Molecular Epidemiological Characteristics of Human Rhinovirus Circulating in Shanghai, 2012 - 2020

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Abstract

Background

Human Rhinoviruses (HRVs) are a common respiratory virus causing acute exacerbations of asthma, chronic obstructive pulmonary disease (COPD) and other related diseases. The sequence of 5' untranslated region (UTR) and VP4/VP2 coding region of the genome are usually analyzed to classify HRV as HRV-A, HRV-B and HRV-C. Given the interest for new viral strains, we identified the phylogenetic characteristics and molecular epidemiological analysis of human rhinovirus in Shanghai from 2012 to 2020.

Methods

6711 throat swabs and sputum samples from patients with acute respiratory infection (ARI) were collected, then the real-time reverse-transcriptase polymerase chain reaction (RT-PCR) method was used to detect HRV. And the fragment of 5' untranslated region (5'UTR) and the VP4/VP2 region were amplified for sequencing and gene typing.

Results

In total, 480/6711(7.2%) HRV-positive samples were detected from 2012–2020, in which 160 samples were further genotyped to HRV-A (109/160, 68.13%), HRV-B (32/160, 20.00%), HRV-C (19/160, 11.88%). We found 13 samples with unclassified results using 5'UTR. Then, these unclassified sample were genotyped to HRV-A and HRV-C by sequencing the VP4/VP2 region. The high incidence of HRV infection occurs in the fall and it is more prevalent in children than in adults.

Conclusion

Our study suggests that it is very significant to continuously detect and analyze the genotype distribution and epidemic trend of HRV, which can provide data support for disease prevention and treatment. And the 5' UTR and VP4/VP2 should be combined for HRV genotyping accurately.

Introduction

Human Rhinoviruses (HRVs) are positive-sense, single-stranded ribonucleic acid (RNA) viruses belonging to the *Enterovirus* genus in the *Picornaviridae* family[1]. HRVs are one of the most common respiratory pathogen associated with both upper and lower respiratory tract illness[2].

The HRV genome is approximately 7.2 kb in length, a single open reading frame capped by a 5' untranslated region(5'UTR) and a short viral primer protein (VPg). The viral genome consists of a single gene encoding 11 proteins: four capsid proteins and seven non-structural proteins. Four structural proteins: VP1, VP2, VP3 and VP4 together participate in the assembly of viral capsids[3]. Rhinovirus was first isolated from the specimens of patients with respiratory tract infection by Pelon et al in 1956 by means of tissue culture[4]. HRVs have

extensive genetic diversity of RNA viruses and classified phylogenetically into 3 species (HRV-A, HRV-B, HRV-C) [5]. Types A and B were discovered in the early 1990s, while HRV-C was detected until 2006 due to cannot be cultured in histocyte [6]. With the development of molecular biology technologies, multiple new rhinovirus subtypes have been found[7, 8]. Currently, more than 150 rhinoviruses serotypes have been identified and without a uniform antigenic epitope, which make the discovery of HRV vaccines difficult [9].

In recent years, increasing number of studies have shown that in addition to causing acute respiratory tract diseases, HRV infection could also lead to the aggravation of asthma in children and chronic obstructive pulmonary disease (COPD) in middle-aged and elderly people[9]. Epidemiological investigation of the virus is a powerful tool to control the spread of disease.

In this study, we identified the genotypes of HRV from clinical samples collected in Shanghai from sentinel hospitals, during 2012 to 2020. HRV-positive samples were typed by analyzing the nucleotide sequence of 5'UTR and VP4/VP2 region.

