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Association between human rhinovirus C and severity of acute asthma in children

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ABSTRACT: A new and potentially more pathogenic group of human rhinovirus (HRV), group C (HRVC), has recently been discovered. We hypothesised that HRVC would be present in children with acute asthma and cause more severe attacks than other viruses or HRV groups.

Children with acute asthma (n=128; age 2–16 yrs) were recruited on presentation to an emergency department. Asthma exacerbation severity was assessed, and respiratory viruses and HRV strains were identified in a nasal aspirate.

The majority of the children studied had moderate-to-severe asthma (85.2%) and 98.9% were admitted to hospital. HRV was detected in 87.5% and other respiratory viruses in 14.8% of children, most of whom also had HRV. HRVC was present in the majority of children with acute asthma (59.4%) and associated with more severe asthma. Children with HRVC (n=76) had higher asthma severity scores than children whose HRV infection was HRVA or HRVB only (n=34; p=0.018), and all other children (n=50; p=0.016). Of the 19 children with a non-HRV virus, 13 had HRV co-infections, seven of these being HRVC.

HRVC accounts for the majority of asthma attacks in children presenting to hospital and causes more severe attacks than previously known HRV groups and other viruses.

KEYWORDS: **Acute asthma, children, exacerbation severity, human rhinovirus C, rhinovirus**

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Asthma exacerbations are a leading cause of hospitalisation for children in developed countries and the majority of these exacerbations are associated with viral respiratory infections (VRIs), particularly with human rhinovirus (HRV) [1, 2]. HRV can infect and replicate within the lower respiratory tract where the host inflammatory response may potentiate an asthma attack [3]. Approximately 60% of asthma exacerbations have been estimated to be associated with an HRV infection [1].

Detection and typing of HRV was originally based on viral culture in cell lines and human serum antibody responses, which identified 101 classical serotypes [4] that belong to HRV groups A (HRVA) or B (HRVB). Subsequently, these techniques were replaced by more sensitive and specific detection methods using RT-PCR. These methods have been responsible for the recent detection of many new HRV strains, the majority of which have been tentatively classified into a phylogenetically distinct group of HRV strains referred to as HRVC [5–22]. The importance of HRVC in respiratory disease is still unclear, as its community prevalence and clinical role have not been investigated in detail, but the recent detection of HRVC in hospitalised children has led to the suggestion that HRVCs may be more pathogenic than other HRVs [10].

One of the recently developed molecular assays is the Respiratory Multicode-PLx Assay (RMA), which is sensitive and specific, and involves multiplex PCR and flow cytometry to simultaneously detect HRV and other common respiratory viruses [23]. When coupled with semi-nested PCR, cloning and sequencing to type HRV strains, 26 new HRV strains were discovered in infants with a VRI [13]. The large number of new strains detected with this approach is likely to result from advances in primer design based on the 5′ noncoding region (NCR) sequences from all 101 classical serotypes. The region covered by each primer sequence is completely conserved in 99 out of the 101 serotypes and has only one single mismatch in two serotypes (HRV33 and HRV78). These improved methods have the

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potential to enhance HRV detection and identify new strains in children with respiratory symptoms, thereby increasing prevalence estimates.

Therefore, using these new detection methods [13, 23], we aimed to: 1) determine the prevalence of infection with HRV, including HRVC, and other respiratory viruses in children presenting to a hospital emergency department with acute asthma; and 2) investigate the relationship between HRVC and acute asthma. We hypothesised that we would detect a higher prevalence of HRV, including HRVC, than previous studies and that HRVC would be associated with more severe asthma.

MATERIALS AND METHODS

Study participants

Children studied were participants of the Perth Childhood Acute Asthma Study (PCAAS), for which subjects aged 2–16 yrs with acute asthma were recruited on presentation to the Emergency Department (ED) of Princess Margaret Hospital for Children, Perth, Australia since February 2002 [24]. Recruitment occurred without regard to season. PCAAS was approved by the hospital's Human Ethics Committee, with parental/guardian written informed consent prior to participation. Participants were diagnosed with acute asthma, treated and the requirement for hospitalisation evaluated by ED doctors using criteria in accord with previous and current published international guidelines [25], and standard hospital protocols were used to determine treatment with supplemental oxygen, inhaled salbutamol, ipratropium bromide and oral prednisolone.

