandemic was 248 cases per month but increased to 752 in the post-pandemic period due to the policy and prevalence alteration of COVID-19. The male was counted for 55.01% with a gender ratio of 1.22:1. The median age was 2 years old. The overall detection rate of MP was 27.29% (4,707/17,247). During the COVID-19 pandemic, the detection rate of MP was 8.34% (372/4,461) which was significantly lower than the post-pandemic period with a rate of 33.90% (4,335/12,786, $p < 0.001$).

3.2 Seasonality

The seasonality profile and the epidemiological trends of MP infection from 2021 to 2024 are shown in Figure 1. The annual positive rates of MP from 2021 to 2024 were 5.91% (118/1,996), 10.30% (254/2,465), 38.03% (2,880/7,573) and 27.91% (1,455/5,213) respectively. During the COVID-19 pandemic, there is no obvious seasonality but a distinct reduction from April 2022 to June 2022. And the positive rate rises slowly from July 2022 to September 2022. It is suspected that there should be a small peak of MP in early summer to autumn but was disrupted by the lockdown policy in Shanghai stopping the spread of COVID-19. After the COVID-19 pandemic, the positive rate of MP increased relatively slowly from March to June 2023 and surged afterward. An outbreak has occurred since early summer, peaked in October, declined at a lowest level in March 2024, and risen again as the climate gets warmer. The positive rate of MP was the lowest in June 2022 (1.72%) and the highest in October 2023 (59.63%).

3.3 Age distribution of MP

During the COVID-19 pandemic, the detection rates for the five different age groups (≤ 28 d, ~12 m, ~3 y, ~6 y, and >6 y) were 0.00% (0/962), 2.25% (38/1,688), 7.85% (61/777), 23.43% (108/461), and 28.80% (165/573), respectively. After the pandemic, the rates were 0.42% (4/954), 7.44% (179/2,407), 25.27% (702/2,778), 48.29% (1,352/2,800), and 54.51% (2,097/3,847), respectively. Detection rates in newborns were the lowest, and school-age children over 6 years old were the highest. As age increased, the detection rate of MP gradually increased in two periods. Except the ≤28 d group, the positive rates of the post-pandemic period was higher than that of the pandemic in each age group ($p < 0.001$; Figure 2). The median age of MP-positive cases during the pandemic was 6 years old with the range of 0.15 to 17. In the post-pandemic period, the median age was 6 years old too but with the range of 0.02 to 17.

3.4 Gender distribution of MP

As shown in Table 1, the total detection rate of MP in female patients (30.30%) was higher than that in male patients (25.31%, $p < 0.001$). During and after the pandemic, the female proportions were both higher than the male patients, with statistical differences ($p < 0.001$) and the pandemic had no impact on the gender distribution of MP (Table 1).

3.5 Co-infection

The co-infections in MP-infected patients were common and complex. Among the 4,707 positive samples from 2021 to 2024, a total of 1,972 samples were detected with multiple pathogens, accounting for 41.90% (1,972/4,707). The co-infection rate during the post-pandemic period (14.63%, 1,870/12,786) was higher than that during the pandemic (2.29%, 102/4,461, $p < 0.001$). Duplex infections were the most common accounting for 79.97%, with the highest incidence

