Original Article

Association of Different Human Rhinovirus Species with Asthma in Children: A Preliminary Study

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Abstract

Background: Human rhinoviruses (HRVs) are divided into three genetic species: HRV-A, HRV-B, and HRV-C. The association of different HRV species with asthma in children in China has not yet been evaluated. This preliminary study aimed to assess the associations between different HRV species, particularly HRV-C, and asthma in young children in China.

Methods: A total of 702 nasopharyngeal aspirates were obtained from 155 children with asthma (asthma group), 461 children with acute respiratory infection (ARI) without asthma (nonasthma ARI group), and 86 children from the control group. Semi-nested polymerase chain reaction (PCR) was used to detect HRVs, and PCR products were sequenced for species identification. Epidemiological characteristics of HRV-positive cases were analyzed.

Results: HRVs were the most common pathogen (15.4%; 108/702) in the patients in this study. The prevalence of HRV was significantly different ($F = 20.633$, $P = 0.000$) between the asthma (25.8%) and nonasthma ARI groups (11.1%). Phylogenetic analysis indicated that in the 108 cases positive for HRVs, 41 were identified as HRV-A, 8 as HRV-B, and 56 as HRV-C. Comparing the asthma with the nonasthma ARI group, Spearman's rank correlation analysis revealed an association between HRV-A ($P < 0.05$) and C ($P < 0.01$) and asthma, confirmed by regression analysis, with odds ratios of 2.2 (HRV-A) and 4.2 (HRV-C).

Conclusions: Our data revealed a high prevalence of HRVs in children in China, regardless of clinical status. HRV-C was the dominant species and may be one of the key factors in the association of HRVs with asthma.

Key words: Asthma; Children; China; Human Rhinovirus

Introduction

Asthma is a chronic inflammatory lung disease, characterized by recurrent wheezing, and is the leading cause of morbidity and mortality in children worldwide.[1] Infectious agents are the predominant triggers that drive disease and airway pathobiology. Most infants who experience wheezing episodes also exhibit evidence of an ongoing viral respiratory infection. The viral agents respiratory syncytial virus (RSV), influenza A (Flu A), and particularly human rhinovirus (HRV) appear to be the most prevalent and recurring threats.[2]

HRVs are members of the genus Enterovirus within the *Picornaviridae* family. HRVs have been noted as pathogens of the common cold for over 50 years; however, recent advances in viral molecular diagnostics have led to an appreciation of their role in more significant respiratory diseases. These include asymptomatic infections, frequent common colds, and severe lower respiratory infections, especially infantile bronchiolitis, childhood pneumonia, and acute exacerbations of chronic respiratory diseases, such as asthma.[3,4]

HRVs are highly genetically and antigenically diverse. Based on nucleotide sequence homology, HRVs can be divided into three genetically distinct species: HRV-A, HRV-B,[5] and the recently identified HRV-C.[6] A proposed

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classification protocol based on the sequence of the HRV capsid genes revealed approximately 78 A types, 30 B types, and 51 C types.[7] As with many other viruses, the specific strain or genotype of HRV has an impact on pathogenesis.[4] Genetically, HRV-C is most closely related to HRV-A and HRV-B, but even small genetic variance can result in significant differences in clinical impact. It is well-known that HRVs are a major trigger for asthma exacerbations, and HRV-C is now under investigation for its potential role in the development of asthma.[8] This is a preliminary study to assess the associations between different HRV species, particularly HRV-C, and asthma in young children in China.

Methods

Patients and specimens

Nasopharyngeal aspirates (NPAs) were obtained for respiratory virus screening from three groups of patients who visited the Children's Hospital Affiliated to the Capital Institute of Pediatrics in Beijing, China. These groups included: (1) asthma group: the inclusion criteria were pediatric patients younger than 16 years of age, diagnosed with asthma characterized by recurrent episodes (≥3) of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. The exclusion criteria were pediatric patients diagnosed with bronchiolitis or other diseases with symptoms of wheezing, breathlessness, chest tightness, and coughing. (2) Nonasthma, acute respiratory infection (ARI) group: the inclusion criteria were pediatric patients younger than 16 years of age, diagnosed with ARI, including rhinitis, laryngitis, tonsillitis, tracheitis, bronchitis, bronchiolitis, and pneumonia. The exclusion criterion was pediatric patients diagnosed with asthma. (3) Control group: the inclusion criteria were pediatric patients with underlying surgical therapy. The exclusion criterion was pediatric patients confirmed with respiratory infections within the 10 days prior to collection of NPA.