Data and sample collection

For each child, a detailed questionnaire and medical records were used to provide information on asthma and respiratory infections. An asthma exacerbation severity score (see online supplement) was assigned at presentation using a modified National Institutes of Health score [24, 26] of clinical parameters that was corrected to baseline: mild (score of 0–2), moderate (3–6) or severe (7–10). A specimen of nasal secretions was collected from children recruited between April 2003 and February 2010, either by per-nasal aspirate using suction into a mucus trap and diluted in 2 mL saline, or by a flocked swab.

Virus detection

A nasal secretion specimen from each child was tested for respiratory viruses by direct fluorescent antibody testing using virus-specific monoclonal antisera (Meridian Bioscience Inc., Cincinnati, OH, USA and Dako Diagnostics Ltd, Glostrup, Denmark), and/or by RNA extraction, cDNA synthesis and the RMA [23]. In brief, the RMA uses 18 sets of virus-specific primers for multiplex amplification of conserved regions of the genomes of nine respiratory viruses: HRV, respiratory syncytial virus (RSV), adenovirus, influenza A and B, parainfluenza 1–4b, metapneumovirus, enterovirus (EnV), coronavirus, and bocavirus. PCR products were labelled with virus-specific tags and site-specific biotins by target-specific extension (TSE). The virus-specific tags were then hybridised to complementary oligonucleotides on the surfaces of microspheres, which were distinguished by cytometry (Luminex LabMap 100 cytometer; Luminex, Brisbane, Australia) and read in association with TSE product-specific fluorescence signals.

HRV typing

Nasal secretion specimens were also analysed with a HRV molecular typing assay to determine which HRV strains were present and differentiate the closely related EnV from HRV. This molecular assay determines the HRV strain using a 260-bp variable sequence in the 5′ NCR of the HRV genome [13, 23]. Briefly, cDNA made from extracted viral RNA was amplified using specificallydesigned RT-PCR primers. PCR products were sequenced either directly, or after being cloned into plasmid vectors and transformed into *Escherichia coli*. For each cloned sample, three or more plasmids were sequenced. Sequences were then used to type the HRVs by comparing them with sequences of 101 classical serotypes and recently identified strains (prefixed with W) using phylogenetic tree reconstruction analysis with ClustalX (University College Dublin, Dublin, Ireland) software [13]. The genetic grouping of the new strains was then confirmed by analysing their 420-bp viral protein (VP)4/VP2 sequences.

Statistical analyses

Independent-samples t-tests were employed to analyse differences in severity between new HRVC strains, and HRVA/B serotypes, all other viruses and all children not infected with new HRVC strains. An independent-samples t-test was also used to investigate relationships between age and HRVC infection. Linear regression was used to adjust for confounding factors. A p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

Nucelotide sequence accession numbers

The 5′ NCR sequences of new strains found in this study were deposited in the GenBank sequence database, accession numbers FJ968439 to FJ968447.

RESULTS

Population demographics

Table 1 lists the demographics for the 128 children with acute asthma.

Respiratory virus detection

In children with acute asthma, a respiratory virus was detected in 118 (92.2%) out of 128 cases and an HRV in 112 (87.5%) out of 128 cases (table 2). We observed a high prevalence of HRV co-infection and few children (six (4.7%) out of 128 cases) that

TABLE 1 Population demographics of the Perth Childhood Acute Asthma Study

Cases n	128
Age at recruitment yrs	6.4±3.3
Males	79 (61.7)
Admitted to hospital#	93 (98.9)
Acute asthma severity¶	5.0±2.2
Oxygen saturation+ %	93.2±5.4

Data are presented as mean±SD or n (%), unless otherwise stated. #: n=94 cases with data available; ¶: acute asthma severity scores were assigned (online supplement) and corrected to baseline; scores range from 0 (mild) to 10 (severe) exacerbations; +: n=110 cases with data available.

were positive for a virus that was not a HRV. In the 112 HRV-positive nasal secretion specimens, 114 HRVs were detected (two cases of dual HRV infection) and 112 were successfully typed. For the children with a non-HRV respiratory virus detected, 13 were co-infected with HRV.