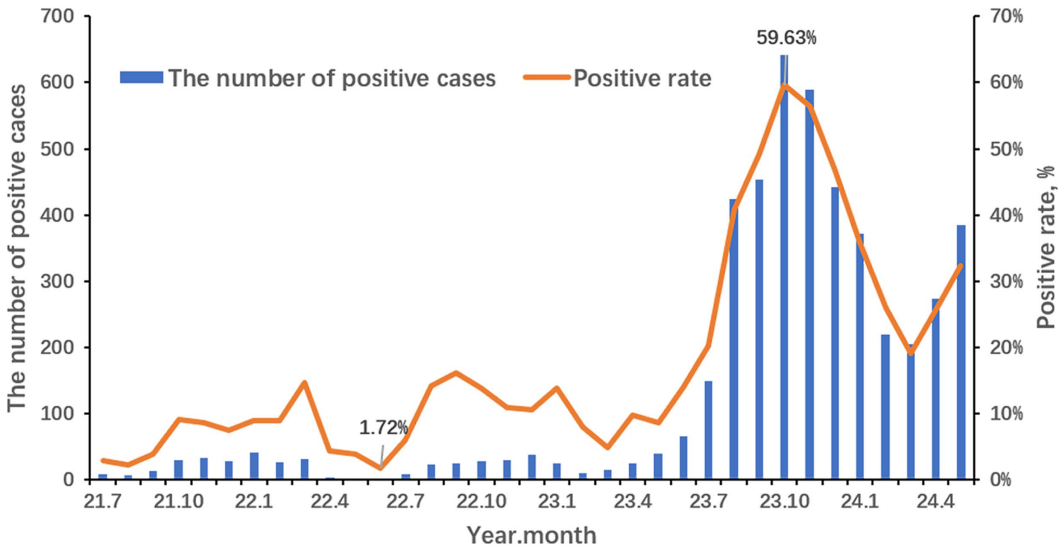

FIGURE 1
Seasonal distribution of MP from July 2021 to May 2024.

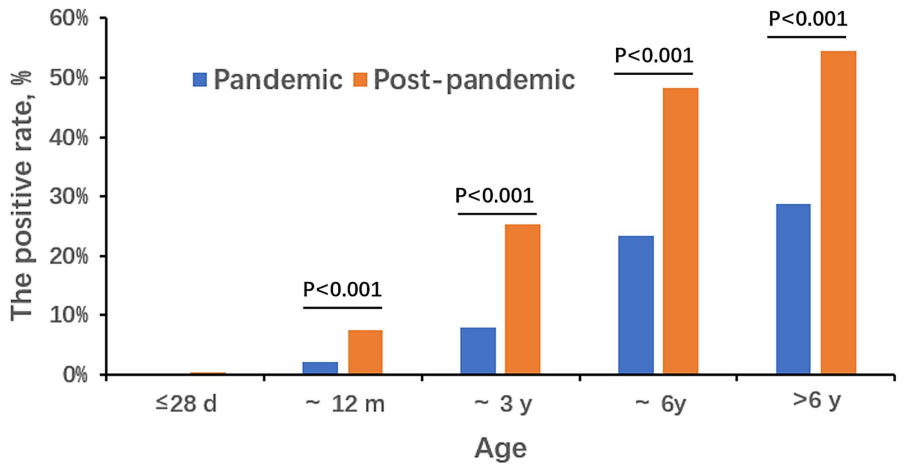

FIGURE 2
The positive rate of MP in five age groups during the pandemic and the post-pandemic.

TABLE 1 The gender distribution of MP.

Gender	the pandemic	Post-pandemic	Total
Male	177/2,510 (7.05%)	2,179/6,799 (32.05%)	2,356/9,309 (25.31%)
Female	195/1,951 (9.99%)	2,156/5,809 (37.11%)	2,351/7,760 (30.30%)
P	0.00	0.00	0.00

with HRV, followed by ADV, RSV, H3N2, and HCOV. Triple infections accounted for 17.19%, with the most common infection combinations being MP, HRV, and ADV. The co-infections with four and five pathogens accounted for 2.69% and 0.15%, respectively. The details of the co-infection situations are listed in Table 2.

3.6 P1 genotypes

A total of 835 samples were P1 genotyped. 731 samples (87.54%) were classified as type 1, and type 2 was identified in 104 cases (12.46%). From 2021 to 2024, the genotyped numbers were 111, 180, 364, and 180, respectively, and all of which were dominated by type 1. The proportion of type 1 has been increasing annually, reaching 66.67% in 2021, 83.89% in 2022, 92.86% in 2023, and 93.33% in 2024. The proportion of Type 2 has been decreasing yearly, reaching 33.33% in 2021, 16.11% in 2022, 7.14% in 2023, and 6.67% in 2024, respectively. The rate of MP P1 genotype during post panel (93.01%, 506/544) is higher than that during COVID-19 pandemic (77.32%, 225/291, $p < 0.001$; Figure 3).

3.7 Macrolide resistance-associated mutations

A total of 261 samples were sequenced successfully. A point mutation of A to G at position 2063 in domain V of the 23S rRNA gene was identified in 251 (96.17%) clinical samples. And no other mutations were found in all samples. From 2021 to 2024, 16, 78, 134, and 33 cases were successfully sequenced, with a resistance rate of 95.74% (90/94) during the pandemic and 96.41% (161/167) in the post. There was no significant difference in the macrolide-resistant associated mutations between the two groups ($p = 1.000$; Table 3).

