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RESEARCH ARTICLE

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Epidemiological analysis and follow-up of human rhinovirus infection in children with asthma exacerbation

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To determine the prevalence of human rhinovirus (HRV) infection in children with acute asthma exacerbations, investigation of HRV viral load and severity of asthma exacerbations is also required. Nasopharyngeal aspirates and swabs were collected and assessed for respiratory viruses. HRV-positive samples were sequenced to identify types and determine viral load. Outpatients with asthma exacerbations underwent follow-up evaluations, their swabs were collected and clinical outcomes were recorded at their next clinic visit 4 weeks later. One hundred forty-three inpatients and 131 outpatients, including 88 patients with asthma exacerbations and 43 controls with stable asthma were recruited. HRV-A was mainly detected in September and February (45.5% and 33.3%, respectively), while HRV-C was mainly detected in November and April (70.0% and 55.6%, respectively). HRV-C was the primary type and was primarily found in inpatients with severe asthma exacerbations. HRV-A viral load in the group of inpatients with severe exacerbations was higher than in the mild and moderate groups ($P < 0.001$ and $P = 0.022$). The HRV-A viral load of both inpatients and outpatients was higher than that of HRV-C ($P < 0.001$ and $P = 0.036$). The main genotypes were HRV-C53 and HRV-A20 among inpatients, and this genotype caused more severe clinical manifestations. HRV persisted for no more than 4 weeks, and their symptoms or signs of disease were well-controlled well. HRV-C was most frequently detected in asthma exacerbations. HRV-A with high viral load led to severe asthma exacerbations.

KEYWORDS
asthma exacerbations, children, human rhinoviruses, type, viral load

1 | INTRODUCTION

Asthma is a major childhood health risk. Acute asthma exacerbations remain a significant cause of morbidity in children and can lead to an accelerated decline in lung function,[1–3] which emphasises the importance of finding an appropriate preventive treatment. Epidemiologic studies have detected viral infections in more than 80% of school children with asthma exacerbations.[4] Of the respiratory viruses known to cause asthma exacerbations, between 60% and 70% are types of human rhinoviruses (HRV).[5,6] Moreover, one recent study showed that HRV was associated with a significantly increased risk of day-to-day asthma symptoms in children.[7] Understanding the role of HRV in acute asthma exacerbations may lead to interventions that will improve asthma management.

HRV is a single-stranded RNA virus belonging to the genus *Enterovirus* and the family *Picornaviridae*. There are more than 100 classical HRV serotypes, which are divided into groups A (HRV-A) and B (HRV-B). Because of developments in molecular methods, recent

studies have discovered over 50 new HRV strains referred to as HRV-C.[8–14] Molecular epidemiological studies suggest that the dominant types are HRV-A and HRV-C, while HRV-B is relatively rarely detected.[12–14] HRV-C accounts for the majority of asthma attacks in children and causes more severe attacks than other HRV types and other viruses.[6,15–19] In our previous study, we found that HRV infection was associated with asthma in our region.[20] Some authors have demonstrated a close correlation between the HRV viral load and the disease severity in children with lower respiratory tract infections.[21–23] However, there have been no reports on the correlation of the viral load of different HRV species with the severity of asthma exacerbations.

The objective of the present study is to determine the prevalence of HRV infection in children with acute asthma exacerbations and the virus detection after acute asthma acute attacks as well as to investigate the relationship between different HRV types, viral load and severity of acute asthma exacerbations.

2 | MATERIALS AND METHODS

2.1 | Study participants and sample collection

During the study from March 2012 through November 2015, we enrolled inpatients with acute asthma exacerbations from the Department of Respiratory Medicine and outpatients from November 2014 to December 2015 at the Children's Hospital of Chongqing Medical University in China. Inclusion criteria were: children with asthma exacerbations and symptom onset ≤3 days and children with stable asthma to make up the control group. Exclusion criteria were as follows: children with chronic pulmonary disease, cardiopathy, metabolic and genetic diseases, or immunosuppression. The diagnosis of asthma and the severity of asthma exacerbations were assessed according to the guidelines of the Global Initiative for Asthma.

Nasopharyngeal aspirates (NPAs) and swabs (NPSs) were collected when the patients were admitted to our department, and clinical outcomes were recorded. We conducted a follow-up study among these outpatients with asthma exacerbations and their NPSs were collected, and clinical outcomes were recorded when they visited the clinic again after 4 weeks. This study was approved by the Ethics Committee of the Children's Hospital of Chongqing Medical University. The informed consent for participation in the study was obtained from patients or legal guardians.

2.2 | Definition of the severity of asthma exacerbations

The severity of asthma exacerbations at presentation was determined using a previously validated reference standard for each subject by a paediatrician and divided into mild, moderate and severe groups.[24] Asthma symptom control was assessed with the Childhood Asthma Control Test[25] and divided into well-controlled, not well-controlled and poorly controlled groups according to the clinical symptom score.

2.3 | HRV nucleotide sequence analysis and viral load detection

The 5′-NCR (non-coding region) and a partial sequence of the VP4/VP2 region was amplified by PCR as previously described.[26–28] The PCR products were sequenced by Shanghai Majorbio Bio-Pharm Technology. The nucleotide sequences were compared with reference HRV strains obtained from GenBank for typing and to distinguish human enterovirus (HEV).

