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Epidemiological and genetic characteristics of respiratory syncytial virus infection in children from Hangzhou after the peak of COVID-19

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ABSTRACT

Background: Respiratory syncytial virus (RSV) is one of the main pathogens that causes acute lower respiratory tract infection (ARTI) in infants. During the Coronavirus Disease 2019 (COVID-19) pandemic, although strict interventions have been implemented, RSV infection has not decreased.
Objectives: To study the epidemiological and genetic characteristics of RSV circulating in Hangzhou after the peak of COVID-19.
Methods: A total of 1225 nasopharyngeal swabs were collected from outpatients with ARTIs from July 2021 to January 2022 in The Children's Hospital, Zhejiang University School of Medicine.
Results: A total of 267 (21.79%) of the 1225 samples were RSV positive. There was no gender bias. However, an obvious age preference for infection was observed, and children aged 3-6 years were more susceptible, which was very different from previous RSV pandemic seasons. Phylogenetic analysis of 115 sequenced RSV isolates showed that all the RSV-A viruses belong to the ON1 subtype, which could be clustered into three clusters. While all the RSV-B viruses belong to BA9. Further analysis of the mutations highlights the fixation of ten mutations, which should be given extra attention regarding their biological properties.
Conclusion: The incidence of RSV infection in preschool children reported in this study is high. Phylogenetic analysis showed that the subtype A ON1 genotype was the dominant strain in Hangzhou from July 2021 to January 2022.

1. Introduction

Respiratory syncytial virus belongs to the family *Pneumoviridae* and the genus *Orthopneumovirus* [1]. RSV is a non-segmented negative-stranded RNA virus with a genomic length of approximately 15.2 kilobases. Its virion's diameter ranges from 100 to 300 nm. As one of the most common pathogens that cause acute lower respiratory tract infection in infants, RSV usually infects children under 2 years of age and causes severe cases that require hospitalization or even death [2–8]. Like other viruses, RSV does not have any specific clinical manifestations ranging from fever to chest pain. As a result, RSV infection presents a diagnostic challenge, as it can be confused with infections caused by other viruses or bacteria.

RSV has ten genes that encode eleven proteins named NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2 and L [9]. Among these proteins, adhesion protein G and fusion protein F glycoproteins are the main surface antigens that induce neutralizing antibodies and are usually designed as the targets of vaccines and therapeutic drugs. Additionally, the two antigenic subtypes of RSV (serotype A and serotype B) were divided based on the reactivity of the F and G surface proteins to monoclonal antibodies. Of these two important proteins, the G protein is the most variable protein of RSV. Two hyper-variable regions, which have a close relationship with viral antigenic variation, are located in the G protein. Thus, monitoring the mutations in hyper-variable region of the G protein could provide timely information on viral antigenic drift and help the development of related vaccines.

In this study, we collected over 1225 nasopharyngeal swabs from outpatients with ARTIs from July 2021 to January 2022 in The

Abbreviations: RSV, Respiratory syncytial virus; ARTI, Acute lower respiratory tract infection; NCBI, National Center for Biotechnology Information.

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Children's Hospital, Zhejiang University School of Medicine. In the 1225 swab samples, 107 were identified as RSV-A positive, and 8 were RSV-B positive. Epidemiological analysis revealed that the age of the most susceptible population ranged from 3 years to 6 years (85.8% of all infections). In contrast to other reports in China, the dominant RSV virus in Hangzhou is RSV-A rather than RSV-B. Phylogenetic analysis showed that all of the detected RSV-A viruses belonged to the ON1 subtype, which could be clustered into three clusters. All of the detected RSV-B viruses belonged to BA9. Further analysis of the mutations highlights the fixation of ten mutations, which should be given extra attention regarding their biological properties.