The study was approved by the Ethics Committee of the Capital Institute of Pediatrics. Written informed consent was obtained from the parents or guardians of participants in the control group. The Capital Institute of Pediatrics determined informed consent was not needed for the participants in the asthma and nonasthma ARI groups for using the leftover NPA for respiratory virus screening.

Screening for respiratory viruses

Specimens were processed routinely then centrifuged at 500 ×*g* for 10 min. Supernatants were inoculated onto Madin–Darby canine kidney cell cultures to isolate Flu A and B, onto cultured HEp-2 and Vero cell lines to isolate RSV and human adenovirus (HAdV), and LLC-MK2 cell cultures for parainfluenza virus (PIV) Types 1–3.[9] Cell pellets from all NPA samples were re-suspended and spotted onto an acetone-cleaned slide. Then, individual monoclonal antibody reagents, labeled with fluorescein isothiocyanate (FITC) against RSV, HAdV, Flu A and B, and PIV 1–3, were used for specific virus identification by direct immunofluorescence assay (DFA) with a D^3 Ultra™ DFA Respiratory Virus Screening & ID Kit (Diagnostic Hybrids Inc., Athens, OH, USA). In addition, FITC-labeled monoclonal antibody against human metapneumovirus (HMPV) (Diagnostic Hybrids Inc., Athens, OH, USA) was also used to detect HMPV.

Next, DNA and RNA were extracted from 150 μl of each NPA specimen using TRIzol reagent (Invitrogen Inc., Carlsbad, CA, USA) and solubilized in 30 μl of 8 mmol/L NaOH for DNA or 30 μl of diethylpyrocarbonate-treated water for RNA, according to the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) and PCR assays were performed for detection of PIV Types 1–4, human bocavirus, and WU and KI polyomaviruses.[10-12]

Both RT-PCR and DFA methods can detect PIV Types 1–3 from specimens; however, RT-PCR can detect PIV-4; therefore, the PIV results in this study refer to those of RT-PCR. All remaining specimens were kept frozen at −70°C for further analysis.

Identification of human rhinoviruses

Semi-nested PCR was performed for HRV screening using primers P1 (163–181): 5′-C(A/G)A (G/A)CA CTT CTG T(T/C)(T/A) CCC C-3′, P2 (533–551): 5′-C(A/G)A CTA CTT TGG GTG TCC G-3′, and R2 (1082–1064): 5′-C(T/G/C/A)G G(T/C/A/G)A (A/G)(T/C) T TCC A(C/A)C ACC A-3′, designed according to the conserved VP4/VP2 region of HRV (GenBank sequence L24917), resulting in amplification of a 920-bp fragment in the first run with primers P1 and R2 and a 550-bp fragment in the second run with primers P2 and R2. All PCR products were analyzed by agarose gel electrophoresis. A previously published protocol was used,[13] and all procedures, including specimen processing, RNA and DNA extraction, RT-PCR/PCR amplification, and gel electrophoresis, were performed in separate areas of the laboratory to avoid PCR contamination.

Sequencing and analysis of second run polymerase chain reaction products

Amplified products from the second semi-nested PCR run were sequenced by Invitrogen Inc., and phylogenetic analysis performed via an NCBI BLAST search (http://blast.ncbi.nlm.nih.gov) and the MEGA version 6.0 software package,[14] to identify HRV species. Phylogenetic trees were constructed with the neighbor-joining method and maximum composite likelihood model, using HRV sequences from our study and from GenBank. A discrete gamma distribution used to model evolutionary rate differences among sites (1 category, +G) was constructed with MEGA 6.0. Bootstrap resampling (1000 replications) was used to assess the reliability of individual nodes in each phylogenetic tree.