Amplification of HRV RNA by real-time fluorescence quantitative PCR (RT-PCR) was performed with the 5′-NCR primers and probe.[29] RT-PCR was performed using a TaKaRa OneStep PCR kit (Takara Biotechnology, Dalian, China).

2.4 | Phylogenetic analysis

Phylogenetic analysis of HRV-positive strains was performed as described previously using the neighbour-joining (NJ) method,[27,30,31] and the reliability of the tree was estimated with 1000 bootstrap replications. We performed a phylogenetic tree of the VP4/VP2 coding region and 5′-NCR using the CLUSTAL W program and MEGA 5.05 software. Sequences of reference strains were obtained from the GenBank database.

2.5 | Detection of other respiratory viruses

Respiratory syncytial virus types A and B (RSVA, RSVB), influenza virus A, B and C (IFVA, IFVB, IFVC), human coronaviruses (HCoV-229E, HCoV-OC43), human metapneumovirus (HMPV) and parainfluenza virus (PIV types 1-4) were screened using nested PCR assays.[32,33] Adenovirus (ADV) was detected by means of multiplex PCR, and human bocavirus 1 (HBoV1) was screened using RT-PCR.[34,35] All the specimens were analyzed using a commercial detection kit (TaKaRa Biotechnology, Dalian, China), according to the manufacturer's instructions.

2.6 | Statistical analysis

Data were analyzed using the SPSS 17.0 software package. Categorical variables were compared using the chi-squared or Fisher's exact test, and continuous variables were compared using Student's *t*-test or the nonparametric Mann-Whitney *U*-test. Two-sided *P*-values of <0.05 were considered statistically significant.

3 | RESULTS

3.1 | Population demographics

A total of 143 inpatients with acute asthma exacerbations and another 131 outpatients, including 88 patients with asthma exacerbations and 43 controls with stable asthma were enrolled in this study. The age of inpatients ranged from 17 to 170 months (median, 45 months), and the male to female ratio was 84:59. The age of outpatients ranged from 22

to 144 months (median, 64 months), and the male to female ratio was 81:50. The percentage of mild asthma exacerbations was 21.7% (31/143), moderate was 53.1% (76/143) and severe was 25.2% (36/143) of inpatients. In outpatients, 20.5% (18/88) of exacerbations were mild, 58.0% (51/88) were moderate and 21.6% (19/88) were severe.

3.2 | Virus detection

Among inpatients, at least one virus was detected in 121 (84.6%) cases. HRV was identified in 72 samples (50.3%), and 65.3% (47/72) of the cases were identified as single HRV infections. Other common viruses included 36 RSV cases (25.2%), 13 PIV cases (9.1%), 12 HBoV1 cases (8.4%), 9 IFVA cases (6.3%), 6 ADV cases (4.2%), 2 HMPV cases (1.4%), and 8 HEV cases (5.6%). Among outpatients, respiratory viruses were detected in 45 (34.4%) subjects, and 40 were single infections. The rate of viral infections was 43.2% (38/88) in the group with asthma exacerbations. The HRV-positive rate in the group with asthma exacerbations was 15.9% (14/88). The detection rate of both total virus and HRV of inpatients was significantly higher than that of outpatients (84.6% vs 43.2%; $P < 0.001$ and 50.3% vs 15.9%; $P < 0.001$). The detection rate of both total virus and HRV of inpatients was significantly higher than that of the stable asthma group (84.6% vs 16.3%; $P < 0.001$ and 50.3% vs 2.3%; $P < 0.001$). Furthermore, both the rate of total virus and HRV-positive status in outpatients with asthma exacerbation was higher than that of the stable asthma group (43.2% vs 16.3%; $P = 0.002$ and 15.9% vs 2.3%; $P = 0.045$) (Table 1).

TABLE 1 Specific detected virus among all patients

Virus	Inpatients	Outpatients	
	Asthma exacerbation ($n = 143$) N (%)	Asthma exacerbation ($n = 88$) N (%)	Stable asthma ($n = 43$) N (%)
HRV	72 (50.3)	14 (15.9)	1 (2.3)
HRV-A	25 (17.5)	3 (3.4)	1 (2.3)
HRV-B	5 (3.5)	1 (1.1)	0 (0)
HRV-C	42 (29.4)	9 (10.2)	0 (0)
Untyped	0 (0)	1 (1.1)	0 (0)
RSV	36 (25.2)	4 (4.5)	0 (0)
RSVA	23 (16.1)	3 (3.4)	0 (0)
RSVB	13 (9.1)	1 (1.1)	0 (0)
PIV	13 (9.1)	9 (10.2)	3 (7.0)
PIV3	8 (5.6)	5 (5.7)	3 (7.0)
PIV4	5 (3.5)	4 (4.5)	0 (0)
IFV	9 (6.3)	3 (3.4)	1 (2.3)
IFVA	9 (6.3)	1 (1.1)	0 (0)
IFVB	0 (0)	2 (2.3)	1 (2.3)
HBoV1	12 (8.4)	3 (3.4)	2 (4.7)
ADV	6 (4.2)	1 (1.1)	0 (0)
HMPV	2 (1.4)	1 (1.1)	0 (0)
HCoV	0 (0)	1 (1.1)	0 (0)
HEV	8 (5.6)	2 (2.3)	0 (0)
Total	121 (84.6)	38 (43.2)	7 (16.3)

HRV, human rhinovirus; RSV, respiratory syncytial virus; PIV, parainfluenza virus; IFV, influenza virus; HBoV1, human bocavirus type 1; ADV, adenovirus; HMPV, human metapneumovirus; HCoV, human coronaviruses; HEV, human enterovirus.