4 Discussion

Since COVID-19 was first discovered in December 2019 and spread all over the world quickly then, people got infected and affected by the large waves of outbreaks over the years and suffered a lot (Zhu et al., 2020). Though the clinical presentation caused by the Omicron strain is usually mild and at a low risk of hospitalization and death, long COVID and immune disorders have become more concerning issues (Klein et al., 2023). Meanwhile, the pandemic and the derived kinds of NPIs have had a huge impact on the epidemiology of other respiratory pathogens as well like influenza virus and RSV (Liu et al., 2021; Ye and Liu, 2022). In our study, the amounts of specimens for multiple-pathogen detection during the pandemic period are lower than the post as well as the MP-positive rate which means the pandemic influenced the circulation of MP negatively within the community.

It's reported that the MP outbreaks occur every 3 to 7 years and the seasonal trend varies according to geography and temperature (Yan et al., 2016). In our study, the lowest monthly positive rate was in June 2022 right in the lockdown period in Shanghai. After that, the positive rate increased slowly until September 2022. It was suggested that there might be a small peak in the summer and autumn seasons in 2022 but was disrupted by the lockdown policy to stop COVID-19 from spreading. Since the "10 measures" policy was published in December 2022, kinds of NPIs were canceled. Respiratory pathogens start circulating in communities. At the onset of the summer of 2023, the MP-positive rates increased. Afterward, the MP outbreak occurred and swept through the children in Shanghai with the highest positive rate (59.63%) in October 2023 and still lasting till the spring of 2024. This means when an MP outbreak occurs, the prevalent period gets longer.

Consistent with many other studies (Kutty et al., 2019; Cheng et al., 2022), the most susceptible age group was in school-age children in our study both in the pandemic period and the post-pandemic. The reason may be that children in school, such a closed and concentrated place, are more likely to get infected by MP. It is worth noting that during the post-pandemic period, really young children even neonates get MP infection, while there were no cases in the pandemic period in the <28 d age group. This indicates that when an MP outbreak occurs, the susceptible age gets younger. As for the gender distribution, the positive rate of female patients is higher than that of males both in the two periods.

TABLE 2 The co-infection types and proportions of MP.

Co-infection types	Number	Proportions
2 pathogens	1,577	79.97%
MP + HRV	624	31.64%
MP + ADV	343	17.39%
MP + RSV	114	5.78%
MP + H3N2a	93	4.72%
MP + HCOV	78	3.96%
MP + MPV	73	3.70%
MP + BOCA	49	2.48%
MP + INFB	39	1.98%
MP + INFB	6	0.46%
MP + CH	6	0.30%
MP + H1N1b	2	0.10%
3 pathogens	339	17.19%
MP + HRV + ADV	69	3.50%
MP + HRV + PIV	41	2.08%
MP + HRV + RSV	20	1.01%
MP + HRV + BOCA	20	1.01%
MP + ADV + PIV	16	0.81%
MP + HRV + MPV	15	0.76%
Others	158	8.01%
4 pathogens	53	2.69%
5 pathogens	3	0.15%

[a]Influenza virus of H3N2 subtype; [b]Influenza virus of H1N1 subtype.

Previous studies showed that MP is likely to be co-infected with other respiratory pathogens, especially with viruses leading to a longer duration of fever, severe pneumonia, and poor response to the stepwise treatment of MPP (Gao et al., 2020; Choo et al., 2022; Li et al., 2022). In our study, the most common coinfected virus is HRV, which corresponds to the Soojeong Choo's from Korea (Choo et al., 2022). ADV is another common and important virus co-infected with MP. According to the study of Shen Jun et al., children with severe CAP have high mixed detection rates of *Mycoplasma pneumoniae* and adenovirus in alveolar lavage fluid (ALF) samples (Li et al., 2022).

P1 genotyping, dividing MP into two subtypes of type 1 and type 2, is one of the most common methods of monitoring the molecular epidemiology of MP. A study from Japan showed that a type shift phenomenon occurs every 8–10 years and the type shift from one group to another requires 2–3 years (Kenri et al., 2008). In our study, the proportion of type 1 during 2021–2024 was 66.67%, 83.89%, 92.86%, and 93.33% respectively, which was increasing year by year. Correspondingly, the proportion of type 2 has been decreasing from 33.33% in 2021 to 6.67% in 2024. Thus, we predicted that the year 2021 to 2024 coincided with the period of type shift of MP and that type 1 will continue to dominate in the next few years.