Materials And Methods

Clinical samples collection

From January 2012 to April 2020, 6711 throat swabs or sputum samples of patients were collected from 6 sentinel hospitals in Shanghai (Shanghai Public Health Clinical Center, Shanghai Xinhua Hospital, Shanghai Tongren Hospital, Shanghai East Hospital, Shanghai No.5 People's Hospital and Shanghai Pediatric Hospital). Clinical information related to patients was recorded. All of the specimens were kept in virus transport medium and sent to the laboratory immediately. The study was approved by the ethic committee of Shanghai Public Health Clinical Center (2018-S045-02)

Laboratory detection and subtype classification of rhinovirus

Viral RNA was extracted from specimen by using Nucleic acid extraction and purification kit (BioGerm, Shanghai). The extracted nucleic acids can be directly detected using One Step PrimeScript RT-PCR Kit (TaKaRa®, Japan) for the Real-Time polymerase chain reaction (RT-PCR). RT-PCR was performed at 42°C for 10min, 95°C for 1min followed by 40 cycles at 95°C for 10s, and at 55°C for 1min.

HRV positive samples were screened out and transcribed to cDNA by using the SuperScript® III first-strand synthesis system (Invitrogen, USA). Human rhinovirus cDNA samples were then amplified using 5'UTR or VP4/VP2 primers. The lengths of 5'UTR and VP4/VP2 amplicons were 390bp and 610bp respectively forming by Platinum™ SuperFi™ DNA Polymerase kits (Invitrogen, USA). PCR was performed at 98°C for 30 s followed by 45 cycles at 98°C for 10 s, at 55°C for 10 s, at 72°C for 30 s, and at 72°C for 10 min for a final extension. Primers targeting the amplification sequences and RT-PCR of HRV were listed in Table 1. DNA products were extracted from agarose gels by using QIAquick Gel Extraction Kit (Qiagen), and were linked with pZero-Blunt vector. The plasmid pZero-Blunt was provided by Key Laboratory of Medical Molecular Virology, Shanghai Medical College, Fudan University, Shanghai, China.

Table 1
HRV specific primes used for RNA amplification and detection.

PCR	Name	Sequence
RT-PCR	HRV-F	5'-CTCCGGCCCCTGAATRYGGCTAA-3'
	HRV-R	5'-TCIGGIARYTTCCASYACCAICC-3'
Sequencing 5'UTR	HRV-5'UTR -F	5'-CAAGCACTTCTGTTWCCCC-3'
	HRV-5'UTR -R	5'-ACGGACACCCAAAGTAGT-3'
Sequencing VP4/VP2	HRV- VP4/VP2 -F	5'-GCCCCTGAATGYGGCTAA-3'
	HRV- VP4/VP2 -R	5'-GGTAAYTTCCACCACCANCC-3'

Sequence and statistical analysis

The genomic sequences were assembled using SnapGene software. The alignments were compared, and the phylogenetic tree was generated using MEGA software (version X). Identities between the strains were calculated using BioEdit. Statistical analysis and data management were implemented using the IBM SPSS Statistics software (version 20.0, IBM Corporation, Armonk, NY, USA) and GraphPad Prism (version 8.01, GraphPad Software, La Jolla, CA, USA). Quantitative data were tested by chi-square test and the p-value of < 0.05 was considered statistically significant.

Result

Respiratory virus detection

From January 2012 to April 2020, 6711 respiratory specimens were detected by RT-PCR for HRV. A total of 480 samples were HRV positive samples, and 160 samples were screened out after removing repetitive ones from the same patients. Of the 160 patients included in this study, 89(55.6%) were male and 71(44.4%) were female. We compared whether there were differences in genotypes between the sexes($\chi^2 = 3.070, P = 0.215 > 0.05$). (Table 2)

Table 2
Positive results of HRV detection in Shanghai from 2012 to 2020, the GBD 2000.