2. Materials and methods

2.1. Patients and samples

Nasopharyngeal swabs were collected from pediatric patients with ARTIs in The Children's Hospital, Zhejiang University School of Medicine (Hangzhou, Zhejiang), from July 2021 to January 2022. The inclusion criteria were as follows: fever (body temperature≥37.5°C), accompanied by one or more symptoms of ARTIs including cough, runny nose, sputum, and sore throat. Information including demographic data, case history, symptoms, and clinical results of each patient was collected. The throat swabs were stored in 2.5 ml of viral transport medium (KaiBiLi, Hangzhou, China). RSV and five common respiratory viruses were screened: influenza virus-A, influenza virus-B, human parainfluenza virus-1, human parainfluenza virus-3 and human adenovirus.

2.2. Nucleic acid extraction and screening for respiratory viruses

Viral RNA and DNA were extracted from 300 μL nasopharyngeal swab using a Nucleic Acid Extraction Kit (Catalog Z-ME-0044, Shanghai Zhijiang biotechnology Ltd. Co, China) according to the manufacturer's instructions. Common respiratory viruses and RSV were screened by real-time PCR with the Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The thermocycling protocol was reverse transcription and included 10 min at 45 °C, 5 min at 95 °C and 45 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 45 s. All experimental operations were strictly performed in accordance with the manufacturer's instructions.

2.3. Nested PCR for G gene Amplification of RSV-A and RSV-B

The RSV-positive samples were selected and reverse transcription of the extracted viral RNA was performed by using the MonScript RTIII Super Mix with dsDNase (Monad Biotech Co..Lid, China). The second hypervariable region of the G protein gene was the target for the external and seminested PCRs [10]. First, external PCR was carried out with the forward primer ABG490 and the reverse primer F164. Four microliters of cDNA were added to 21 μl PCR reagents (2 × Taq MasterMix, CWBio Co., Ltd, China). Amplification was carried out at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 4 min. Four microliters of the external PCR products were used for the seminested PCRs. Second, AG655 for group A (450bp) and BG517 for group B (585bp) were used as forward primers, and F164 was used as the reverse primer for the seminested PCR (primers are listed in Table 1). Amplification was carried out at 94 °C for 2 min,followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 4 min. Finally, the PCR products were sent to TSingKe Biological Technology Co., Ltd. (Hangzhou, China) for sequencing.

Table 1
Primers used for RSV G gene amplification.

Primers	Sequence(5'-3')
ABG490	5'-ATGATTWYCAYTTTGAAGTGTTC-3'
AG655	5'-GATCYCAAACCTCAAACCAC-3'
BG517	5'-TTYGTTCCCTGTAGTATATGTG-3'
F164	5'-GTTATGACACTGGTATACCAACC-3'

2.4. Statistical analysis

SPSS 22.0 was used for statistical analysis. The Chi-square test was used to compare the detection rates of RSV among different groups, and $P<0.05$ indicated statistically significant differences.

2.5. Phylogenetic analysis

The nucleotide sequences obtained from within the second hypervariable region of the G gene of subgroups A and B were compared to the available RSV sequences from GenBank. All sequences were aligned by using MAFFT v7.4.1 [11]. Phylogenetic trees were constructed by using the IQTREE v2.1.3 [12] and visualized with R package ggtree [13]. Analysis of complete gap deletion or missing data was also performed. Genetic distances were calculated using the Kimura 2-Parameter method in MEGA-X [14].

2.6. Nucleotide sequence accession numbers

Representative nucleotide sequences of RSV group A and B genotypes were submitted to GenBank and given accession numbers OP310836-OP310942 and OP310945-OP310952.