Macrolides are used as the first-line antibiotics for treating MMP in children, China. Anne et al. reported an MP outbreak from October to December, in Denmark, detecting a low resistant proportion of less than 2% (Nordholm et al., 2024). However, in our study, the total macrolide-resistance rate over the 3 years was high to 96.17%. The extremely high drug resistance rate poses a serious challenge to the treatment of MPP. It's vitally important to use macrolides reasonably and other treatments timely for MP-resistant patients.

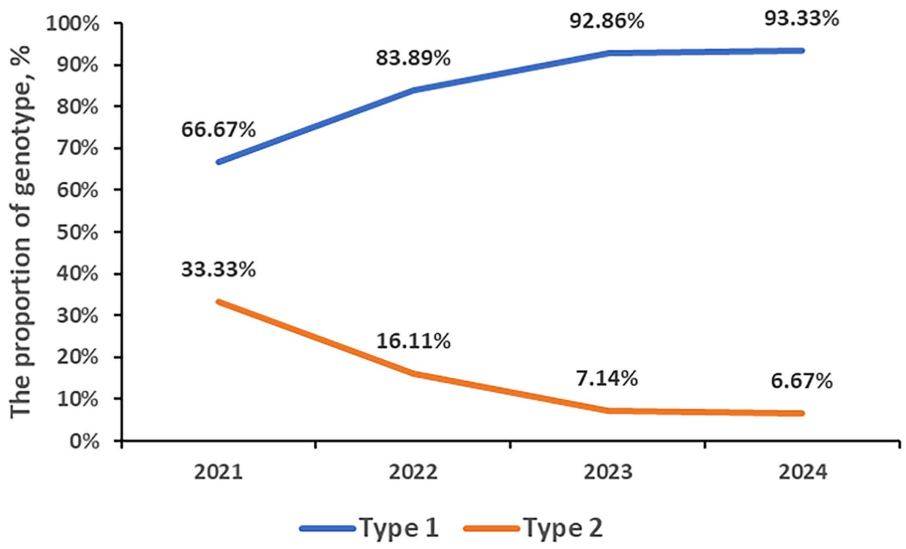

FIGURE 3
The prevalent trends of the P1 genotype.

TABLE 3 The sequencing results of macrolide resistance-associated mutations.

Mutation point	The pandemic		Post-pandemic		*P*
	2021	2022	2023	2024	
A2063G	16 (100%)	74 (94.87%)	128 (95.52)	33(100%)	
No mutation	0 (0%)	4 (5.13%)	6 (4.48%)	0 (0%)	
Total of A2063G	90(95.74%)		161(96.41%)		1.000

There are several limitations in our study. Firstly, it's a single-center and retrospective study, only radiating to Shanghai and its surrounding provinces and not representing the overall situation in China, though it is the largest children's hospital in Shanghai. Secondly, due to the limited time of detecting nuclide acid of multiple respiratory pathogens, the study has a short period and needs further surveillance in the future.

In conclusion, we found that the macrolide-resistant mutations, the gender and age distribution did not have apparent changes. Immunity debt and high rate of macrolide-resistance may take responsibility for this MP epidemic and the large population mobility after the COVID-19 pandemic may take effects to the spread of MP. Continuous surveillance of MP and other respiratory pathogens is necessary to help to alert the prevalence of kinds of pathogens.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Children's Hospital of Fudan University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

XuZ: Writing – original draft, Data curation, Investigation, Methodology, Resources, Software, Supervision, Visualization, Writing – review & editing. PL: Writing – review & editing, Methodology, Resources. HY: Writing – review & editing, Investigation, Resources. LW: Writing – review & editing, Investigation, Resources. HZ: Writing – review & editing, Data curation, Investigation, Methodology. MX: Writing – review & editing, Methodology, Resources, Software. LL: Writing – review & editing, Methodology, Project administration. RJ: Writing – review & editing, Investigation, Methodology, Resources, Software. LS: Writing – review & editing, Investigation, Resources. LC: Writing – review & editing, Data curation, Resources. XiZ: Writing – review & editing, Data curation, Resources. YW: Writing – review & editing, Data curation, Resources. JX: Writing – review & editing, Resources, Supervision, Validation.

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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

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