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ORIGINAL ARTICLE

Molecular epidemiology and clinical characterization of human rhinoviruses circulating in Shanghai, 2012-2020

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Abstract

Human rhinoviruses (HRVs) cause acute upper and lower respiratory tract infections and aggravation of asthma and chronic obstructive pulmonary disease. The 5' untranslated region (5' UTR) and the VP4/VP2 region are widely used for genotyping of HRVs. Members of the species *Rhinovirus A* and *Rhinovirus C* have been reported to be more frequently associated with severe disease than members of the species *Rhinovirus B*. We report the clinical and molecular epidemiological characteristics of HRVs circulating from 2012 to 2020 in Shanghai. A total of 5832 nasopharyngeal swabs from patients with acute respiratory infections were collected. A real-time reverse transcription polymerase chain reaction assay was used for virus detection. The 5' untranslated region and VP4/VP2 region were amplified and sequenced for genotyping and phylogenetic analysis. The overall rate of rhinovirus detection was 2.74% (160/5832), with members of species A, B, and C accounting for 68.13% (109/160), 20.00% (32/160), and 11.88% (19/160) of the total, respectively. A peak of HRV infection was observed in autumn (5.34%, 58/1087). Patients in the 3- to 14-year-old age group were the most susceptible to HRV infection ($\chi^2 = 23.88$, $P = 0.017$). Influenza virus and *Streptococcus pneumoniae* were detected more frequently than other pathogens in cases of coinfection. Recombination events were identified in 10 strains, which were successfully genotyped by phylogenetic analysis based on the 5' UTR-VP4/VP2 region but not the 5' UTR region alone. We observed a high degree of variability in the relative distribution of HRV genotypes and the prevalence of HRV infection in Shanghai and found evidence of recombination events in the portion of the genome containing the 5' UTR and the VP4/VP2 region between HRV-C strains and HRV-A-like strains. This study is important for surveillance of the spread of HRVs and the emergence of new variants.

Introduction

Human rhinoviruses (HRVs) are positive-sense, single-strand RNA viruses belonging to the family *Picornaviridae*, genus *Enterovirus* [1]. Their genome is approximately 7.2 kb in size, with a single open reading frame joined to a 5' untranslated region (5' UTR), which is capped with a short viral primer protein (VPg). The viral genome encodes 11 proteins, including four structural proteins (VP1, VP2, VP3, and VP4) and seven nonstructural proteins. The structural proteins participate in the assembly of the viral capsid. The seven nonstructural proteins are involved in virus replication and the host cell immune response [2]. The first HRV was isolated in 1956 via tissue culture from a patient with a respiratory tract infection [3]. HRVs are among the most diverse RNA viruses and are classified into three species (*Rhinovirus A, B,* and *C*) [4]. HRV-A and B were discovered in the early 1960s in clinical specimens, whereas HRV-C was identified in 2006 [5]. Advances in molecular biological technology have allowed a deeper classification of rhinovirus genotypes. The highly conserved nucleotide sequences of the 5' UTR and VP4/VP2 regions are widely used for HRV subtyping [6, 7], and currently, more than 150 rhinovirus subtypes have been identified, making it difficult to develop effective universal vaccines against all HRV serotypes [8].

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HRVs are common respiratory pathogens, causing upper and lower respiratory tract infections [9]. Rhinoviruses can infect bronchial epithelial cells to cause cytopathology and induce proinflammatory responses [10]. Numerous studies have shown that HRV infection aggravates asthma in children and chronic obstructive pulmonary disease (COPD) in middle-aged and elderly people in addition to causing acute respiratory infections (ARIs) [8]. It has been reported that pneumonia patients infected with HRVs are more likely to be immunocompromised than those infected with influenza viruses [9]. Studies have also shown that HRV-C is more frequently associated with lower respiratory tract infections and severe illness than HRV-A and HRV-B [10, 11], but no relationship has been found between clinical type and HRV species [12]. Rhinoviruses can be detected throughout the world and throughout the year. The detection rate varies in different countries and regions with different climatic conditions. In China, the detection rate of HRV infection in ARI cases has been reported to range from 3.7% to 17.9% [13–15]. The peak of HRV infection occurs in the spring and fall, but HRV infection causes more-severe disease in the winter [16]. Although rhinoviruses can infect individuals of all ages, children are more susceptible to this virus and tend to develop more-severe illnesses leading to hospitalization [11].

In this study, we launched an 8-year-long molecular epidemiology surveillance of common respiratory viruses in Shanghai, China, to investigate the prevalence and diversity of HRV strains and clinical characteristics of HRV infection. Our study showed a high degree of variation in HRV subtypes and the prevalence of HRV-A in Shanghai. In addition, we observed evidence of recombination events in the region linking the 5' UTR and VP4/VP2 region between HRV-C strains and strains related to HRV-A. These findings suggest that it is better to use the 5' UTR-VP4/VP2 region than the 5' UTR alone to classify HRV strains and to identify novel recombinants. This study is important for surveillance of the spread of HRVs and the emergence of new variants.

Materials and methods

Sample collection

From January 2012 to April 2020, 5832 nasopharyngeal swabs from patients with symptoms of acute respiratory infection (ARI) who consulted any of the six sentinel hospitals were collected. The six sentinel hospitals included Shanghai Public Health Clinical Center, Shanghai Xinhua Hospital, Shanghai Tongren Hospital, Shanghai East Hospital, Shanghai No. 5 People's Hospital, and Shanghai Pediatric Hospital, whose serving areas cover five of the 16 administrative districts of Shanghai. The definition of ARI symptoms includes fever (>37.3°C), cough, and other symptoms listed in Table 1.

Swabs were immediately stored in virus transport medium (VTM) and transported in a cold chain to the laboratory at the Shanghai Public Health Clinical Center for virus detection. The clinical data of patients were recorded.