Items	HRV positive samples	Frequency (%)	HRV-A	HRV-B	HRV-C	χ^2	P
Gender							
Male	89	55.63	56	22	11	3.070	0.215
Female	71	44.38	53	10	8		
Season							
Spring	20	12.50	16	4	0	17.908	0.004
Summer	38	23.75	34	3	1		
Fall	58	36.25	36	12	10		
Winter	44	27.50	23	13	8		
Age group(year)							
0 ~ 2	3	1.88	1	1	1	23.880	0.017
3–14	58	36.25	41	7	10		
15–29	25	15.63	21	2	2		
30–44	26	16.25	16	8	2		
45–59	17	10.63	11	6	0		
60–69	15	9.38	8	6	1		
70–79	12	7.50	10	1	1		
≥ 80	4	2.50	1	1	2		

Seasonal dynamics of HRV

The detection frequency of HRV in different seasons were 12.5% (20/160) in spring, 23.7% (38/160) in summer, 36.3% (58/160) in autumn, and 27.5% (44/160) in winter. The peak incidence of HRV infection occurred mainly in autumn, with statistical differences in HRV genotype detection rates between seasons. ($\chi^2 = 17.908$, $P = 0.004 < 0.05$). (Table 2) We also compared the HRV detections in different months from 2012–2020, which showed total HRV in 2012 and 2020 is higher compared to other years and the highest detection frequency in October. (Fig. 1,2)

Distribution of age groups

The minimum age of the collected HRV-positive specimens was 2 years and the maximum age was 87 years, and they were divided into 8 groups of 0–2, 3–14, 15–29, 30–44, 45–59, 60–69, 70–79, and ≥80 years. The

detection rates were 1.88%,36.25%, 15.63%, 16.25%, 10.63%, 9.38%, 7.50%, and 2.50%, respectively, with the highest HRV detection rate in the 3–14 years age group, which may be attributed to the larger number of specimens collected from children. (Fig. 3) (Table 2) ($\chi^2 = 23.880, P = 0.017 < 0.05$)

Co-infection of HRV

There were 52 cases of mixed other respiratory viral infections in all HRV-positive samples, among which the highest number of cases of HRV co-infection with influenza was 12, accounting for 23.1%, followed by bocavirus or adenovirus, accounting for 17.3%. (Table 3)

Table 3
Co-infections of HRV with other respiratory viruses.

Co-infections	Infection cases	Frequency(%)
HRV	160	100
Flu + HRV	12	7.50
RSV + HRV	5	3.13
BOV + HRV	9	5.63
PIV + HRV	5	3.13
RSV + HRV	7	4.37
ADV + HRV	9	5.62
RSV + ADV + HRV	4	2.50
HMPV + BOV + HRV	1	0.63

Phylogenetic analysis of 5'UTR

The sequencing results were compared and analyzed with the reference sequences selected from Genbank. Multiple sequence alignment and phylogenic tree were generated by MEGA-X program and ClustalW (Fig. 4). The evolutionary tree showed that the detected HRV-positive samples were divided into 3 genetic clusters A, B and C, accounting for 61.25% (98/160), 20.00% (32/160) and 10.62% (17/160), respectively. The HRV-A, B, C clusters had identities of 66.2%~100%, 76.7%~100% and 65.0%~100% for nucleotide sequences within individual groups, which indicate that HRV-A, C was highly variable than B. On the contrary, the variation of HRV-B sequence was relatively stable. In the phylogenetic tree of 5'UTR, 6 of the HRV-A genotypes and 5 of the HRV-C genotypes were present in the same branch. We analyzed the identities of these 11 samples (78.9%-100%), which was higher than the corresponding genotypes (Fig. 5) (Table 3).

Unclassified samples

In the genotyping using 5'UTR sequences, there were 13 samples unclassified, and we performed VP4/VP2 genome sequencing of these samples, which 10samples were all HRV-A type, others were HRV-C. (Fig. 6) One particular strain was distributed under HRV-A clade in the 5' UTR phylogenetic tree and under HRV-C clade in

the VP4/VP2 phylogenetic tree. This samples that grouped as C in VP4/VP2 but as A in 5'UTR are denoted as C/A[10].