Statistical analysis

Chi-square and analysis of variance were used to compare the prevalence of the three HRV species in each study group. To identify the correlation of different species of HRV infection with asthma, Spearman's rank correlation was used to compare the positive rates of HRV-A, B, or C between the asthma group and control group or nonasthma ARI group.

Using odds ratio (*OR*) as an indicator, regression analysis was used to determine the effect of different species of HRV infection on asthma. All tests were two-tailed, and $P < 0.05$ was considered statistically significant. All analyses were conducted in SPSS statistics version 22.0 (IBM, NY, USA).

Results

Respiratory virus screening

Between October 2013 and November 2014, 155 young children with a mean age 2.7 ± 1.5 years, 116 (74.8%) male, and 39 (25.2%) female were enrolled in the asthma group, 461 children with a mean age 3.0 ± 3.3 years, 288 (62.5%) male, and 173 (37.5%) female were enrolled in the nonasthma group. Between August 2014 and March 2015, 86 children with a mean age 3.0 ± 2.3 years, 70 (81.4%) male, and 16 (18.6%) female were enrolled in the control group. A total of 702 patients were enrolled.

The viral pathogen screening for all patients [Table 1] revealed that HRVs were the most common pathogens, with a prevalence of 15.4% (108/702). Similarly, in the asthma group, HRVs were the most common pathogens, with a prevalence of 25.8% (40/155). In the nonasthma ARI group, 11.1% (51/461) patients were positive for HRV. In the control group, 19.8% (17/86) of patients were HRV-positive. The prevalence of HRV was significantly different ($F = 10.680$, $P < 0.001$) among the three study groups (25.8%; 11.1%; 19.8%). While no significant difference was found between the asthma and control groups ($F = 1.113$, $P = 0.293$), there was a significant difference between the asthma and nonasthma ARI groups ($F = 20.633$, $P = 0.000$).

Sequencing and phylogenetic analysis of human rhinoviruses

In the 108 samples positive for HRV by semi-nested PCR, there were 105 (97.2%) specimens with sufficient PCR product for sequencing. By sequencing the 105 PCR products, partial VP4/VP2 nucleotide sequences were obtained, then BLAST-searched against available sequences of HRV-A, HRV-B, and HRV-C from GenBank. Figure 1 shows these 105 sequences from the three patient groups were divided into three HRV species Groups (A, B, and C). The HRV-A cluster was closer to HRV-B than HRV-C. The HRV-C cluster, with 56 of the 105 sequences, is the largest one of these three clusters, revealing HRV-C as the dominant species in the study population. The HRV-A cluster, with 41 sequences, is the second largest, and the HRV-B cluster, with eight sequences, is the smallest. The sequences belonging to different HRV species from the different patient groups were stratified into different subclusters, together with sequences of different HRV serotypes from GenBank, and there are sequences from different patient groups clustered into one subcluster [Figure 1].

The sequence analysis results showed that 41 cases (5.8%, 41/702) were confirmed as HRV-A, including 22 from the nonasthma ARI group (4.8%, 22/461), 13 from the asthma group (8.4%, 13/155), and six from the control group (7.0%, 6/86), eight cases (1.4%, 8/702) were confirmed as HRV-B, including four from the nonasthma ARI group (0.8%, 4/461), one from the asthma group (0.6%, 1/155), and three from the control group (3.5%, 3/86), and 56 (8.0%, 56/702) were confirmed as HRV-C, including 22 from the nonasthma ARI group (4.8%, 22/461), 26 from the asthma group (16.8%, 26/155), and 6 from the control group (9.3%, 6/86). The overall proportion of HRV-A and HRV-C positive samples in the study were similar ($\chi^2 = 2.171$, $P > 0.05$), and both were higher than that of HRV-B ($\chi^2 = 21.654$, $P < 0.01$ and $\chi^2 = 36.164$, $P < 0.01$, for HRV-A and HRV-C, respectively).

Correlation between human rhinovirus species and asthma

In the nonasthma ARI group, there were significant correlations between HRV-A and HRV-C and asthma, with *R* values of 0.08 ($P = 0.037$, $P < 0.05$) and 0.20 ($P < 0.001$), respectively [Table 2]. No significant correlations were identified for HRV-A, B, and C infections between the asthma group and control group ($P > 0.05$).