Table 1 Clinical and demographic characteristics of enrolled patients from 2012 to 2020

	All patients	HRV-positive (n = 160) (%)	χ^2	*P*-value
Age group (years)				
0~2	279	4 (1.43)		
3-14	889	59 (6.64)	61.376	**0.000***
15-29	1155	22 (1.90)		
30-44	1147	25 (2.18)		
45-59	820	18 (2.20)		
60-69	703	15 (2.13)		
70-79	523	11 (2.10)		
≥80	316	5 (1.58)		
Gender				
Male	3076	89 (2.89)	0.548	0.459
Female	2756	71 (2.58)		
Patient type				
Outpatient	3142	94 (2.99)	1.573	0.210
Inpatient	2690	66 (2.45)		
Clinical manifestation				
Fever	4975	138 (2.77)		
Cough	4007	97 (2.42)		
Rhinorrhea	1289	51 (3.96)		
Sore throat	1686	40 (2.37)		
Expectoration	2217	47 (2.12)		
Chest pain	290	13 (4.48)		
Wheezing	666	10 (1.50)		
Dyspnea	486	4 (0.82)	30.039	**0.002***
Headache	838	21 (2.51)		
Loss of activity	812	14 (1.72)		
Stomachache	64	2 (3.13)		
Diarrhea	95	1 (1.05)		
Diagnosis				
URTI	1641	69 (4.20)	5.963	0.113
LRTI				
Bronchiolitis	185	5 (2.70)		
Pneumonia	1331	44 (3.31)		
COPD/AECOPD	381	7 (1.84)		

Abbreviations: URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; COPD/AECOPD, chronic obstructive pulmonary disease/acute exacerbation of chronic obstructive pulmonary disease; HRV, human rhinovirus

*Statistical significance: $p < 0.05$. The bold values represent significant results with $P < 0.05$.

The study was approved by the Ethics Committee of Shanghai Public Health Clinical Center (2018-S045-02).

Virus detection

Viral RNA was extracted from the samples using a nucleic acid extraction and purification kit (BioGerm, Shanghai), following the user's guide. cDNA was synthesized and virus-specific real-time PCR was performed using a One Step PrimeScript RT-PCR Kit (TaKaRa, Japan). The sequences of the primers and TaqMan probes are listed in Table 2 and Supplementary Table S1. The reaction conditions were 42°C for 10 min, followed by 95°C for 1 min and 40 cycles of 95°C for 10 s and 55°C for 1 min.

The viral genomes of HRV isolates were transcribed to cDNA using a SuperScript® III First-Strand Synthesis Kit (Invitrogen, USA). Then, the 5' UTR and VP4/VP2 fragments were amplified using Platinum™ SuperFi™ DNA Polymerase (Invitrogen, USA). The sizes of the 5' UTR and VP4/VP2 amplicons were approximately 390 bp and 610 bp, respectively. The PCR reaction conditions were 98°C for 30 s for initial denaturation, followed by 45 amplification cycles with denaturation for 10 s at 98°C, annealing for 10 s at 55°C, and elongation for 30 s at 72°C, and a final extension at 72°C for 10 min. The sequences of primers used to amplify the 5' UTR and VP4/VP2 regions are shown in Table 2. PCR products were examined and extracted from agarose gels using a QIAquick Gel Extraction Kit (QIAGEN). The DNA fragments of each isolate were inserted into the pZero-Blunt vector, and three to five individual clones were selected and sent to Sangon Biotech, Shanghai, for Sanger sequencing. The plasmid pZero-Blunt was provided by the Key Laboratory of Medical Molecular Virology, Shanghai Medical College, Fudan University, Shanghai, China.

Table 2 Primers used for sequencing and real-time PCR

Name	Primer	Sequence (5'→3')
HRV-5'UTR	5'UTR -F	CAAGCACTTCTGTTWCCCC
	5'UTR -R	ACGGACACCCAAAGTAGT
HRV-VP4/VP2	VP4/VP2 -F	GCCCCTGAATGYGGCTAA
	VP4/VP2 -R	GGTAAYTTCCACCAC-CANCC
HRV real-time PCR	HRV -F	CTCCGGCCCCTGAATRYG GCTAA
	HRV -R	TCIGGIARYTTCCASYAC-CAICC
	HRV -P	**FAM-**TCCTCCGGCCCCTGA ATGYGGCTAA-**BHQ1**

Sequence analysis

Sequencing results for the 5' UTR and VP4/VP2 fragments were verified and the vector sequences were removed using SnapGene software. Consensus sequences derived from the multiple clones were used for analysis. All of the sequences were subjected to a BLAST search against rhinovirus reference sequences in the NCBI Nucleotide GenBank database (https://blast.ncbi.nlm.nih.gov/) for preliminary genotyping.

Phylogenetic analysis was performed using MEGA software (version X) [17]. Genetic saturation analysis was performed using Data Analysis in Molecular Biology and Evolution (DAMBE) software [18, 19]. A phylogenetic tree was constructed by the maximum-likelihood (ML) method, using the general time-reversible (GTR) model. The phylogenetic tree was visualized using Adobe Illustrator software. Sequence identity values were determined using BioEdit (version 7.0).

Excel and SPSS software (version 20.0, IBM Corporation, Armonk, NY, USA) were used for statistics analysis. Differences between groups were analyzed using a chi-square test or Fisher's exact test. Statistical significance was set at $p < 0.05$.

Recombination analysis

Recombination analysis was performed using Recombination Detection Program (RDP) (version 5.05) with the RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq algorithms [20]. Potential recombination events were further evaluated using bootscan and similarity analysis in SimPlot software (version 3. 51).

Results

Prevalence of HRV infection

From January 2012 to April 2020, 5832 nasopharyngeal swabs from ARI patients were collected and tested for common respiratory viruses by real-time PCR. The detection results for all respiratory viruses are shown in Supplementary Figure S1. Influenza virus (12.07%, 704/5832) was the most frequently detected respiratory virus, followed by human rhinovirus (2.74%,160/5832), adenovirus (2.21%,129/5832), respiratory syncytial virus (RSV) (1.69%,99/5832), bocavirus (1.27%,74/5832), coronavirus (CoV) (0.87%, 51/5832), human metapneumovirus (0.86%,50/5832), and parainfluenza virus (PIV) (0.69%, 40/5832).

Phylogenetic analysis of HRV strains

All 160 HRV strains were first classified based on the sequence of the 5' UTR. A substitution saturation test revealed that the sequences were phylogenetically diverse (Iss<Iss.c and $p < 0.05$).

Phylogenetic analysis based on 5' UTR sequences showed that most of the isolates grouped into three genetic clades, A, B, and C, accounting for 101, 32, and 3 of the 160 isolates, respectively (Fig. 1). The nucleotide sequence identity values for HRV-A, B, and C within the individual clades were 66.2%-100%, 76.7%-100%, and 78.1%-85.4%, respectively. However, five strains (212-2020.6, 227-2020.7, 260-2020.8, 5744-2013.9, 9554-2017.6) that were most similar to HRV-A reference strains in BLAST analysis and six strains (171-2020.1, 299-2020.9, 4217-2012.10, 4234-2012.10, 10384-2019.1, 7089-2014.10) that were most similar to HRV-C reference strains in BLAST analysis mixed in cluster 1. The intra-cluster sequence identity in cluster 1 was 74.3%-100%. Three strains (4204-2012.10, 5980-2013.12, 7135-2014.11) that were most similar to HRV-A reference strains in BLAST analysis and 10 strains (102-2019.12, 165-2020.1, 286-2020.9, 291-2020.9, 282-2020.9, 350-2020.10, 316-2020.10,4360-2012.11, 7344-2015.1, 9854-2018.1) that were most similar to HRV-C reference strains in BLAST analysis were grouped in cluster 2. The intra-cluster sequence identity in cluster 2 was 75.5%-100%. Hence, 24 strains in total could not be completely classified in the tree based on the 5' UTR sequences.