HRV typing

The 112 identified HRVs from 110 samples clustered into 56 different strains: 32 HRVs detected belonged to 24 previously known serotypes (22 HRVA and two HRVB) and 80 HRVs belonged to 32 new strains (one new HRVA strain and 31 HRVC strains) (table 3). Two children had an untypeable HRV. Although not statistically significant, children infected with an HRVC strain tended to be younger than children who were not infected with HRVC (mean age 5.9 and 7.0 yrs, respectively; p=0.064). Nine strains (W2, W3, W4, W16, W29, W30, W43, W44 and W46) were novel and have been deposited in the GenBank sequence database. No one strain was predominant, yet the majority (76 (67.9%) out of 112 strains) of HRVs identified were HRVC.

TABLE 2 Frequency of common respiratory viruses detected in per-nasal aspirates from 128 children with an asthma exacerbation

Viral respiratory infections	Cases n (%)
Virus-positive	118 (92.2)
HRV	112 (87.5)
New strains	79 (61.7)
HRVC	76 (59.4)
HRVA#	3 (2.3)
Classical serotypes, HRVA# or B	31 (24.2)
Unidentified	2 (1.6)
Other common respiratory viruses¶	19 (14.8)
RSV	2 (1.6)
PIV	3 (2.3)
InfV	1 (0.8)
AdV	3 (2.3)
hMPV	5 (3.9)
EnV	5 (4.8)
CoV	1 (1.0)
BoV	1 (1.0)
Multiple infections	14 (10.9)
HRV co-infection+	13 (10.2)
Dual infection§	11 (8.6)
Triple infectionƒ	3 (2.3)

HRV: human rhinovirus; RSV: respiratory syncytial virus; PIV: parainfluenza virus; InfV: influenza virus; AdV: adenovirus; hMPV: human metapneumovirus; EnV: enterovirus; CoV: coronavirus; BoV: bocavirus. #: HRV infection was only with HRVA strain; ¶: two cases had two non-HRV viruses; only 104 cases were tested for EnV, CoV and BoV; +: HRV co-infection does not include dual HRV infection; §: HRV/hMPV (n=3), HRV/AdV (n=2), HRV/RSV (n=2), HRV/PIV (n=2), HRV/EnV (n=1) and HRV/HRV (n=1; classical and new strain); ƒ: HRV/EnV/CoV (n=1), HRV/hMPV/InfV (n=1) and HRV/HRV/AdV (n=1; new HRV strains).

Frequency of HRVC infection

The majority (76 (59.4%) out of 128 cases) of children with acute asthma were infected with an HRVC strain compared with 34 (26.6%) out of 128 cases whose only HRV infection was with a strain from the HRVA or B group (one child had dual infection with a new HRV and a classical HRV) (table 2 and fig. 1).

HRVC and asthma severity

Asthma severity scores for the 76 children infected with HRVC (mean exacerbation severity score 10.4, 95% CI 10.0–10.9) were higher than: 1) those whose HRV infection was only with HRVA or B serotypes (n=34; mean score 9.5, 95% CI 8.7–10.3; p=0.028); 2) those with a viral infection that was not a new HRVC strain (n=40; mean score 9.4, 95% CI 8.7–10.1; p=0.013); or 3) all other children who were not infected with an HRVC strain (n=50; mean score 9.4, 95% CI 8.7–10.2; p=0.015). These differences in severity remained significant after adjustment for age and sex (p=0.018, 0.009 and 0.016, respectively) (fig. 2). Similar statistical significance remained if the one child with dual (new and classic) HRV infection was excluded from these analyses. The high detection rate of HRV precluded analyses of clinical phenotypes between HRV-infected and noninfected children, as well as children with any VRI *versus* children without a VRI detected. In addition, the high admission rate of subjects with acute asthma meant that HRV infection rates and types between children admitted and not admitted could not be compared. There was no statistically significant difference between children infected with HRVC and the other groups of children for additional variables that may indicate severity, relapse and atopy, which included: 1) the number of hours from hospital presentation to discharge; 2) number of asthma admissions since the last study visit; 3) atopic status by skin prick test (SPT); and 4) the number of positive SPTs (data not shown).