Discussion

Human rhinovirus (HRV) is one of the most important respiratory viruses causing influenza-like illnesses, and HRV also causes pediatric asthma, chronic obstructive pulmonary disease (COPD), acute otitis media, sinusitis, pneumonia, and bronchopneumonia[9, 11, 12], posing a serious risk to human health. Studies have shown that HRV types A and C cause more severe clinical symptoms compared to HRV type B and are strongly associated with exacerbation of asthma disease in children[13]. As a result, drug and vaccine development for HRV may should focus mainly on the two subtypes A, C. Huang T and coworkers roundly analyzing HRV genotypes, they found that more than half of HRV-C types contain a variable length gene fragment identical to the 5' end of HRV-A, and HRV-C was divided into two new subspecies: HRV-Cc and HRV-Ca. It suggests that viral recombination may be the result of co-infection with A and C[14], and that HRV-C and C/A subtypes are more likely to be detected in the asthmatic patients of preschool age [10], and the recombination between A and C subtypes provides an opportunity for continuous mutation in HRV, but creates challenges for disease treatment and research.

In this study, the nucleotide sequence of 5'UTR region was efficiently amplified by PCR and ligated into the related vector, which is high specific and sensitive and can eliminate the interference of other pathogens in the sequencing process. Although, there were 13 samples detected as unclassified, we also further analyzed their sequences using the VP4/VP2 regions. The detection rate of rhinovirus among respiratory viruses was 7.2%, and among the 160 HRV-positive samples, the proportions of genotypes A, B, and C were 68.13%, 20.00%, and 11.88%, respectively, which indicated that HRV-A was the main prevalence of rhinovirus in Shanghai during 2012–2020. This is broadly in line with the results of related studies[15–17].

The detection rate of HRV is related to seasonal, age, gender and regional differences.

In the previous study, HRV outbreak pattern was identified that occurred during the spring and fall seasons [5]. It was noticed that the presence of HRV was detected in all season, with the highest prevalence in fall (36.25%;58/160), which was consistent with previous studies.

Among the several age groups, the highest HRV detection rate of 36.25% was found in the 3–14 years group, which may be due to the higher number of children samples collected. The morbidity of HRV is high in school-aged children and respiratory viral infections are an important cause of mortality in children under 5 years of age [18], which may be associated with an underdeveloped adaptive immune response system in infants and young children. Positive-sense RNA genomes contain a short viral priming protein(VPg), which plays essential functions in evading host recognition[19]. The mechanisms involved in viral VPg remain to be investigated.

Among the 160 positive samples, influenza was the most common co-infecting respiratory virus. High incidence of both influenza and parainfluenza has been reported during the onset of HRV[20]. Co-infection with other viruses or bacteria may increase disease severity[21]. HRV promotes bacterial endocytosis, thereby increasing the susceptibility of pathogens to invade host cells[22]. Therefore, in the prevention and control of respiratory virus infection, we should strengthen the monitoring of other pathogens.

The results of phylogenetic analysis showed that during 2012–2020, three genotypes of HRV-A, B, C were co-prevalent, with A predominant and B, C following. In our phylogenetic tree, two HRV-A types (4204,5980) appeared among the HRV-C group. This phenomenon can occur when some HRV-Cs have 5'UTRs with similar phylogenetic origins in HRV-A[23]. The existence of multiple branches under the same genotype in the phylogenetic tree indicates that there is high variation within the same genotype. Recombination between different subtypes may offer an opportunity for virus evolution[24]. RNA viruses mutate through recombinant variation to improve their fitness and evade the host immune system[25–27]. Type A and C recombination is mostly found in the 5'UTR and 2A protease gene regions[28], and it is uncertain whether recombination in this region is useful for virus entry into host cells and relevant for HRV vaccine and drug development.