3. Results

3.1. Epidemiological characteristics of RSV-positive cases

As was shown in Table 2, 267 (21.79%, 267/1225) among the 1225 samples were found to be RSV positive. Among the 267 positive patients, 130 (10.61%, 130/1225) were male, and 137 (11.18%, 137/1225) were female; the difference was insignificant ($\chi^2 = 2.242$, $P = 0.134$). There were 2 cases aged from 0 to 6 months old (0.16%, 2 / 1225), 20 cases aged from 6 months to 1 year old (1.63%, 20/1225), 104 cases aged from 1 to 3 years old (8.49%, 104/1225), 125 cases aged from 3 to 6 years old (10.21%, 125/1225), and 16 cases aged from 6 years to 18 years old (1.3%, 16/1225). The positive detection rates from the age of 1 to 3 years age group ($\chi^2 = 9.599$, $P = 0.002$) and 3 to 6 years age group ($\chi^2 = 26.586$, $P<0.001$) were significant higher compared with the other groups, while there was no difference between the two age groups ($\chi^2 = 0.907$, $P = 0.341$). Adenovirus was the most common co-infecting virus in RSV-positive cases (7.49%, 20/267).

3.2. Phylogenetic analysis of genotype

For the 267 RSV-positive samples, 115 were successfully sequenced. Of these 115 sequences, 107 were identified to be the RSV-A genotype, and 8 were detected to be the RSV-B genotype using genotype-specific primers. To show their evolutionary history, phylogenetic analysis was used to analyze all the sequenced RSV-A and RSV-B. Forty-four representative RSV-A sequences, and 24 RSV-B sequences were selected by clustering all the RSV-A sequences and RSV-B sequences in the National Center for Biotechnology Information (NCBI) with CD-HIT [15]. Phylogenetic trees of the second hyper-variable region of G gene in RSV-A and RSV-B were built with both sequenced sequences and related representative sequences, respectively (Figs. 1 and 2). All the sequenced RSV-A viruses belonged to the genotype ON1 and could be clustered into 3 clusters. For the phylogenetic analysis with 8 sequenced RSV-B viruses and 24 representative sequences (Fig. 2), all these 8 sequences were classified as the BA9 genotype. For children with ON1 or BA9 RSV infection, Fever, Cough and Coarse breath sound were most common

Table 2
Epidemiologic characteristics of 267 children with RSV infection.

Age Gender	≤6m	≤1y	≤3y	≤6y	>6y	Totle positive case	χ^2	*P*
Male	1(0.08%)	13(1.06%)	47(3.84%)	61(4.98%)	8(0.65%)	130(10.61%)	2.242	0.134
Female	1(0.08%)	7(0.57%)	57(4.65%)	64(5.23%)	8(0.65%)	137(11.18%)		
Totle positive case	2(0.16%)	20(1.63%)	104(8.49%)	125(10.21%)	16(1.3%)	267(21.79%)		
χ^2	0.363	1.8	9.599	26.586	2.798			
P	0.547	0.18	0.002*	<0.001*	0.094			

The chi-square (χ^2) test was performed using SPSS.A *P*-value<0.05 is indicated by * and is considered statistically significant.