Sex and age were not evenly distributed, with more male cases and more young patients in all groups; therefore, the distribution of sex and age in different groups was adjusted prior to regression analysis [Table 3]. The *OR* between the asthma group and nonasthma ARI group for HRV-A and HRV-C were 2.2 (95% confidence interval [*CI*], 1.065–4.409) and 4.2 (95% *CI*, 2.262–7.906), respectively, which confirmed patients with HRV-C infection were 4.2 times more likely to have asthma ($P < 0.001$), and patients with HRV-A infection were 2.2 times more likely to have asthma ($P < 0.05$).

Table 1: Results of viral detection in different patient groups

Patient groups	Number of tested	Number of positive for DFA and virus isolation (%)					Number of positive for PCR (%)							
		RSV	HAdV	Flu A	Flu B	HMPV	HRVs	HBoV	WU	KI	PIV1	PIV2	PIV3	PIV4
Nonasthma ARI	461	77 (16.7)	8 (1.7)	11 (2.4)	5 (1.1)	2 (0.4)	51 (11.1)	18 (3.9)	4 (0.8)	8 (1.7)	2 (0.4)	2 (0.4)	15 (3.3)	5 (1.1)
Asthma	155	26 (16.8)	0	2 (1.3)	0	2 (1.3)	40 (25.8)	4 (2.6)	5 (3.2)	0	1 (0.6)	0	3 (1.9)	1 (0.6)
Control	86	4 (4.7)	0	0	0	0	17 (19.8)	0	0	0	0	0	2 (2.3)	0
Total	702	107 (15.2)	8 (1.1)	13 (1.9)	5 (0.7)	4 (0.6)	108 (15.4)	22 (3.1)	9 (1.3)	8 (1.1)	3 (0.4)	2 (0.2)	20 (2.8)	6 (0.9)

ARI: Acute respiratory infection; RSV: Respiratory syncytial virus; HAdV: Human adenovirus; Flu A: Influenza virus A; Flu B: Influenza virus B; HMPV: Human metapneumovirus; HRVs: Human rhinoviruses; HBoV: Human bocavirus; WU: WU polyomavirus; KI: KI polyomavirus; PIV1, PIV2, PIV3, and PIV4: Parainfluenza viruses 1, 2, 3, and 4; DFA: Direct immunofluorescence assay; PCR: Polymerase chain reaction.