These 24 isolates were then subjected to RT-PCR for amplification of the entire 5' UTR-VP4/VP2 region, and 13 of them yielded amplicons, which were sequenced for further genotyping (GenBank ID: OM976042-OM976054). Of these, 10 were from cluster 1 and three were from cluster 2 in the previous analysis. Another two ML trees, based on the VP4/VP2 or 5' UTR-VP4/VP2 region, were constructed (Figs. 2 and 3). The results showed that eight of the isolates were HRV-A and five were HRV-C. The remaining 11 strains were judged to be HRV-C based on BLAST results. Thus, of the 160 HRV isolates, 109 were HRV-A, 32 were HRV-B, and 19 were HRV-C.

Recombination analysis

To investigate why the 5' UTR alone was not sufficient for HRV genotyping based on phylogenetic analysis, recombination analysis was performed. The 5' UTR-VP4/VP2 sequences of 13 isolates (HRV-A or HRV-C) and those of the corresponding reference strains were imported into RDP software to identify the potential parent strains of each isolate. Potential recombination events were then further evaluated based on Bootscan and Similarity analysis (Fig. 4). The entire 5' UTR-VP4/VP2 fragment of all 13 strains produced three sub-fragments in Bootscan analysis. The lengths of the sub-fragments varied among the 13 strains. The first sub-fragment ended at nt position 240-360. The second sub-fragment started from nt position 240-360 and ended at nt position 440-660. The third sub-fragment started at position nt 440-620. The second sub-fragment showed very low similarity to either of the parental strains. A switch with high probability of sequence permuting (>95%) to parent strains was observed in the third sub-fragment in the Bootscan plots of 10 isolates, which indicated that a recombination event was likely to have occurred. As a consequence, five strains (171-2020.1, 299-2020.9, 4217-2012.10, 4234-2012.10, 10384-2019.1) were classified as HRV-A based on the 5' UTR but as HRV-C based on the VP4/VP2 region, and the other five strains (212-2020.6, 227-2020.7, 260-2020.8, 5744-2013.9, 9554-2017.6) were classified as HRV-C based on the 5' UTR but as HRV-A based on the VP4/VP2 region.

Seasonality of HRV infection

Although sample collection was interrupted for 9 months in 2016 due to a human resource problem and restarted in 2017, the seasonality of HRV from 2012 to 2020 was still analyzed (Fig. 5). Rhinovirus could be detected each year from 2012 to 2020. HRV circulated throughout the year but was detected most frequently in autumn (September-November) (5.34%, 58/1087), followed by summer (June-August) (3.29%, 38/1155), winter (December-February), (2.09%, 44/2106), and spring (March-May) (1.35%, 20/1484). The peak of HRV infection mainly occurred in autumn. HRV-A was the predominant type circulating in Shanghai (Fig. 6). In addition, there was a peak of HRV-positive samples in October due to a larger number of samples being collected during that period (Figs. 5 and 6).

Age distribution of HRV infection

Of the 160 HRV-positive samples, 89 (2.89%, 89/3076) were from male patients and 71 (2.58%, 71/2756) were from female patients. The clinical and demographic characteristics of HRV-infected patients are shown in Table 1. The patients, aged from 2 to 87 years old, were grouped into eight age groups. The HRV detection rate for the groups of 0-2, 3-14, 15-29, 30-44, 45-59, 60-69, 70-79, and ≥80 years was 1.43%, 6.64%, 1.90%, 2.18%, 2.20%, 2.13%, 2.10%, and 1.58%, respectively. Statistical analysis showed that the 3- to 14-year-old group was the most susceptible to HRV infection ($\chi^2 = 23.88$, $P = 0.017$) (Table 1). Furthermore, there was a significant difference in the prevalence of species B and