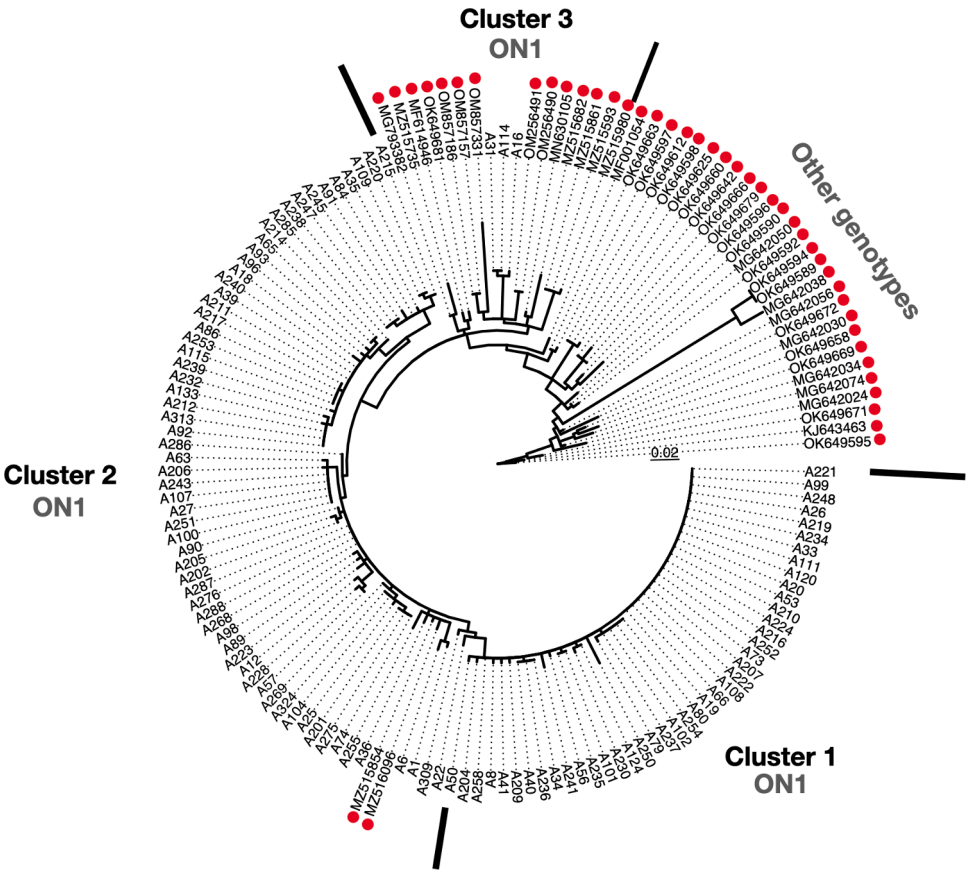

Fig. 1. G gene phylogenetic trees of RSV A strains. The phylogenetic trees of RSV A strains (A) derived in the study during 2021-2022 based on the G gene. The phylogenetic trees were constructed using the maximum likelihood method with 1,000 bootstraps. Reference genotypes downloaded from the GenBank are labeled with red dots.

clinical symptoms with the proportion above 85% (Table 3).

According to the phylogenetic analysis of the partial G gene fragment to the RSV strain, the A strains isolated in the present study was similar to the strain (MZ515854) found in United Kingdom, the percentage identity at nucleotide and amino acid(aa) levels between them were 97.93% and 95.71%. 107 ON1 strains were classified into 3 clusters. Cluster 1 consisted of 42 strains and cluster 2 include 62 strains which were similar to the strains from the United Kingdom (MZ515854, MZ516096). Whereas 3 ON1 strains were similar to the strains from Beijing, China (OM256491 and OM256490). The BA9 strains isolated in the study were similar to the strain (MZ516135) found in Netherlands, the percentage identity at the nucleotide and amino acid (AA) levels between them were 98.65% and 97.03%, respectively.

3.3. Amino acid analysis

All amino acid mutations of RSV-A were shown in Table S1. Compared with the reference strain MZ515854, we identified the specific amino acid substitution in cluster 1 of the ON1 strain of RSV-A: P230T, E232G, T241P, G284S, S299N; specific amino acid substitution in cluster 2 of the folLlowing virus strains: V225A (23 strains), E232G/R, Y273H (28 strains), L274P (57 strains), L298P (36 strains), Y304H (10 strains), L310P (19 strains); and specific amino acid substitutions in cluster 3: E224A, V225A, E232G, L247P, Q258H, L266H, L274P, T282I, G296S, L298P, Y304H, L310P, S311P, L314P and T320A. All three clusters had an amino acid substitution at position 232, and there were the same or different amino acid substitutions but the same mutation site among the three clusters. For example, cluster 2 had the same amino acid substitution as cluster 1 at sites 284 (2 strains) and 299 (2 strains). The difference is that although both clusters had mutations at sites 230 and 300, 4 strains of cluster 2 were P230H, and 6 strains were P300Q. The same situation occurred at site 258, and there were 2 strains of Q258R in cluster 1, but the substituted amino acids in cluster 3 were different. Other mutations found in this study include T239A, E286K, T245A, L248F, P256Q, E257K, T264I, E271K, L289I, T293S, S294T and S313Y (Fig. 3).