3.3 | HRV types and asthma exacerbations

In hospitalized children, the VP4/VP2 region was successfully amplified in 56 samples: HRV-A was identified in 35.7% of samples (20/56), HRV-B in 8.9% (5/56), and HRV-C in 55.4% (31/56). The number of HRV-C-positive cases was more than those of HRV-A and HRV-B ($P = 0.037$ and $P < 0.001$). The HRV-C detection rate in severe groups was significantly higher than that of the mild group (36.1% vs 6.5%; $P = 0.004$). The rates of HRV-A and HRV-B-positivity rated showed no different in the three severity groups ($P = 0.596$ and $P = 0.731$). The VP4/VP2 sequence was amplified in one outpatient, while others could not be obtained because of low amplicon yield, and 5′-NCR sequences from the remaining HRV-positive specimens were used for typing. The majority of those in the group with acute asthma exacerbations (64.3%; 9/14) was infected with an HRV-C, 21.4% (3/14) was infected with HRV-A, only one patient was infected with HRV-B and one was untyped (Table 2). Because of the small number of outpatients with HRV in the different severity groups, comparisons were not performed.

3.4 | Clinical features of HRV positive and negative patients

The percentage of severe acute asthma exacerbations in the HRV-positive group was higher than that of the HRV-negative group ($P = 0.032$), while the number of patients with fever was less than that in the HRV-negative group ($P = 0.007$). The white blood cell counts and neutrophil percentages were both significantly higher in the HRV-positive group ($P = 0.041$ and $P = 0.043$, respectively). The other features between the HRV-positive and HRV-negative groups had no statistically significant difference. All the clinical features between single HRV-A-positive and HRV-C-positive groups had no statistically significant difference (Table 3). The percentage of familial eczema and neutrophils were significantly higher in the group of outpatients with HRV infection than in the group without HRV infection ($P = 0.049$ and $P = 0.030$, respectively). The clinical characteristics of HRV-A and HRV-C-positive outpatients showed that the percentage of allergic rhinitis in the HRV-C-positive group was significantly higher than that of the HRV-A group ($P = 0.045$) (Table 4).

3.5 | Seasonality of HRV infections and asthma exacerbations

The frequency of asthma exacerbations among HRV-positive inpatients was calculated as 48.0% (12/25) in spring, 44.0% (11/25) in

TABLE 2 HRV types and severity of acute asthma exacerbations in children

Inpatients (n = 143) N (%)				Outpatients (n = 88) N (%)			
Severity	HRV-A	HRV-B	HRV-C	Severity	HRV-A	HRV-B	HRV-C
Mild (n = 31)	6 (19.4)	1 (3.2)	2 (6.5)	Mild (n = 18)	0 (0)	0 (0)	4 (22.2)
Moderate (n = 76)	9 (11.8)	2 (2.6)	16 (21.1)	Moderate (n = 51)	1 (2.0)	1 (2.0)	3 (5.9)
Severe (n = 36)	5 (13.9)	2 (5.6)	13 (36.1)*	Severe (n = 19)	2 (10.5)	0 (0)	2 (10.5)

HRV indicates human rhinovirus.
*There were more HRV-C positive inpatients in the severe asthma exacerbation than in the mild exacerbation group (P = 0.004).

summer, 60.6% (43/71) in autumn, and 27.3% (6/22) in winter during the study period by merging the same month. HRV-A was mainly detected in September and February (45.5% and 33.3%, respectively), while HRV-C was mainly detected in November and April (70.0% and 55.6%, respectively) (Fig. 1).

3.6 | HRV viral load and severity of asthma exacerbations

The genome viral load of 62 inpatients infected with HRV has been detected, and 10 cases have not been detected using the 5′-NCR primers and probe. The median age of these inpatients was 45 months, and the male to female ratio was 32:30. The genome viral load ranged from 2.2 to 2.9×10^7 copies/mL. However, there was no significant difference of HRV total viral load among the different severities of asthma exacerbations (Fig. 2A). The viral load of HRV-A in the severe group was higher than that in the mild group (mean, 2.5×10^5 copies/mL vs 1.7×10^3 copies/mL; $P < 0.001$) and the moderate group (mean, 2.5×10^5 copies/mL vs 2.1×10^3 copies/mL; $P = 0.022$) (Fig. 2B). However, the HRV-C viral load among the three groups showed no significant difference (Fig. 2C). Moreover, the viral loads of HRV-A and HRV-B were significantly higher than the HRV-C viral load regardless of the severity (mean, 8.5×10^3 copies/mL vs 2.5×10^2 copies/mL; $P < 0.001$; mean, 1.6×10^4 copies/mL vs 2.5×10^2 copies/mL; $P = 0.014$) (Fig. 2D). The viral load of HRV-A was higher than HRV-C in the group with severe asthma exacerbations (mean, 2.5×10^5 copies/mL vs 1.6×10^2 copies/mL; $P < 0.0001$) (Fig. 2E). Because of the small number of outpatients with HRV in the different severity groups, the comparisons between different HRV types viral load and severity of disease were excluded. Even so, the total viral load of HRV was compared. And the HRV-A total viral load was significantly higher than that of HRV-C (mean, 9.4×10^3 copies/mL vs 7.1×10^2 copies/mL; $P = 0.036$).