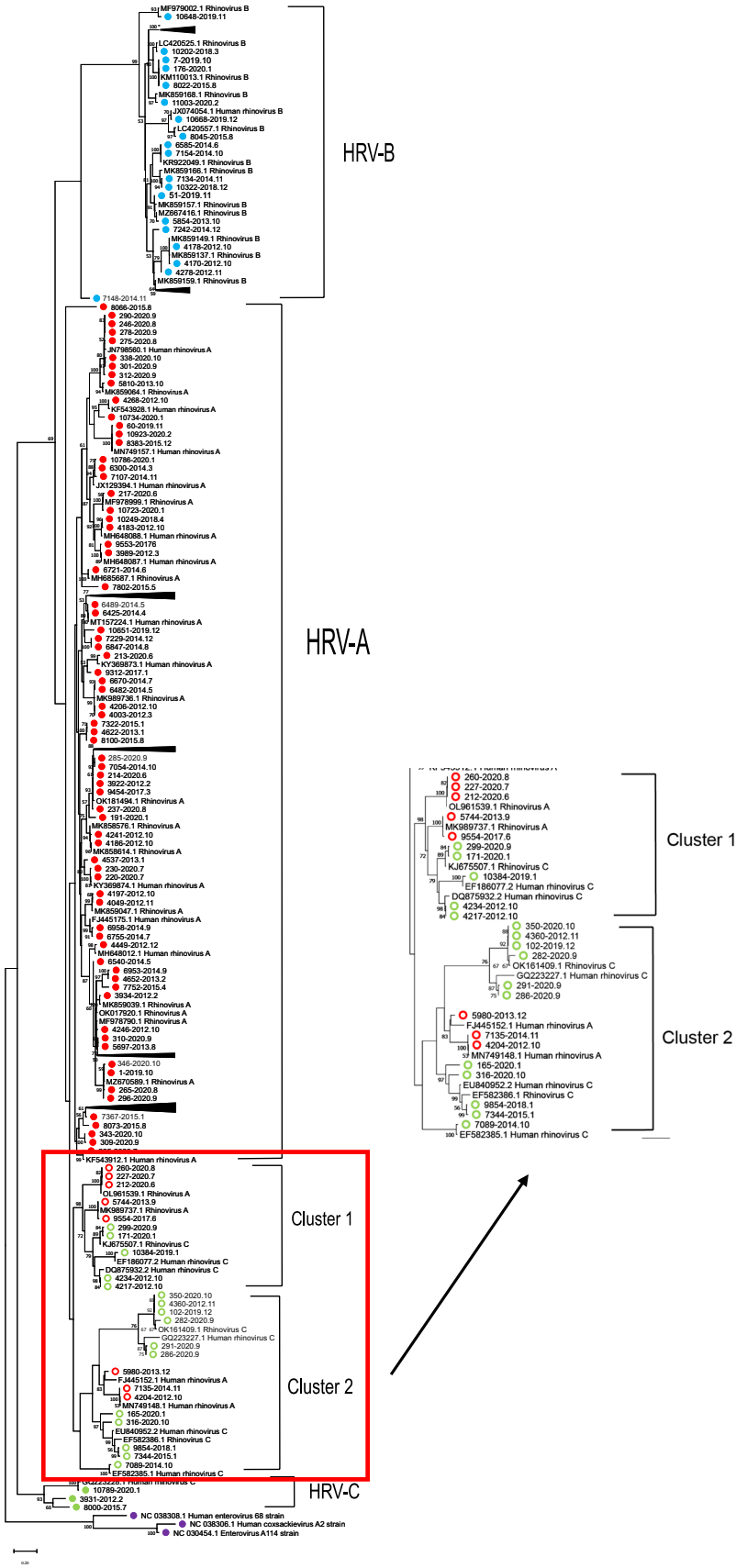


Fig. 1 Phylogenetic tree based on the 5' UTR region of HRV, constructed by the maximum-likelihood method with 100 bootstrap iterations. Tree branches are proportional to genetic distance, and all bootstrap values greater than 50 are shown at the branches. Reference sequences are represented by GenBank ID, and strains from clinical samples are indicated by a sample ID number and the detection date. Black triangles indicate sequences with high similarity (95.1%-100% identity) at the merged branch ends. (HRV-B: 7274-2015.1, 7190-2014.12, 7574-2015.2, 7754-2015.4, 4691-2013.3, 7196-2014.12, 322-2020.10, 10316-2018.11, 157-2020.1, 7742-2015.4, 7395-2015.1, 4291-2012.11, and 359-2020.10. HRV-A: 221-2020.7, 5677-2013.8, 4367-2012.11, 5697-2013.8, 310-2020.9, 4202-2012.10, 219-2020.6, 292-2020.9, 300-2020.9, 231-2020.7, 271-2020.8, 298-2020.9, 255-2020.8, 235-2020.7, 216-2020.6, 215-2020.6, 3983-2012.3, 4282-2012.11, 4525-2012.12, 6273-2014.3, 174-2020.1, 6353-2014.4, 6875-2014.8, 6966-2014.9, 7225-2014.12, 7226-2014.12, 7321-2015.1, 7677-2015.3, and 9567-2017.6) Strains most similar to HRV-A reference strains are shown in red, strains most similar to HRV-B reference strains are shown in blue, and strains most similar to HRV-C reference strains are shown in green. The strains in clusters 1 and 2 are indicated by circles. Purple dots indicate the outgroup reference strains.

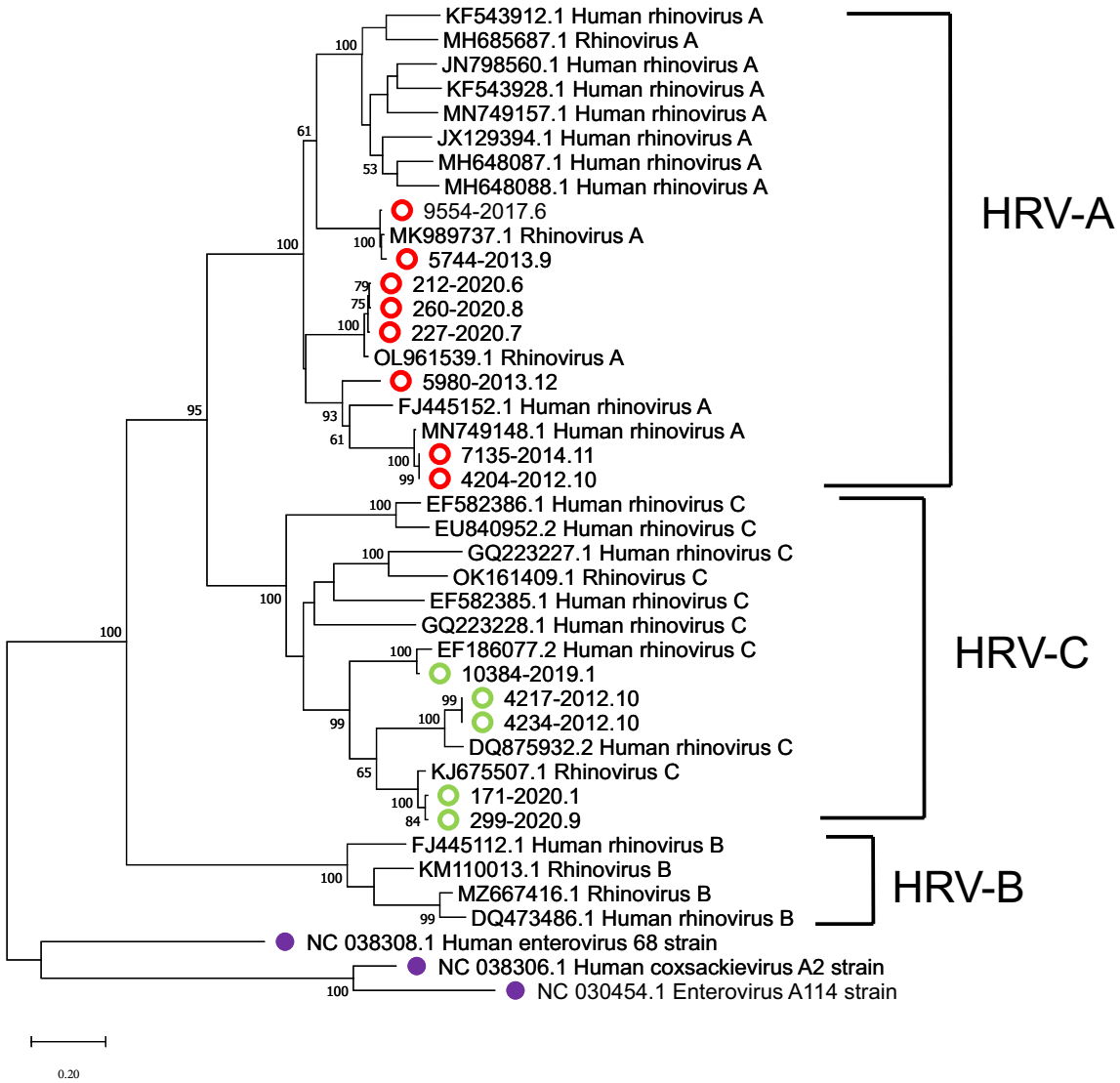


Fig. 2 Phylogenetic tree based on the VP4/VP2 region of HRV, constructed by the maximum-likelihood method with 100 bootstrap iterations. Tree branches are proportional to genetic distance, and all bootstrap values greater than 50 are shown at branches. Reference sequences are represented by GenBank ID, and strains from clinical sample are indicated by a sample ID number and the detection date. Strains most similar to HRV-A reference strains are shown in red and strains most similar to HRV-C reference strains are shown in green. The strains in clusters 1 and 2 in Figure 1 are indicated by circles. Purple dots indicate the outgroup reference strains.