All amino acid mutations of RSV-B were shown in Table S2. In comparison with the reference strain MZ516135, we found that there

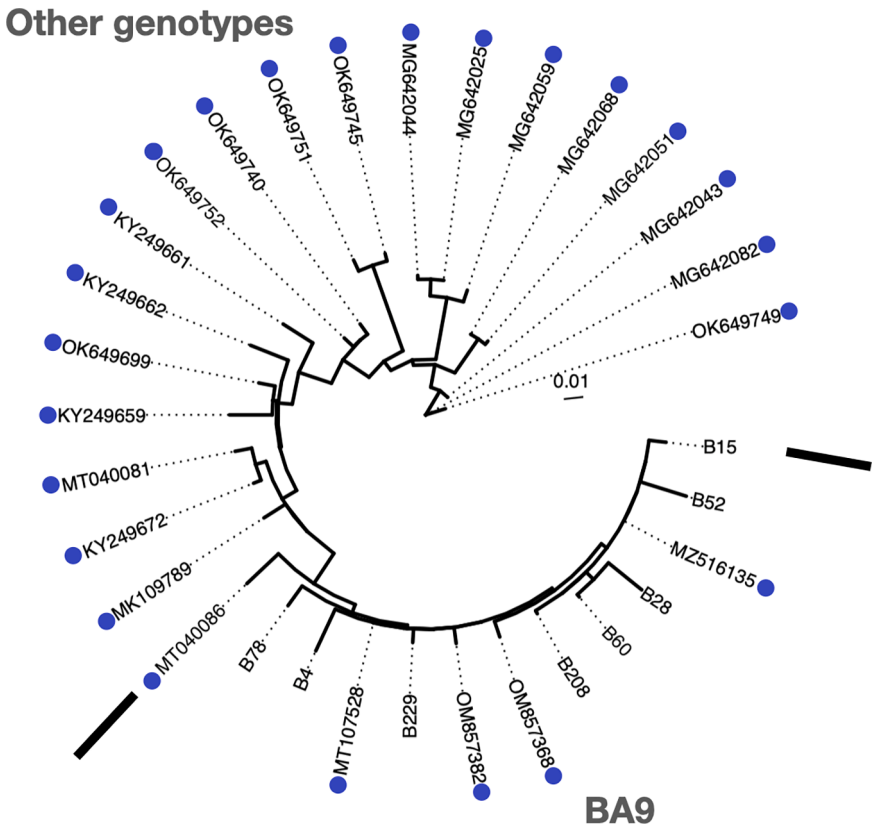

Fig. 2. G gene phylogenetic trees of RSV B strains. The phylogenetic trees of RSV B strains (B) derived in the study during 2021-2022 based on the G gene. The phylogenetic trees were constructed using the maximum likelihood method with 1,000 bootstraps. Reference genotypes downloaded from the GenBank are labeled with blue dots.

Table 3
Clinical symptoms of children with RSV infection.

Characteristics	Overall (n = 115)	Group		*P*-value
		ON1 (n = 107)	BA9 (n = 8)	
Age, mean (SD)	3.1 (1.5)	3.2 (1.5)	2.5 (1.1)	0.200
Female, N (%)	67 (58.3)	63 (58.9)	4 (50.0)	0.905
Fever, N (%)	114 (99.1)	106 (99.1)	8 (100.0)	1.000
Cough, N (%)	104 (90.4)	97 (90.7)	7 (87.5)	1.000
Expectoration, N (%)	63 (54.8)	57 (53.3)	6 (75.0)	0.411
Pharyngeal congestion or pharyngalgia, N (%)	99 (86.1)	93 (86.9)	6 (75.0)	0.682
Coarse breath sound, N (%)	104 (90.4)	99 (92.5)	5 (62.5)	0.031
Abdominal pain, N (%)	2 (1.7)	2 (1.9)	0 (0.0)	1.000
Diarrhea, N (%)	1 (0.9)	1 (0.9)	0 (0.0)	1.000
Vomit, N (%)	6 (5.2)	4 (3.7)	2 (25.0)	0.074
Pant, N (%)	2 (1.7)	1 (0.9)	1 (12.5)	0.312
Anhelation, N (%)	1 (0.9)	1 (0.9)	0 (0.0)	1.000
Otalgia, N (%)	2 (1.7)	2 (1.9)	0 (0.0)	1.000