DISCUSSION

The current study has shown that the newly identified HRVC group of viruses is responsible for not only the majority of acute asthma attacks in children, but also that this group causes more severe attacks than previously known viruses. We used a technique that optimised viral detection and typed HRV to demonstrate a higher HRV detection rate in children presenting to hospital with acute asthma than previously reported [1, 2], but more strikingly, the newly discovered HRVC strains: 1) accounted for the majority of asthma attacks in children presenting to hospital; and 2) were associated with higher acute asthma severity scores than previously known HRV serotypes and other respiratory viruses. Also, most cases of infection with other respiratory viruses were HRV co-infections. Hence, we have established that the unique and close relationship between HRV and acute asthma in children is underpinned by infection with the new HRVC strains. These novel findings are of fundamental importance to the understanding of asthma in children.

The study has two major findings. First, new HRVC strains were detected in more than half of all asthma exacerbations. This rate was much higher than in previous studies in which HRV strains were typed, as in these, HRVC was identified in less than one-third of children studied [7–14]. The differences may be due to our cohort having more severe asthma exacerbations than children in previous studies, as the great

TABLE 3 Frequency and type/strain of 112 human rhinoviruses (HRVs) identified in per-nasal aspirates from 128 children with acute asthma

HRVA strains	n	HRVB serotypes	n	New HRVC strains	n
R1A	1	R3	1	W1	1
R2	1	R69	1	W2	6
R9	1			W3	3
R12	1			W4	1
R15	2			W7	1
R16	1			W8	2
R21	1			W9	1
R24	1			W10	2
R28	1			W12	3
R29	1			W13	1
R33	1			W15	2
R34	3			W16	6
R44	1			W17	2
R49	5			W19	1
R53	1			W20	2
R55	1			W21	4
R57	1			W23	5
R61	1			W24	5
R66	1			W25	2
R75	1			W26	3
R78	2			W29	1
R81	1			W30	2
W28	4			W31	5
				W32	5
				W35	1
				W36	1
				W38	3
				W43	1
				W44	1
				W46	2
				W47	1
Total	34		2		76

Dual HRV infections: R49/W25 and W3/W28. R: classical serotype; W: new strain.

majority of our subjects required admission to hospital. Improved detection methods may also have contributed to our higher detection rate. The second discovery was that these new HRVC strains were associated with more severe asthma attacks than other HRV serotypes and other respiratory viruses. The finding that HRVCs are more pathogenic than other HRVs in acute asthma is consistent with a recent study of children hospitalised with symptoms of a respiratory infection: those infected with HRVC were more likely to require supplemental oxygen than those infected with HRVA [10]. Although many current authorities view HRV as having an important role in asthma exacerbations, our data suggest that even this view underestimates the true importance of HRV in asthma and the significant contribution (in terms of both prevalence and pathogenicity) of the new group of HRVs. Overall, HRVCs appear to represent a species of rhinovirus

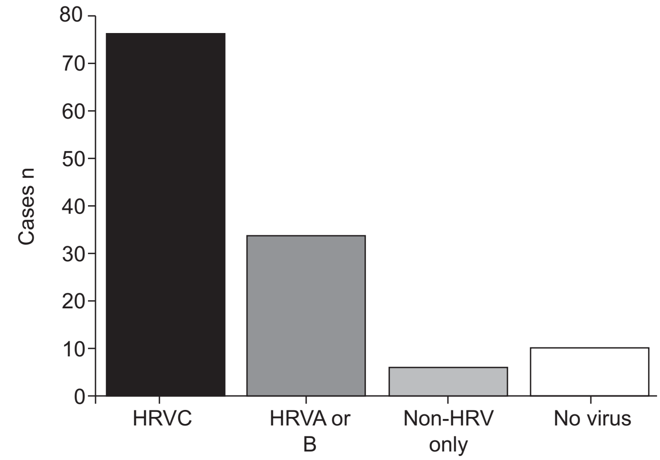

FIGURE 1. Frequency of human rhinovirus (HRV) and other common respiratory viruses identified in 128 children with an asthma exacerbation. HRV strains were identified, hence children were categorised into those with new HRVC strains or those whose HRV infection was only with other HRV serotypes (HRVA or HRVB); two children had unidentified HRV infection. Other common respiratory viruses tested for were respiratory syncytial virus, adenovirus, influenza A and B, parainfluenza 1–4b, metapneumovirus, enterovirus, coronavirus and bocavirus.

that has a greater clinical relevance and prognostic significance for respiratory disease than known HRVs.