TABLE 3 Comparison of clinical features between HRV-positive and HRV-negative inpatients with asthma exacerbations

	HRV N (%)			HRV single infection N (%)		
Variable	Single infection (n = 47)	Negative (n = 71)	P	HRV-A (n = 16)	HRV-C (n = 29)	P
Male:female	23:24	47:24	0.062	5:11	16:13	0.124
Age (median, month)	40	43	0.658	48	37	0.109
History of eczema	13 (27.7)	26 (36.6)	0.311	4 (25.0)	8 (27.6)	1.000
History of allergic conditions	8 (17.0)	18 (25.4)	0.285	3 (18.8)	5 (17.2)	1.000
Familial asthma	28 (59.6)	43 (60.6)	0.858	10 (62.5)	16 (55.2)	0.634
Cough	47 (100)	71 (100)	-	16 (100)	29 (100)	-
Rhinorrhea	8 (17.0)	12 (16.9)	0.986	5 (31.3)	3 (10.3)	0.177
Expectoration	34 (72.3)	47 (66.2)	0.481	11 (68.8)	21 (72.4)	1.000
Wheezing	41 (87.2)	58 (81.7)	0.422	14 (87.5)	26 (89.7)	1.000
Fever	14 (29.8)	39 (54.9)	**0.007**	5 (31.3)	9 (31.0)	1.000
Severe exacerbation	16 (34.0)	12 (16.9)	**0.032**	5 (31.3)	10 (34.5)	0.826
Length of hospitalization (median, day)	5 (3-8)	5 (2-16)	0.388	5 (3-6)	5 (4-8)	0.250
White blood cell($\times 10^9$/L)	12.38 ± 5.13	10.77 ± 4.87	**0.041**	12.16 ± 5.71	12.58 ± 5.05	0.868
Neutrophils (%)	64.70 ± 20.27	56.44 ± 21.95	**0.043**	64.50 ± 21.18	65.31 ± 19.85	0.915
Lymphocyte (%)	30.60 ± 18.50	39.03 ± 21.19	**0.031**	29.63 ± 18.42	30.62 ± 18.47	0.859
CRP > 8 mg/L (%)	6 (12.8)	8 (11.3)	0.805	3 (18.8)	3 (10.3)	0.737
Platelet ($\times 10^9$/L)	333 ± 102	321 ± 94	0.308	319 ± 106	337 ± 102	0.887

HRV indicates human rhinovirus. Categorical variables were compared using the chi-squared test, and the continuous variables were compared using the Student's t-test or the nonparametric Mann-Whitney U-test. Two-sided P-values of < 0.05 were considered statistically significant.

TABLE 4 Comparison of clinical features between HRV-positive and HRV-negative outpatients with asthma exacerbations

	HRV *N* (%)			HRV-positive *N* (%)		
Variable	**Positive (*n* = 14)**	**Negative (*n* = 74)**	***P***	**HRV-A (*n* = 3)**	**HRV-C (*n* = 9)**	***P***
Male:female	8:6	49:25	0.729	2:1	5:4	1.000
Age (median, month)	60	64	0.941	54	56	0.926
History of allergic rhinitis	12 (85.7)	49 (66.2)	0.257	1 (33.3)	9 (100)	**0.045**
History of eczema	5 (35.7)	24 (32.4)	1.000	1 (33.3)	4 (44.4)	1.000
Familial allergic rhinitis	3 (21.4)	10 (13.5)	0.723	1 (33.3)	2 (22.2)	1.000
Familial eczema	3 (21.4)	3 (4.1)	**0.049**	1 (33.3)	2 (22.2)	1.000
Familial asthma	1 (7.1)	12 (16.2)	0.641	0 (0)	1 (11.1)	1.000
Passive smoking	6 (42.9)	18 (24.3)	0.271	0 (0)	5 (55.6)	0.310
Cough	13 (92.9)	71 (95.9)	0.507	3 (100)	8 (88.9)	1.000
Expectoration	6 (42.9)	22 (29.7)	0.513	2 (66.7)	3 (33.3)	0.735
Wheezing	13 (92.9)	57 (77.0)	0.324	3 (100)	8 (88.9)	1.000
Fever	1 (7.1)	1 (1.4)	0.294	0 (0)	1 (11.1)	1.000
Tachypnea	6 (42.9)	14 (18.9)	0.107	0 (0)	5 (55.6)	0.310
White blood cell ($\times 10^9$/L)	10.22 ± 3.66	9.85 ± 3.06	0.698	9.49 ± 2.76	11.26 ± 3.47	0.307
Neutrophils (%)	56.87 ± 13.93	46.95 ± 12.63	**0.030**	60.67 ± 17.21	58.43 ± 11.39	0.540
Eosinophils (%)	6.26 ± 3.68	6.48 ± 3.94	0.791	8.33 ± 6.81	5.39 ± 2.30	0.757
FEV_1% predicted	91.68 ± 12.78	92.69 ± 14.12	0.913	92.70 ± 0	95.30 ± 12.89	0.655
PEF% predicted	93.35 ± 11.39	80.25 ± 19.47	0.147	75.30 ± 0	96.20 ± 12.08	0.180
Positive skin tests	7 (50.0)	42 (58.1)	0.431	1 (33.3)	6 (66.7)	0.735

FEV_1, forced expiratory volume in one second; PEF, peak expiratory flow.
Two-sided *P*-values of < 0.05 were considered statistically significant.