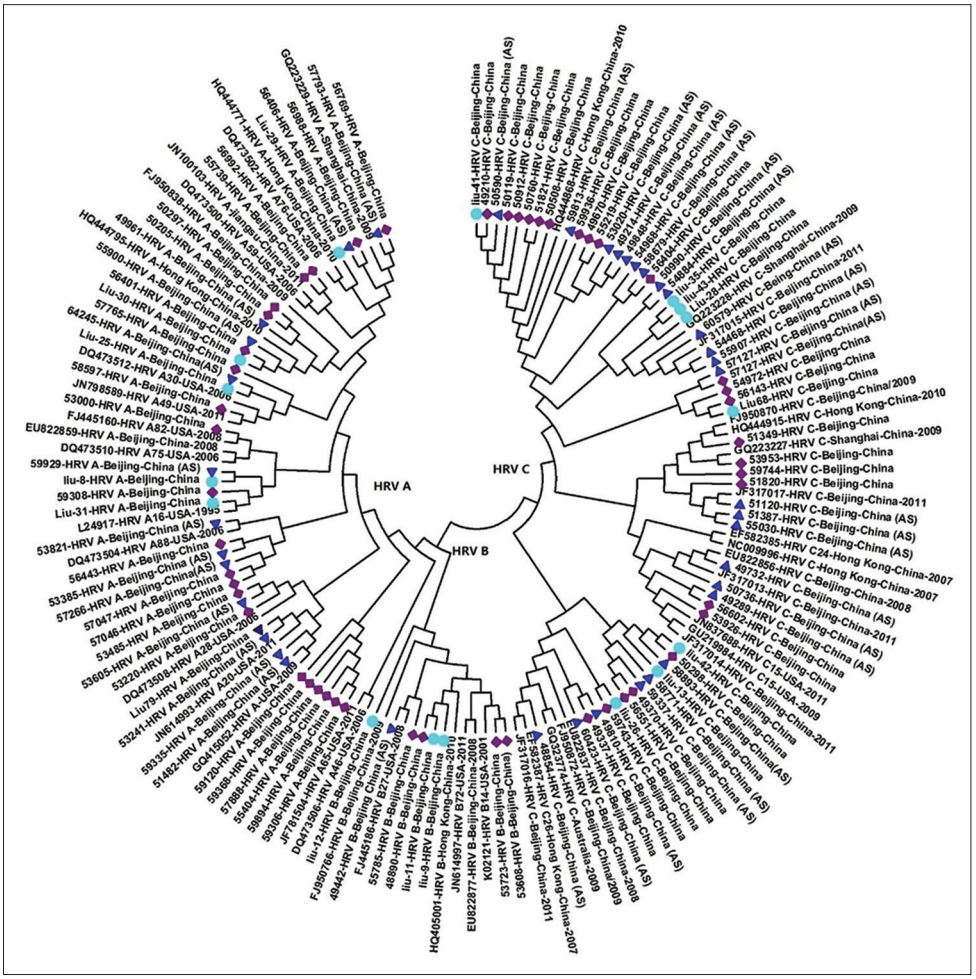

Figure 1: Phylogenetic analysis for HRV sequences from a sample of 702 children in Beijing. The 105 partial VP4/VP2 gene sequences from this study and HRV species A, B, and C sequences from GenBank were used for tree construction. Turquoise circles denote HRV sequences from the control group, blue triangles those from the asthma group, and purple diamonds the sequences from the nonasthma group. HRV: Human rhinovirus.

Table 2: Correlation between different species HRV infection with asthma

Groups compared with asthma group	HRV species	Spearman's *R*	*P*
Control group ($n = 86$)	HRV-A ($n = 6$)	0.036	0.581
	HRV-B ($n = 3$)	−0.107	0.099
	HRV-C ($n = 8$)	0.095	0.141
Nonasthma ARI group ($n = 461$)	HRV-A ($n = 22$)	0.084	0.037
	HRV-B ($n = 4$)	−0.011	0.790
	HRV-C ($n = 22$)	0.197	<0.001

ARI: Acute respiratory infection; HRVs: Human rhinoviruses.

Discussion

In this study, respiratory virus screening (including HRVs) by DFA, virus isolation, and PCR revealed that HRVs were the most common pathogens, with a prevalence of 15.4% (108/702) in all patients, and 25.8% (40/155) in the asthma group, 11.1% (51/461) in the nonasthma ARI group, and 19.8% (17/86) in the control group. The prevalence of HRV was higher in the asthma group, compared with the nonasthma group, which may imply an association between HRVs and asthma.

HRVs are the most common cause of respiratory illness in children and are commonly associated with asthma symptoms, requiring escalation of care and emergency room visits in many patients.[15] A previous study in Beijing reported 19.0% (14/73) of patients diagnosed with asthma were HRV-positive, and wheezing was a common symptom in children with ARI.[16] This suggests that HRV is an important pathogen for children with ARI, particularly those with lower respiratory infections. However, the epidemiology and clinical significance of different species of HRVs, especially HRV-C, are poorly characterized.

Based on the phylogenetic analysis, these HRVs were divided into different species, HRV-A, B, and C. HRV-C cluster was the largest among the three clusters, followed by HRV-A, then HRV-B, identifying HRV-C as the dominant species in the study. HRV-C was also the dominant species in the asthma (16.8%) and control groups (9.3%) but not in the nonasthma ARI group, where the prevalence of HRV-C and HRV-A was equal. In a study aiming to characterize the clinical and demographic features associated with different HRV species in Northeast Brazil, Fawkner-Corbett *et al*.