were different degrees of amino acid mutations in the BA9 type of RSV-B (Fig. 4). The same amino acid substitutions at the same site included I252T (2 strains), V269A and A274T (3 strains), and 2 strains (E290D and E290K) with different amino acid substitutions at the same site. The amino acid substitutions of the BA9 type found in this study were T198P, K199Q, T201H, N202I, K203Q, K211R, T226I, N228G, K231E, K232E, T238A, D251N, T253A, K256E, I268T, S275P, T292I, N294Y, T296K, T300I and A301V.

4. Discussion

RSV is an important pathogen responsible for the development of ARTIs and has attracted the attention of pediatricians. [16,17]. In 2021, although strict interventions were implemented to prevent the transmission of COVID-19, the infection of other respiratory syncytial viruses including RSV did not decrease. In the present study, 1225 throat swabs were collected from the ARTIs of pediatric patients from July 2021 to January 2022, when represented a typical time period after the peak of COVID-19. The positive rate of RSV was 21.79%. This is lower than the infection rate reported in Hangzhou from 2011 to 2013 (34.5%) [18] but higher than the rate in Zhongshan from 2018 to 2020(12.67%) [19]. The focus of our research is the molecular epidemiology, evolution and transmission of RSV to identify epidemic viruses and clinical features, which is important for future epidemiological studies and possible evaluation of future anti-RSV therapy [20].

No gender bias was found in the present study. However, in contrast to previous studies showing that the highest incidence of RSV occurs in children under 1 year old [21,22], we found that the infection rate of children aged 3-6 years old was significantly higher than that of other age groups. A possible explanation might be that adults are required to wear masks in public and wash their hands more frequently due to the outbreak of COVID-19 during the study period, which reduces the risk of respiratory tract infection in adults. As a result, infants and young children under 3 years of age also have a corresponding reduction in the risk of infection. When the outbreak of COVID-19 has been effectively controlled, primary and secondary school children are more active and effective in implementing measures to prevent COVID-19 infection; in contrast, preschool children aged 3-6 years old are slightly weaker in this regard, resulting in an increased risk of infection. RSV is often co-infected with other respiratory viruses, and adenovirus co-infection