3.7 | Phylogenetic analysis

Figure 3 shows the phylogenetic tree of inpatients based on the nucleotide sequences of the VP4/VP2 coding region. The 56 HRV-positive samples from inpatients were grouped into 37 HRV genotypes, 31 HRV-C samples were grouped into 19 HRV-C genotypes, 20 HRV-A samples were grouped into 13 HRV-A genotypes, and 5 HRV-B samples were grouped into 5 HRV-B genotypes. There were six (10.7%) strains which could be designated into novel genotypes and temporarily named HRV-C53 and four (7.1%) HRV-A20 strains, the two predominant genotypes, followed by HRV-C21 three (5.4%), HRV-A49 three (5.4%) (Fig. 3). The clinical characteristics of HRV-C53 and HRV-A20-positive inpatients are summarized in Table 5, the severity of their asthma exacerbations was moderate or severe and there were five cases of dyspnea. Figure 4 shows the phylogenetic tree of outpatients based on the nucleotide sequences of the 5′-NCR, and all the HRV-positive samples were grouped into 15 genotypes. One sample was amplified by the VP4/VP2 region and divided into HRV-C22 (Accession number: JN621242). The distribution of outpatients' genotypes had no obvious regularity and neither HRV-C53 nor HRV-A20 was found.

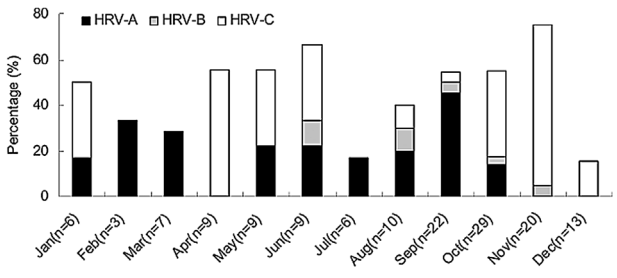

FIGURE 1 Seasonal incidence (rate of positive samples) by month of HRV-A, HRV-B, and HRV-C. Data was combined from 3 years and bar graph represents the detection rate of the three HRV types

3.8 | Follow-up of outpatients

All the HRV-positive outpatients with acute asthma exacerbations participated in the follow-up, and only one case was infected with RSVA within 4 weeks after first the visit. The remaining patients tested negative, and the severity of the disease was well-controlled. Another 23 patients were without HRV during the first visit, while after 4 weeks, five cases of acute asthma exacerbations appeared again and among them, four patients were infected with HRV (Table 6).

4 | DISCUSSION

HRV is an important cause of both upper and lower respiratory illnesses as well as acute exacerbations of childhood asthma. In our study, HRV was the most common viral pathogen that induced asthma