Genotyping of HRV-based sequence similarity directly from clinical specimens replaces cell culture for viral isolation, thus greatly reducing the time to detect the three HRV types. In molecular genetics, due to the highly conserved nucleotide sequences in the 5' UTR, VP4/VP2 regions, these sequences are usually used to classify HRV genotypes[29]. The 5' UTR detection has been used to successfully classify HRV and provides a higher sensitivity compared to VP4/ VP2 region, which is susceptible to a lower binding efficiency[30, 31]. VP4/VP2 sequences can be better used for type A and C identification in epidemiological studies[29]. 5' UTR assay can be used as a powerful tool to address the sensitivity problem regarding capsid region-based genotyping[32]. Although, the 5' UTR did not successfully segregate the unclassified strains into HRV-A and HRV-C, this does not exclude its importance and utility in typing of novel strains of HRV. As a consequence, there are some issues to consider when using the 5' UTR for genetic typing unclassified strains cannot be well segregated, and there will require the joint use of 5' UTR and VP4/VP2 regions for resolution.

This study analyzed the epidemiological variation of rhinovirus in respiratory samples from 2012 to 2020 in Shanghai, which utilize molecular diagnostic assays to provide basic data for the prevention, control and treatment of respiratory diseases.

Declarations

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Author Contributions

WW and ZY conceived and designed the study. WW and ZY provided precious suggestions on the results. HJ performed the experiments, analyzed the data and wrote the manuscript. CY and YL collected and screened the samples. All authors read the manuscript and approved the submitted version.

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

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Tables

Table 4 Sequence Identity Matrix.

The sequence identity matrix of 11 sequence that were present in the same branch were analyzed by Bioedit.

ID	4217	4234	299	171	10384	260	227	212	9554	3983	5744
4217	1										
4234	1	1									
299	0.900	0.900	1								
171	0.903	0.903	0.992	1							
10384	0.861	0.861	0.862	0.854	1						
260	0.842	0.842	0.828	0.830	0.789	1					
227	0.842	0.842	0.828	0.830	0.789	1	1				
212	0.842	0.842	0.828	0.830	0.789	0.997	0.997	1			
9554	0.836	0.836	0.824	0.827	0.821	0.827	0.827	0.827	1		
3983	0.834	0.834	0.822	0.824	0.819	0.825	0.825	0.825	0.997	1	
5744	0.841	0.841	0.829	0.832	0.821	0.827	0.827	0.827	0.992	0.989	1

Figures

Figure 1

Monthly distribution of HRV positive frequency in 160 respiratory samples. The amount of total HRV observed in each month, pooling the years 2012-2020. The year 2009 was excluded, as RV circulation in that year was atypical.

Frequency of HRV-A , HRV-B and HRV-C infections per month

Figure 2

The frequency of viral infections detected monthly is shown for HRV species. The frequency of viral infections detected monthly with total HRV, HRV-A, B and C was analyzed from 2012 to 2020.

Figure 3

Age distribution of different types of Human rhinovirus A, B or C. Different icons to represent type A(HRV-A), HRV-B or HRV-C, and the median and the interquartile range are depicted.

Figure 4

Phylogenetic analysis of the HRV 5'UTR using the Neighbor-Joining method. Multiple sequence alignment and an unrooted phylogenetic tree were generated with neighbor-joining method and bootstrap test (500 replications) using MEGA-X program and ClustalW. This analysis involved 170 nucleotide sequences including 160 samples sequences and 10 reference sequences. Reference sequences were represented by sequence ID of GenBank, and specimens by clinical sample numbers. All positions with less than 60% site coverage were eliminated. The red circles are the different genotypic sequences located under the same branch.

Figure 5

Multiple sequence comparison. The 11 sequences that were present in the same branch were aligned by Clustalw in MEGA X and exported visualization files with Clustalx software. The asterisk on the sequence indicates conserved regions.

Figure 6

Phylogenetic analysis of the unclassified samples of VP4/VP2 using the Neighbor-Joining method. 13 sequences alignment and an unrooted phylogenetic tree were generated with neighbor-joining method and bootstrap test (500 replications) using MEGA-X program and ClustalW. This analysis involved 21 nucleotide sequences including 13 samples sequences and 8 reference sequences. Reference sequences were represented by sequence ID of GenBank, and specimens by clinical sample numbers. All positions with less than 50% site coverage were eliminated.