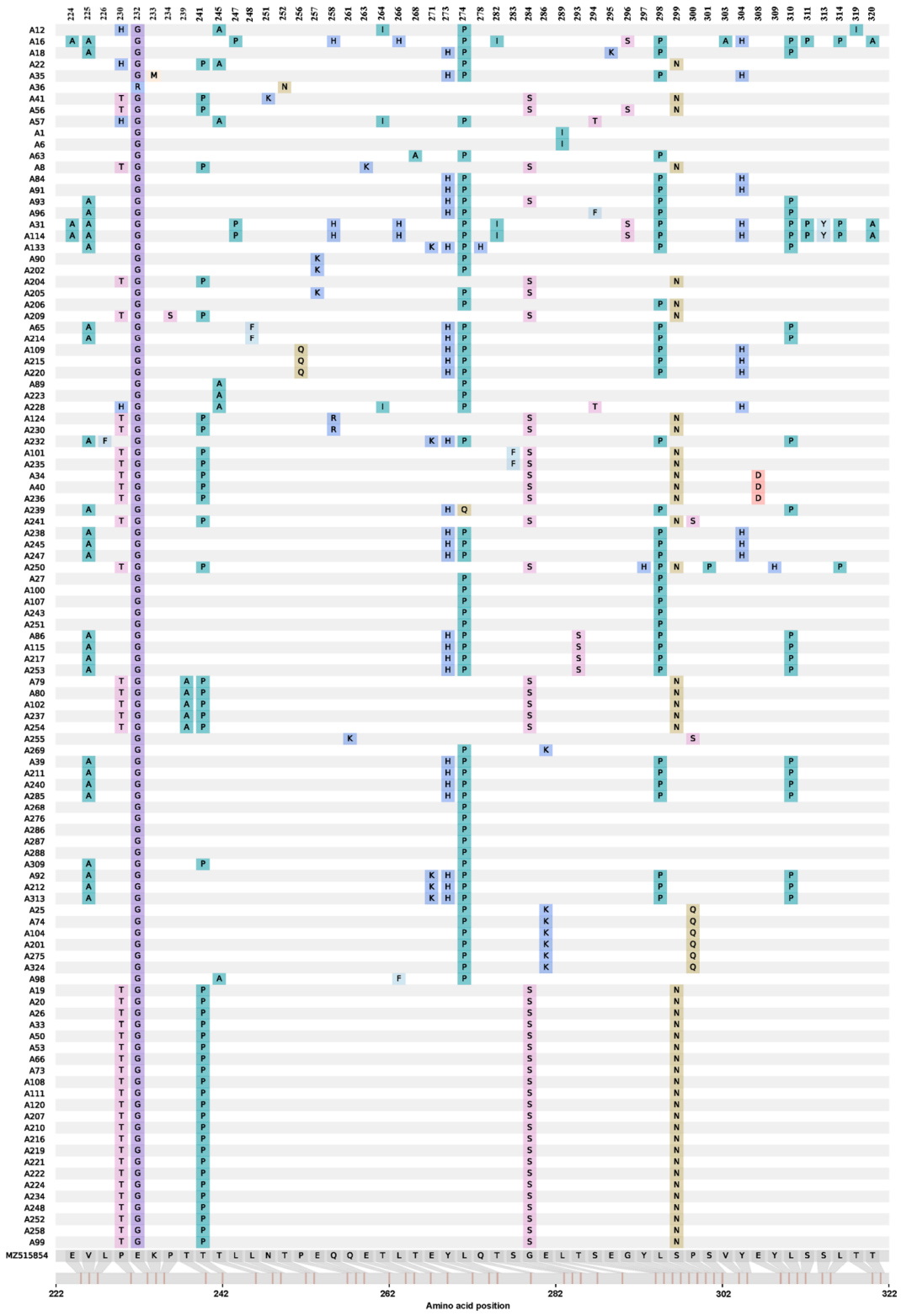

Fig. 3. Deduced amino acid alignments of the G genes from Hangzhou RSV-A strains and reference strains (MZ515854).

was dominant in this study, which is consistent with the research report in Guangzhou [23].

According to reports, the G protein of RSV is highly variable; the extracellular domain of G protein contains two mucin-like regions, which are rich in serine, threonine and proline, as well as N-linked oligosaccharides and O-linked oligosaccharides [24]. These two regions are called hyper variable regions HVR1 and HVR2, HVR1 and HVR2 are separated by a highly conserved region of 13 amino acids [25]. The hypervariable region is a high incidence area of gene mutation and an important area that stimulates the body to produce neutralizing antibodies [26]. From 2009 to 2014, the dominant subtype transformation pattern of RSV in southern Zhejiang was BAABB [27], in which the NA1 genotype was the most common subtype A, followed by the ON1 genotype. The main subtype of RSV transmission in children with ARTIs in