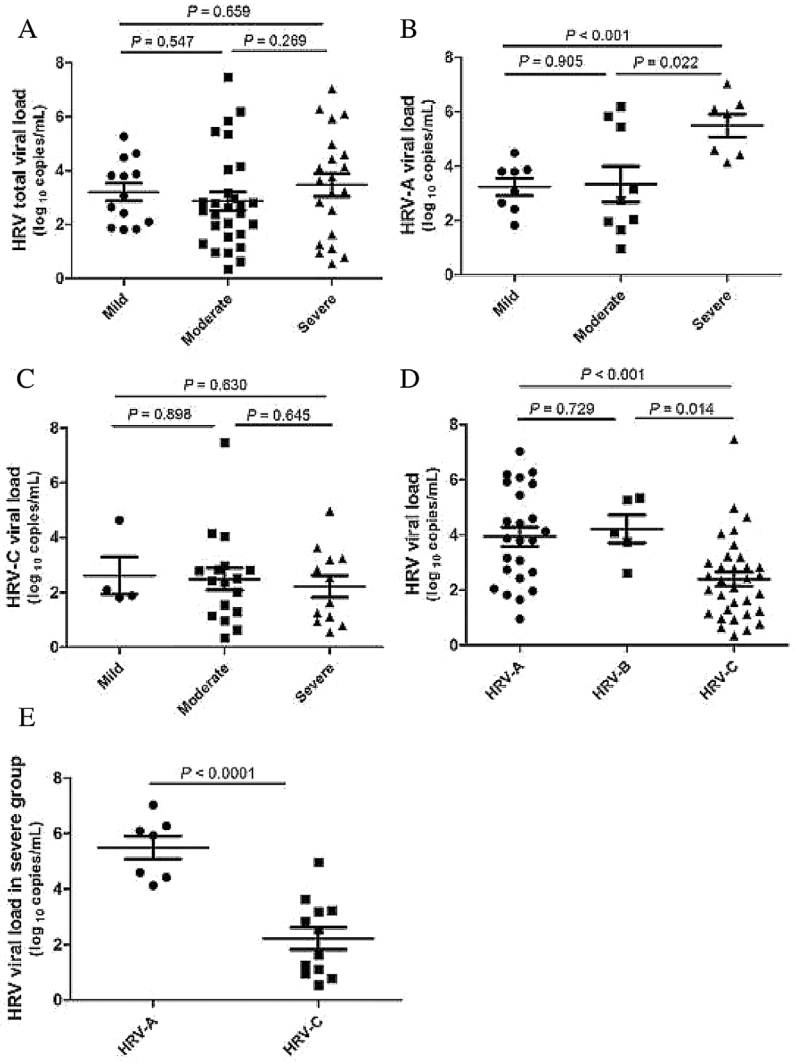

FIGURE 2 Relationship between HRV viral load and severity of asthma exacerbations among inpatients. A: HRV total viral load among the three groups shows no significant difference. B: HRV-A viral load in severe group was significantly higher than in the mild and moderate groups ($P < 0.001$ and $P = 0.022$). C: HRV-C viral load the among three groups shows no significant difference. D: HRV-A and HRV-B viral load were significantly higher than HRV-C regardless of the severity ($P < 0.001$ and $P = 0.014$). E: In the severe group, HRV-A viral load was significantly higher than HRV-C ($P < 0.0001$). The comparison of viral load was conducted by nonparametric Mann-Whitney *U*-test

exacerbations in children. This finding is consistent with previous studies,[6,11,15] and the detection rate of HRV in asthma exacerbation patients was significantly higher than that in the stable asthma group. The detection rate of HRV was 50.3% among inpatients, while some authors reported the rate to be between 60% and 70%. The reason for the difference was that their studies were performed among school-aged children, and the region was different.[5,6] Among the inpatients, 34.7% (25/72) were diagnosed with HRV co-infection, 52% (13/25) of cases were diagnosed as co-infection with RSV, 24% (6/25) of cases were diagnosed as co-infection with HBoV1, 16% (4/25) of cases were diagnosed as PIV, 8% (2/25) of cases were diagnosed as ADV, 8% (2/25) of cases were diagnosed as IFVA and 4% (1/25) of cases were diagnosed as HEV. The median age was 43 months, and 64% (16/25) were in the moderate to severe category groups. The incidence of HRV in outpatients was lower than that in hospitalized children, but the methods of virus detection are consistent, which may be because the severity of outpatients was relatively mild or onset of other causes, such as medication, is irregular. The detection rate of RSV was 25.2% (36/143) among inpatients, and 13 cases were diagnosed as co-infection with HRV, 7 cases were diagnosed as co-infection with other viruses (PIV/ADV/HEV/IFVA) and others were diagnosed as single infections. The median age was 33 months, and 69.5% (25/36) were in the moderate to severe category groups.

Previous studies have demonstrated that neutrophils are the predominant inflammatory cell in the airways of patients with acute asthma exacerbation, and experimental HRV infection has been shown to increase airway neutrophilic inflammation in asthmatic subjects.[36–38] Our clinical and laboratory results, combined with haemograms, indicated that acute viral infections, especially HRV, need to be considered during popular seasons in children with acute asthma attacks, even though the percentage of neutrophils does not exceed 70%.

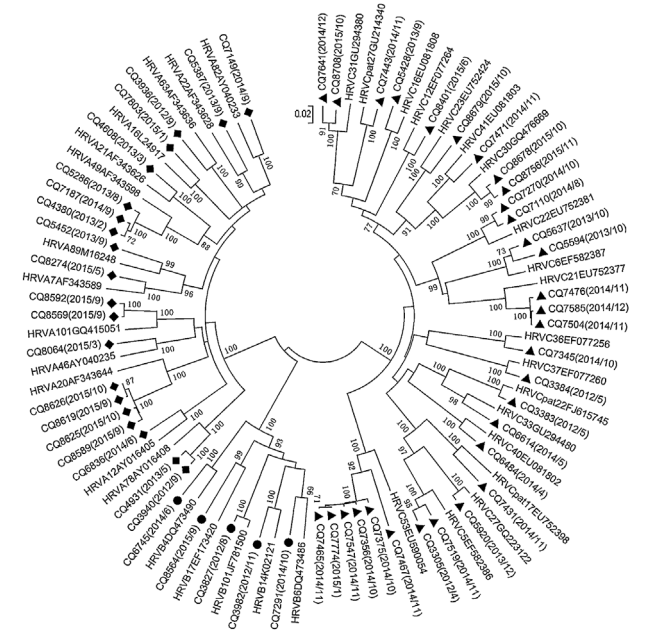

FIGURE 3 Phylogenetic analysis of the HRV VP4/VP2 coding region from inpatients using the neighbour-joining method with 1000 bootstrap replicates with MEGA5.05; branches showing >70% bootstrap support are indicated. The strains in this study are marked with CQ (Chongqing) and HRV-A labeled ♦, HRV-B labeled ●, and HRV-C labeled ▲