C compared to species A in the 3- to 14-year-old age group ($\chi^2 = 23.88$, $P = 0.017$) (Table 3).

Clinical characteristics of HRV infection

Clinical information about the patients and their HRV detection results were analyzed statistically (Table 1). The proportion of outpatients and inpatients was 2.99% (94/3142) and 2.45% (66/2690), respectively, with no significant difference. Among all of the clinical manifestations, dyspnea was the least common ($\chi^2 = 30.039$, $P = 0.002$) (Table 1). Of the 160 patients, 69 (43.125%) had a clinical diagnosis of upper respiratory tract infection (URTI), and 56 (35.00%) had a diagnosis of lower respiratory tract infection (LRTI), with bronchitis, pneumonia, or COPD/AECOPD, and, no statistical difference was found. Furthermore, no statistical difference was observed for the different HRV species.

Coinfecting pathogens

Twenty-four of the HRV-positive patients were coinfected with another pathogen: six (3.75%) with influenza virus, two (3.75%) with RSV, one (3.75%) cases with CoV, and one (3.75%) with PIV (Table 4).

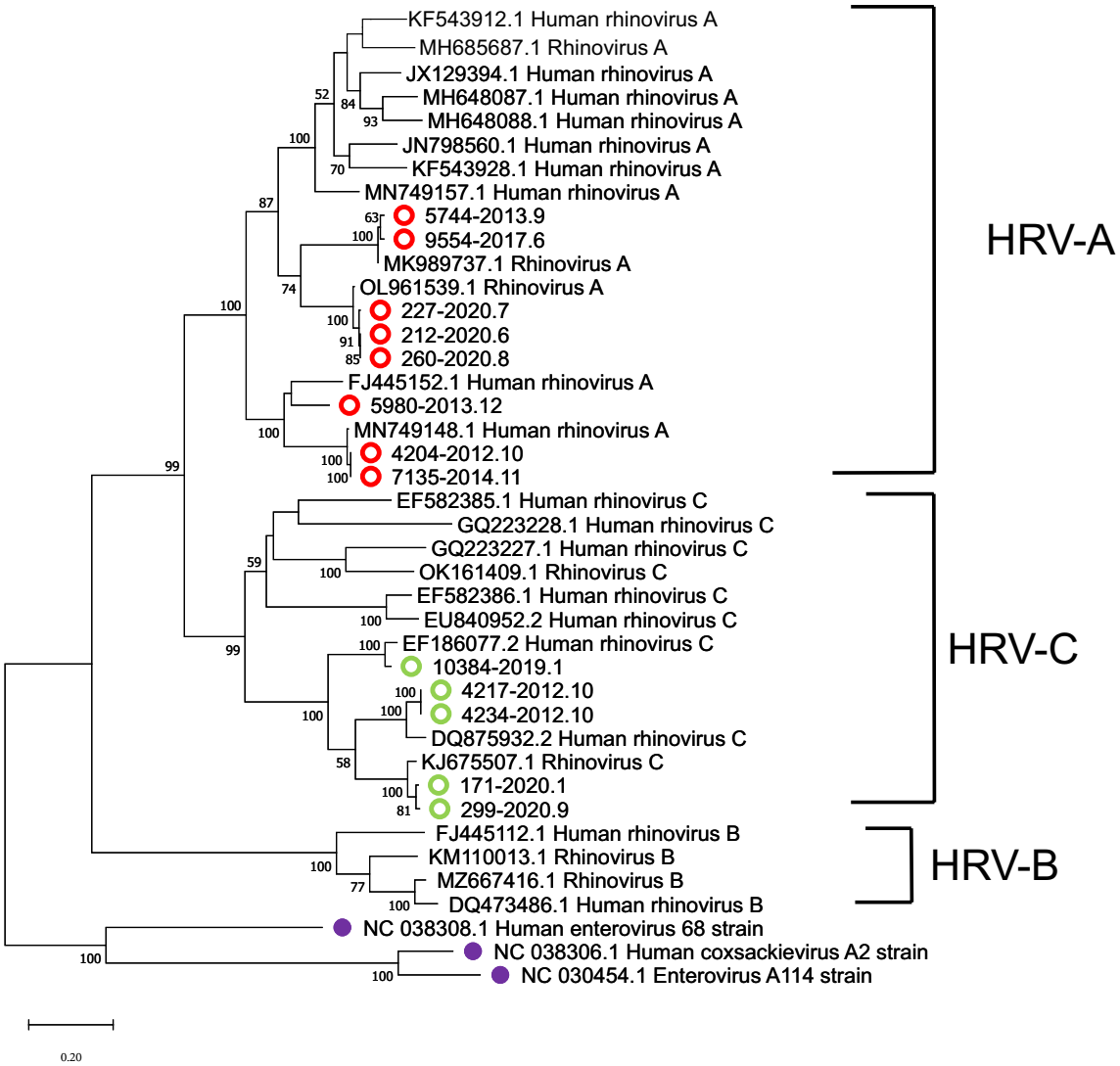


Fig. 3 Phylogenetic tree based on the 5'UTR-VP4/VP2 region of HRV, constructed by the maximum-likelihood method with 100 bootstrap iterations. Tree branches are proportional to genetic distance, and all bootstrap values greater than 50 are shown at the branches. Reference sequences are represented by GenBank ID, and strains from clinical sample are indicated by a sample ID number and detection date. Strains most similar to HRV-A reference strains are shown in red and strains most similar to HRV-C reference strains are shown in green. The strains in clusters 1 and 2 in Figure 1 are indicated by circles. Purple dots indicate the outgroup reference strains.