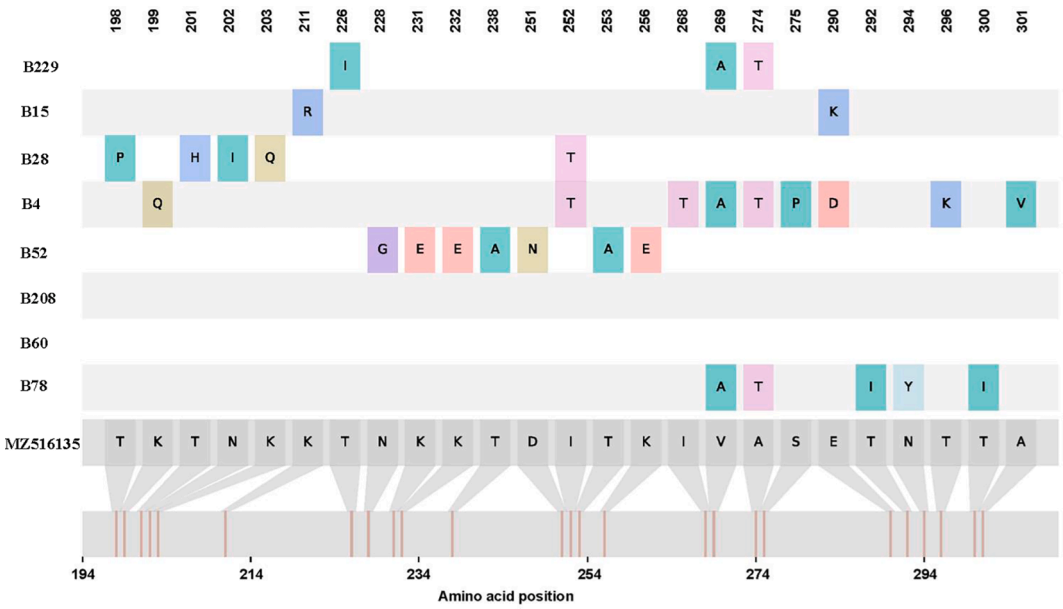

Fig. 4. Deduced amino acid alignments of the G genes from Hangzhou RSV-B strains and reference strains (MZ516135).

Shanghai from 2019 to 2021 was subtype B, and all of them were of the BA9 genotype [28]. While this study shows that subtype A is mainly prevalent in Hangzhou, all of them are ON1 genotype although the BA9 genotype of RSV-B was co-circulation in Hangzhou as well. The diversity of RSV strains and the continuous change in circulating strains in a region make it particularly important to determine the cycle pattern of strains and to understand the relationship between strains and RSV diseases.

According to the amino acid sequence alignment of G gene, it was found that there were different amino acid mutations at different sites between the same genotyped strains of RSV-A and RSV-B, which indicated that there were great differences among different genotypes of different subtypes in the evolution of RSV. The previous study has illustrated that the children who infected with genotype A/NA1 were more easily diagnosed lower respiratory tract infections and were required hospitalization more often than those who infected with genotype A/ON1 [29]. In addition, the clinical severity of RSV-A ON1 was higher in PICU-treated children [30]. In this study, all children were from outpatients who showed mild clinical symptoms.

In summary, our findings describe the epidemiological and genetic changes in RSV infection after the outbreak of COVID-19, and further emphasize the importance of continuous surveillance of RSV in the shadow of COVID-19 locally and globally. It is indispensable to study the relationship between the subtypes of RSV and the caused diseases, to calculate the correlation between the subtypes of RSV and the severity of the disease and clinical symptoms, and to establish preventive diagnosis and treatment programs for specific subtypes, which will also provide ideas for the development of follow-up vaccines.

5. Conclusion

The present study investigated the epidemiological and genetic characteristics of respiratory syncytial virus infection in children in Hangzhou after the epidemic peak of COVID-19. Although some prevention and control measures have been implemented in China, the incidence of RSV was high during this survey period. The incidence rate of preschool children reported in this study was higher, which is different from that described in previous studies. Phylogenetic analysis showed that the subtype A ON1 genotype was the dominant strain in Hangzhou from July 2021 to January 2022.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2022.105354.

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