Table 3: Association between different species of HRV infection and asthma in a sample of 702 children in Beijing

Groups compared with asthma group	HRV species	Category	*P*	*OR*	95% *CI* of *OR*	
					Lower limit	Upper limit
Control group (n = 86)	HRV-A (n = 6)	Reference constant	0.411	1.466		
		Gender*	0.220	1.510	0.782	2.915
		Age*	0.126	0.894	0.774	1.032
		HRV-A	0.584	1.323	0.486	3.603
	HRV-B (n = 3)	Reference constant	0.265	1.691		
		Gender*	0.263	1.458	0.754	2.822
		Age*	0.084	0.879	0.760	1.018
		HRV-B	0.112	0.155	0.016	1.541
	HRV-C (n = 8)	Reference constant	0.543	1.331		
		Gender*	0.193	1.551	0.801	3.005
		Age*	0.132	0.895	0.775	1.034
		HRV-C	0.135	1.912	0.818	4.470
Nonasthma ARI group (n = 461)	HRV-A (n = 22)	Reference constant	0.406	0.781		
		Gender*	0.005	0.557	0.369	0.841
		Age*	0.183	0.956	0.895	1.021
		HRV-A	0.033	2.167	1.065	4.409
	HRV-B (n = 4)	Reference constant	0.467	0.807		
		Gender*	0.006	0.564	0.374	0.849
		Age*	0.199	0.958	0.897	1.023
		HRV-B	0.825	0.778	0.084	7.189
	HRV-C (n = 22)	Reference constant	0.244	0.703		
		Gender*	0.008	0.570	0.376	0.865
		Age*	0.176	0.954	0.892	1.021
		HRV-C	0.000	4.229	2.262	7.906

*Adjusted. HRV: Human rhinoviruses; *CI*: Confidence interval; *OR*: Odds ratio; ARI: Acute respiratory infection.

believed that HRV-A was the dominant species, with a prevalence of 73%, compared with 27% in HRV-C and 0 HRV-B. In addition, HRV-C was detected more frequently than HRV-A in children with asthma or episodic viral wheeze,[17] which supports the association of HRV-C with asthma, identified in the present study that the *OR* between the asthma and nonasthma ARI group for HRV-A and HRV-C confirmed patients with HRV-C infection were 4.2 times more likely to have asthma ($P < 0.001$), and patients with HRV-A infection were 2.2 times ($P < 0.05$). Therefore, HRV-C had the strongest correlation with asthma in the study population.

There was no significant difference in the prevalence of HRV-B among the three groups, which may be because there was no correlation between HRV-B and asthma. However, the proportion of HRV-B in the control group (3.5%, 3/86) is higher than that in the asthma group (0.6%, 1/155) and the nonasthma ARI group (0.8%, 4/461). In addition, there were few HRV-B positive specimens in this study, as in a previous study that nine infants had HRV-A, 13 had HRV-C, and one infant had HRV-B.[18] Therefore, more data are needed to evaluate the association between HRV-B infection and asthma.

There was a high prevalence of HRVs in the control group (19.8%), which may suggest a high prevalence of asymptomatic HRV infection in children. However, this contradicts the association identified between HRV-A and HRV-C infection and asthma. Chen *et al*. reported that the prevalence of HRV species differed with age, and HRV-C infection was more common in children compared with adults, with the majority of adults being infected with HRV-A.[19] Other research has described a high prevalence of HRV-C in children, regardless of clinical status.[20-22] In the present study, compared with 155 patients in the asthma group, there were only 86 patients in the control group. More data are needed to determine the clinical significance of a high prevalence of HRVs in the control group.

The sequences of different clusters from the different patient groups in the phylogenetic analysis were BLAST-searched against HRV serotypes from GenBank, and some patient group sequences were clustered into one subcluster. This revealed that different HRV serotypes were prevalent during the study period. Further studies are needed to understand the relationship of HRV serotype with the severity of illness or specific disease, especially with asthma.

In conclusion, the results in this study suggest there is a high prevalence of HRVs in children in Beijing, regardless of clinical status, and HRV-C may be a key factor in determining an association between HRVs and asthma. These data will help in understanding the relationship between HRVs and asthma.

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Conflicts of interest

There are no conflicts of interest.

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