Among the HRV-bacterial coinfection cases, *Streptococcus pneumonia* (11, 6.875%) was the most frequently detected bacterium (Table 4). Further analysis showed no statistical significance among HRV-A, HRV-B, and HRV-C in the coinfection cases ($\chi^2 = 0.769$, $P = 0.777$) (Supplementary Table S2).

Discussion

Human rhinoviruses (HRV) not only cause common influenza-like respiratory illnesses but also are responsible for other diseases, including pediatric and adult asthma, chronic obstructive pulmonary disease (COPD), acute otitis media, sinusitis, pneumonia, and bronchopneumonia [8, 21, 22]. Studies have shown that members of HRV species A and C cause more-severe infections and are more likely to exacerbate asthma in children and adults than members of species B [23], suggesting that studies on the development of

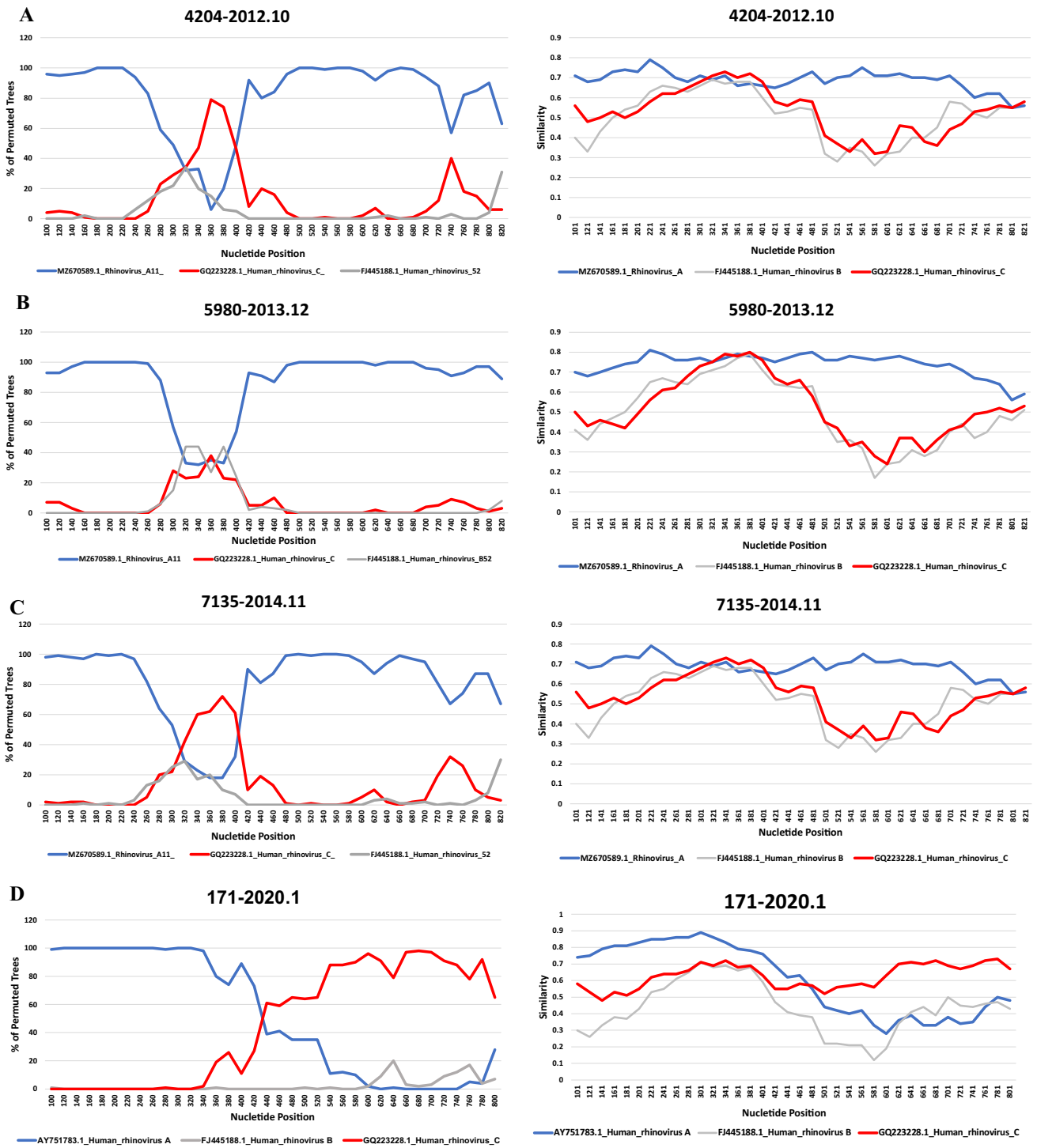


Fig. 4 Bootscan (left) and Similarity (right) plots for recombination analysis. The *y*-axis shows the probability of sequence permuting in Bootscan and the sequence similarity in Simplot plots with a window of 200 base pairs and a step size of 20 base pairs. Thirteen strains are represented by the letters A to M.

antivirals and vaccines should focus more on species A and C [24]. Some previous studies have suggested that HRV-C could be further classified into two subgroups: HRV-Cc and HRV-Ca. The HRV-Ca variants were apparently the result of genetic recombination during coinfection with HRV A and C. The HRV-Cc and HRV-Ca subtypes are more prevalent