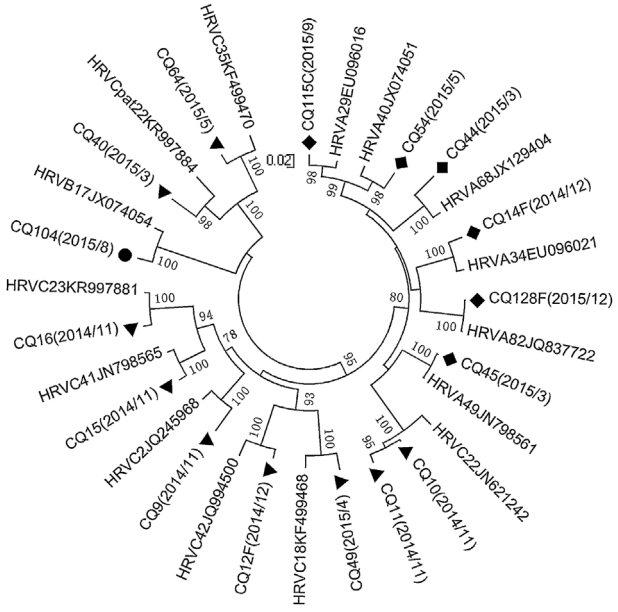

FIGURE 4 Phylogenetic analysis of the HRV 5′-NCR region in outpatients using the neighbor-joining method with 1000 bootstrap replicates with MEGA5.05; branches showing >70% bootstrap support are indicated. The strains in this study are marked with CQ (Chongqing), F (follow-up samples) and C (control group samples) and HRV-A labeled ♦, HRV-B labeled, ● and HRV-C labeled ▲

Although this study only included data from 3 years, we showed both annual and seasonal variation in HRV types. The seasonal prevalence of asthma exacerbations was consistent with HRV infection and occurred mainly in autumn. Our results were similar to those presented in earlier reports, which have associated the September peak in asthma hospitalizations with rhinovirus circulation.[39,40] Our subtropical climate was the reason for the difference.

HRV-C attracts special clinical interest because they can cause more severe illnesses, requiring hospitalization in infants and children, compared with the HRV-A or HRV-B, and are closely associated with acute asthma exacerbations.[15,41,42] Our study has demonstrated that the HRV-C detection rate was greater than 50% among inpatients and outpatients, which supports the hypothesis that HRV-C was to the most common cause of acute asthma attacks in children. Among inpatients, HRV-C was responsible for not only the majority of acute asthma attacks compared to other HRV species or other known viruses, but was also more frequently involved in severe asthma attacks.

TABLE 5 Clinical features of inpatients with HRV-C53 and HRV-A20 infections

Sample	Genotype	Age/sex (year-month)	Sample collection time	Symptoms	Severity
CQ7356	C53	2-7/Male	2014/10	Fever/cough/wheezing/dyspnea	Severe
CQ7375	C53	3-4/Female	2014/10	Fever/cough/expectoration/wheezing	Moderate
CQ7465	C53	2-9/Male	2014/11	Fever/cough/expectoration/wheezing/dyspnea	Severe
CQ7467	C53	2-9/Female	2014/11	Cough/expectoration/wheezing/dyspnea	Severe
CQ7547	C53	2-1/Female	2014/11	Fever/cough/expectoration/wheezing/dyspnea/ diarrhoea	Severe
CQ7774	C53	2-3/Male	2015/01	Cough/expectoration/wheezing	Moderate
CQ8589	A20	7-0/Female	2015/09	Cough/expectoration/wheezing	Moderate
CQ8619	A20	4-0/Female	2015/09	Cough/wheezing/dyspnea	Severe
CQ8625	A20	2-7/Female	2015/10	Cough/expectoration/wheezing	Moderate
CQ8626	A20	3-0/Male	2015/10	Fever/cough/expectoration/wheezing/diarrhoea	Moderate

CQ indicates Chongqing.

TABLE 6 Details of follow-up after 4 weeks in outpatients with asthma exacerbations

Sample	Age/sex (year-month)	First visit pathogen	Follow-up of condition	Follow-up of pathogen
CQ2	1-10/Female	HRV-C	Well controlled	Negative
CQ9	4-0/Male	HRV-C	Well controlled	Negative
CQ10	4-4/Female	HRV-C	Well controlled	Negative
CQ11	5-4/Male	HRV-C	Re-exacerbation	RSVA
CQ15	4-8/Female	HRV-C	Well controlled	Negative
CQ16	8-0/Male	HRV-C	Well controlled	Negative
CQ40	3-8/Female	HRV-C	Well controlled	Negative
CQ49	7-8/Female	HRV-C	Well controlled	Negative
CQ64	7-2/Male	HRV-C/IFVB	Well controlled	Negative
CQ44	3-7/Male	HRV-A	Well controlled	Negative
CQ45	12-11/Female	HRV-A	Well controlled	Negative
CQ54	4-6/Female	HRV-A/HBoV1	Well controlled	Negative
CQ104	5-11/Female	HRV-B	Well controlled	Negative
CQ29	8-7/Male	HRV-untyped	Well controlled	Negative
CQ14	8-0/Female	RSVA	Re-exacerbation	HRV-A
CQ21	12-0/Female	IFVA	Well controlled	Negative
CQ96	7-2/Female	PIV3	Well controlled	Negative
CQ98	11-3/Male	PIV3	Well controlled	Negative
CQ24	2-10/Female	PIV4	Well controlled	Negative
CQ23	5-2/Female	RSVA/PIV4	Well controlled	Negative
CQ12	5-11/Male	Negative	Re-exacerbation	HRV-C/IFVA
CQ120	5-0/Female	Negative	Re-exacerbation	HRV-untyped
CQ128	8-9/Female	Negative	Re-exacerbation	HRV-A/RSVA
CQ31	2-9/Female	Negative	Re-exacerbation	Negative
CQ34	1-5/Male	Negative	Well controlled	Negative
CQ37	4-1/Male	Negative	Well controlled	Negative
CQ42	8-7/Female	Negative	Well controlled	Negative
CQ51	8-7/Female	Negative	Well controlled	Negative
CQ60	3-3/Male	Negative	Well controlled	Negative
CQ72	5-9/Female	Negative	Well controlled	Negative
CQ73	3-9/Female	Negative	Well controlled	Negative
CQ112	5-4/Male	Negative	Well controlled	Negative
CQ116	5-2/Female	Negative	Well controlled	Negative
CQ117	2-5/Male	Negative	Well controlled	Negative
CQ119	4-0/Female	Negative	Well controlled	Negative
CQ125	7-11/Female	Negative	Well controlled	Negative
CQ126	4-7/Female	Negative	Well controlled	Negative