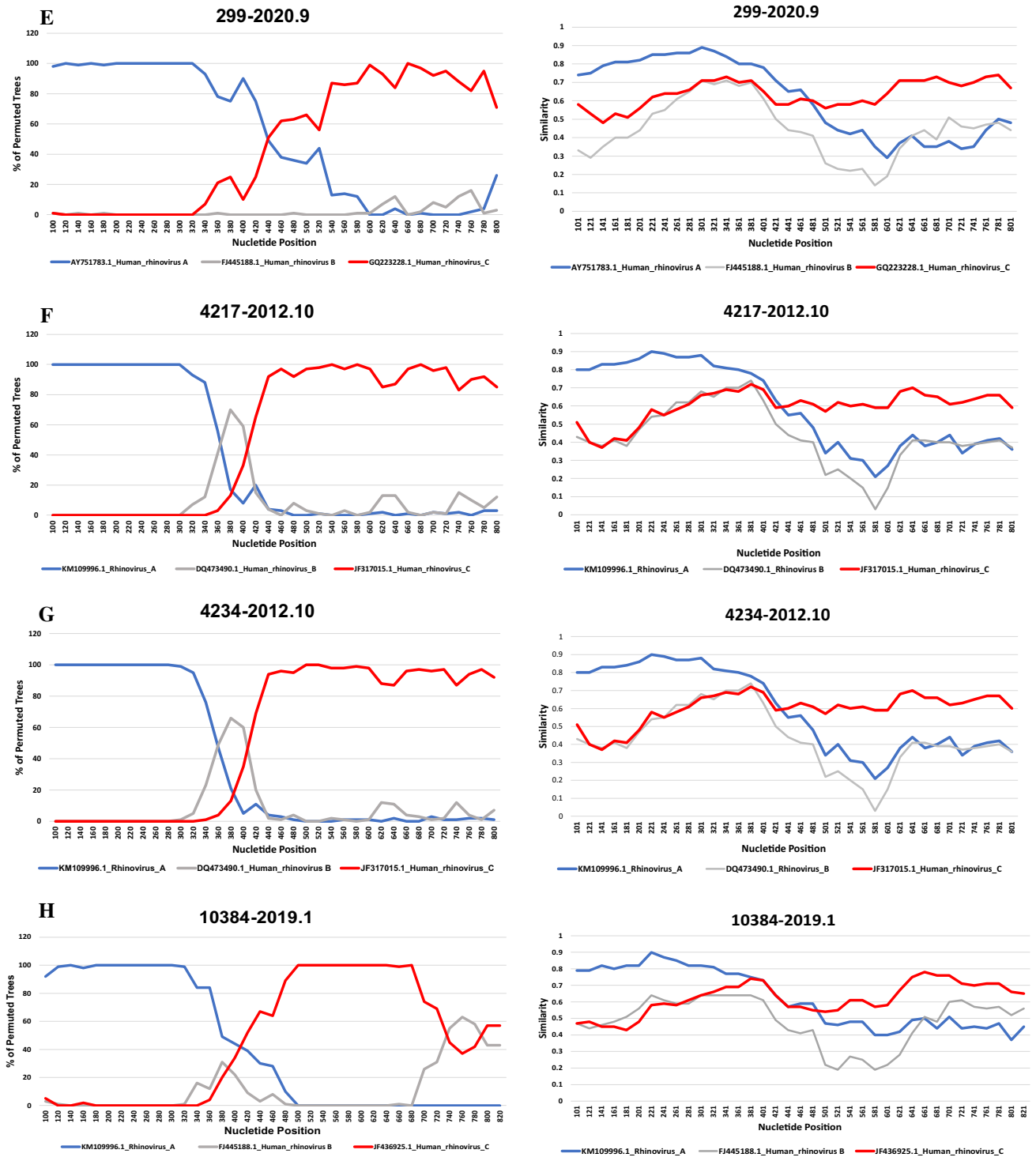
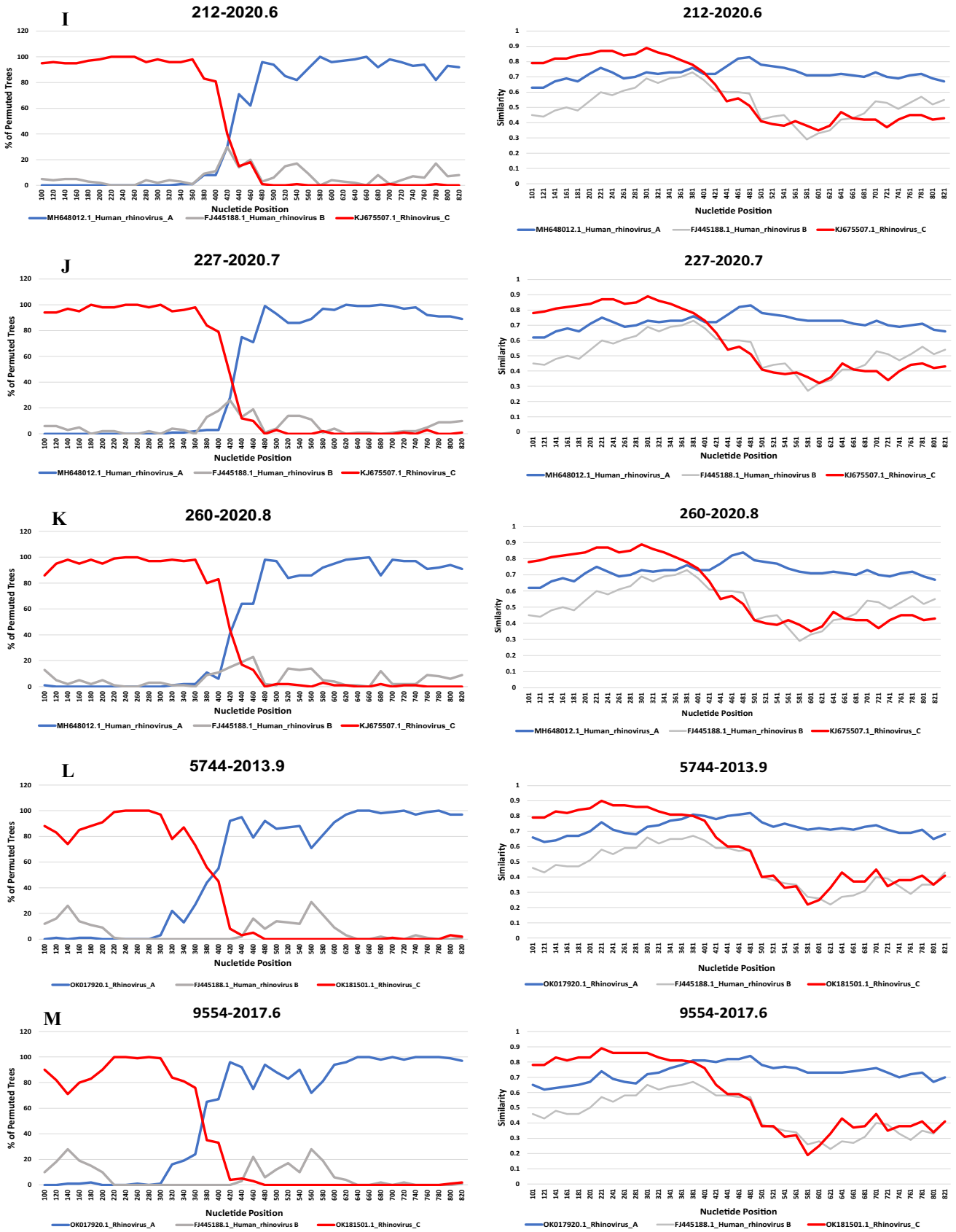


Fig. 4 (continued)

in asthmatic preschool children [25]. Therefore, accurate genotyping of HRV is important for predicting the clinical severity of the infection.

In this study, the overall detection rate of rhinovirus in acute respiratory infection samples collected from 2012 to 2020 was 2.74% (160/5832). HRV-A was the predominant type circulating in Shanghai. A peak of HRV infection was observed in autumn (5.34%, 58/1087). These findings are consistent with those of previous studies [4, 14, 26, 27].