The phylogenetic analysis showed that HRV-C53 and HRV-A20 were the predominant strains among inpatients. The HRV-C53 genotype mainly occurred in October and November 2014, HRV-A20 occurred in September and October 2015 in Chongqing, China and caused more severe clinical manifestations. HRV-C53 was once known as HRV-Cpat10, which was provisionally assigned types (designated "pat"), and we did not find other literature reporting the relationships between asthma attacks and HRV-C53 or HRV-Cpat10.[27,28] However, the two genotypes were not found in outpatients, and these results supported that HRV-C53 and HRV-A20 were more common in severe asthma attacks.

Some observations suggested that people with asthma might be at risk for higher viral loads and symptoms affecting their respiratory tract during HRV infection.[43] Our study confirmed that HRV-A viral load in the severe asthma exacerbations group was significantly higher than that of the mild and moderate groups, while HRV-C was not. This result

indicates that HRV-A viral load is associated with the severity of asthma exacerbations but HRV-C is not. Additionally, in severe asthma exacerbations, the HRV-A viral load was higher than that of HRV-C, which suggested that the mechanisms of the exacerbations were not similar.

We further compared clinical features between single HRV-A positive and HRV-C positive inpatients and no difference was found between the two groups. However, the number of outpatients with allergic rhinitis increased in the HRV-C infection group and whether indicated those asthmatic children who complicated with allergic rhinitis were tend to infect HRV-C even appear exacerbations. The findings highlight the fact that physicians and parents should provide preventive management to those children during the peak season of HRV-C infections.

HRV-C species do not grow in standard cell cultures used for virus isolation, which limits the research on their biological characteristics. According to our understanding of the mechanism, it is possible that the HRV-C receptor is distinct from the intercellular adhesion molecule 1 (ICAM-1) and low-density lipoprotein receptor (LDLR) family members used by HRV-A.[44,45] The latest research has reported that human cadherin-related family member 3 (CDHR3), a member of the cadherin family of transmembrane proteins, mediates HRV-C entry into host cells, and an asthma-related mutation (rs6967330) in this gene is associated with enhanced viral binding and replication. CDHR3 mutations result in susceptibility to childhood asthma with severe exacerbations.[46,47] These findings suggest that CDHR3 is a functional receptor for HRV-C and explain asthma exacerbations are commonly induced by HRV-C. Additionally, different HRV types may have different replications and induce diversified cytokines and chemokines, which contribute to varying illness severity.[48] Further study is needed to investigate the mechanism of HRV-A and HRV-C resulting in different illness severities.

Through the follow-up among outpatients with asthma exacerbations, HRV persisted for no more than 4 weeks in the nasopharynx, and their symptoms or signs of disease appeared well-controlled. Some authors have reported that HRV infections in infants rarely result in persistence of RNA beyond 30 days.[49] Further studies of large samples are required to confirm these findings. Some asthma exacerbation cases may be caused by other respiratory viruses or may present without viral infection at the first visit, while asthma attacks caused by HRV infections may appear again after 4 weeks.

In summary, the virus most frequently detected in asthma exacerbations appears to be HRV, primarily HRV-C, which commonly occurs in severe asthma exacerbations. HRV-A with high viral load leads to severe asthma exacerbations, and HRV-C viral load and disease severity have no significant correlation. The novel genotypes HRV-C53 and HRV-A20 have been found to be predominant strains that cause more severe clinical manifestations. Additionally, we followed up on HRV changes in the airways of asthmatic children, and HRV RNA was found to persist for no longer than 4 weeks. Overall, our results provide some theoretical evidence for the prevention of childhood asthma exacerbations and improving asthma management.

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AUTHORS' CONTRIBUTIONS

E.M.L. conceived and designed this study and revised the manuscripts. S.-Y.Z., L.L.W., L.R., J.L., and W.L. collected the samples and performed the experiments. S.Y.Z. analyzed the data and wrote the paper.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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