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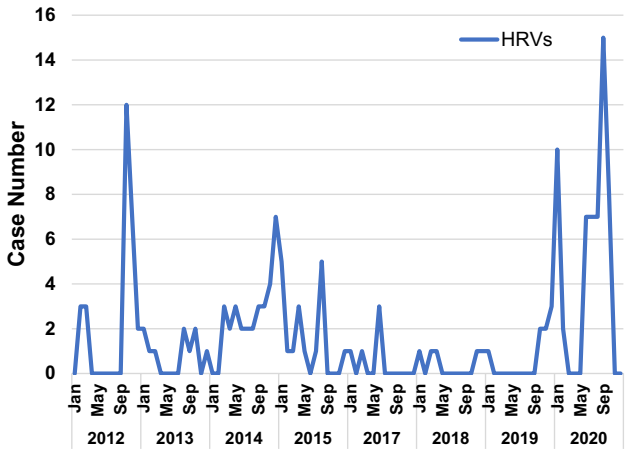

Fig. 5 Monthly distribution of HRV infections from 2012 to 2020. Sample collection was interrupted for 9 months in 2016 due to a human resource problem and was restarted in 2017.

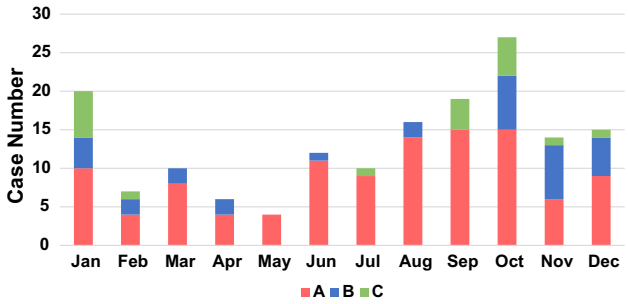

Fig. 6 Monthly distribution of infections with HRV-A, B, and C. Each column represents the combined data from the same month from 2012 to 2020.

Patients in the 3- to 14-year-old age group were found to be the most susceptible to HRV infection ($\chi^2 = 23.88$, $P = 0.017$). HRV has been reported to exacerbate chronic lung disease in children age under 5 years old and cause underdeveloped adaptive immune responses in infants and young children [28]. Thus, HRV infection in young children should be given special attention. Compared to other clinical symptoms, dyspnea was the least frequent in this study, with statistical significance ($\chi^2 = 30.039$, $P = 0.002$).

Twenty-four of the 160 patients had coinfections, with influenza virus and *Streptococcus pneumonia* being the most common coinfecting pathogens. High rates of influenza virus and parainfluenza virus coinfections with HRV have been reported [29]. Coinfections with other viruses or bacteria may increase the severity of HRV infections [30]. HRV increases the risk of bacterial infections by promoting bacterial endocytosis [31]. Therefore, the identification of coinfection is critical for disease treatment.

All 160 isolates were classified into three subtypes, HRV-A, HRV-B, and HRV-C, by phylogenetic analysis. Interestingly, 24 isolates that were most similar to HRV-A or HRV-C reference strains in a BLAST search formed mixed clusters in a phylogenetic tree based on 5' UTR sequences, but 13 of these 24 isolates were completely separated into clades HRV-A and HRV-C in trees based on the VP4/VP2 region or the 5' UTR-VP4/VP2 region. Although both the highly conserved nucleotide sequence of the 5' UTR and that of VP4/VP2 region are widely used for HRV subtyping, the VP4/VP2 region is applied more commonly for classification of HRV types A and C [32]. Our study suggests that the longer fragment 5' UTR-VP4/VP2 should be used for HRV genotyping, since the 5'UTR region alone did not yield adequate results.

To investigate the ambiguous genotyping result, recombination analysis was performed on the 5' UTR-VP4/VP2 region of 13 isolates. The results suggested that recombination events had occurred between the 5' UTR and VP4/VP2 regions in 10 of the 13 isolates. The high variability of this region of the genome may be the reason for the genotyping difficulty. Studies have indicated that mutations

Table 3 Comparison of age distribution of HRV-positive patients

	HRV-positive (n = 160) (%)	HRV-A (n = 109)	HRV-B (n = 32)	HRV-C (n = 19)	χ^2	*P*-value
Age group (years)						
0~2	4 (1.43)	2	1	1		
3-14	59 (6.64)	**41**	**7**	**11**	23.88	**0.017***
15-29	22 (1.90)	19	2	1		
30-44	25 (2.18)	16	7	2		
45-59	18 (2.20)	12	6	0		
60-69	15 (2.13)	8	6	1		
70-79	11 (2.10)	8	2	1		
≥80	5 (1.58)	2	1	2		

*Statistical significance: $p < 0.05$. The bold values represent significant results with $P < 0.05$.

Table 4 Coinfecting pathogens

	Sample number	%
Flu + HRV	6	3.75
RSV + HRV	2	1.25
COV + HRV	1	0.625
PIV + HRV	1	0.625
Sp.+HRV	11	6.875
Mcat.+ HRV	4	2.50
Sa.+HRV	4	2.50
Hi. + HRV	3	1.875
Mpn .+ HRV	2	1.25
Kp.+ HRV	1	0.625

Flu, influenza virus; RSV, respiratory syncytial viral; COV, coronavirus; PIV, parainfluenza virus; *Sp.*, *Streptococcus pneumonia*; *Mcat.*, *Moraxella catarrhalis*; *Sa.*, *Staphylococcus aureus*; *Hi.*,*Haemophilus influenzae*; *Mpn.*, *Mycoplasma pneumoniae*; *Kp.*, *Klebsiella pneumoniae*

in RNA viruses that occur via a recombination mechanism contribute to the ability of these viruses to invade the respiratory system [33–35]. Recombination between members of species A and C mostly occurs in the 5' UTR and in the 2A protease gene [36]. However, the pathogenic and clinical significance of these mutations has not yet been determined.

In conclusion, we report here the clinical and molecular epidemiological characteristics of HRVs circulating from 2012 to 2020 in Shanghai. This study provides information that will be useful for the prevention, control, and treatment of viral respiratory infections in future.

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Author contributions WW conceived and designed the study and revised the manuscript. ZY provided precious advice on cloning experiments. HJ did the cloning experiments and sequence analysis and wrote the manuscript. TY and QZ collected all the samples and carried out the clinical and epidemiological analysis. CY and YL did the virus detection. All authors read the manuscript and approved the submitted version.

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Declarations

Conflict of interest None of the authors have any competing interests to declare.

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