The importance of the role of HRV in acute asthma is further emphasised by the comparatively substantially lower rate of detection of other respiratory viruses, together with the high incidence of HRV co-infection. The techniques we used are

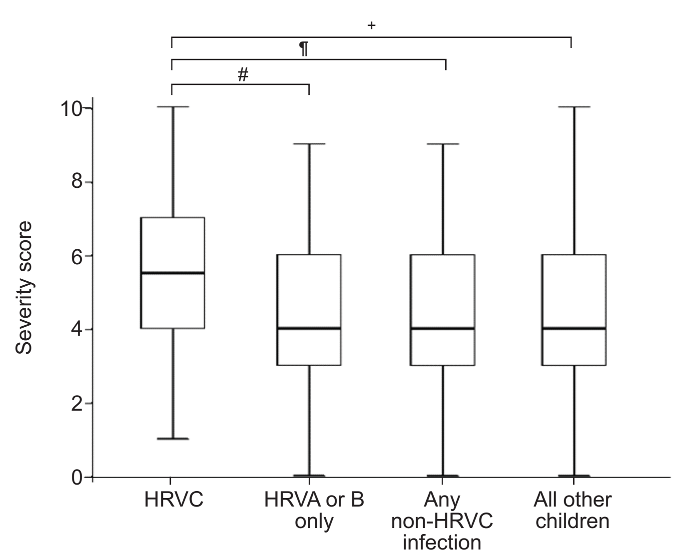

FIGURE 2. Relationship between human rhinovirus (HRV)C infection and severity of asthma exacerbation in 128 children. Following HRV strain identification in all but two cases. Linear regression analyses showed that children with HRVC (n=76) had significantly more severe asthma attacks than the children without HRVC, whether they were analysed as those whose HRV infection was only with HRVA or HRVB serotypes (n=34), those with any respiratory virus other than HRVC (n=40) or all children negative for HRVC (n=50). —: median; box: interquartile range; whiskers: range. #: p=0.018; ¶: p=0.009; +: p=0.016.

likely to be accurate with respect to detection rate of other respiratory viruses, as in the 90 children in whom both detection techniques were used, the detection of non-HRV viruses was very similar (slightly improved with molecular methods). Previous studies have shown that HRV was detected in more infants and children than RSV, including those with severe disease requiring hospitalisation [2, 27]. In addition, in HRV infections induced experimentally, asthmatics have been shown to have relatively defective innate immune and T-helper cell type 1 responses that are likely to lead to increased viral replication and load [28, 29]. These defects in immune responses may explain the higher frequency, severity and duration of lower respiratory tract symptoms in asthmatics with HRV compared with nonasthmatics [30]. Furthermore, a prospective birth cohort of infants with an atopic parent [31] found HRV was the strongest risk factor for the development of asthma at 6 yrs of age; the odds ratio for children diagnosed with asthma by 6 yrs of age was 31.7 ($p<0.0001$) for children who wheezed with HRV in the third year of life *versus* only OR 3.4 for aeroallergen sensitisation. This exceptionally high odds ratio suggests that HRV-induced wheeze at 3 yrs of age and asthma at 6 yrs of age are the same condition, and that HRV infection is the most powerful early environmental factor associated with asthma in mid-childhood identified to date. Our results support and substantially strengthen the view that HRV is the most significant pathogenic factor associated with childhood asthma exacerbations, and other respiratory viruses have a comparatively minor role. Clearly, our data raise many further questions and more studies will be needed to fully define the role and pathogenicity of HRV, particularly the new strains, in asthma in both children and adults as well as in other respiratory illnesses.

The most important finding of this study, the significant relationship between HRVC and attack severity, is independent of the presence of data from a control group. The finding that HRVC was present in over half of the cases of children with acute asthma is also independent of control group data. Indeed, no one control group could adequately account for the many important variables found in such children with acute asthma, but particular attention needs to be paid to determine how often HRVC causes symptoms in those in whom it is detected. Several different ‘‘control groups’’ would be required to provide full context to our findings. A control group of children who are siblings or close friends of study cases, for example, would be useful to demonstrate whether these children in close contact with the subject are also infected with HRV, but suffer comparatively mild respiratory symptoms. In addition, studying a control group of unselected community children that includes subjects who have previously been diagnosed with asthma and are not necessarily unwell at the time of study would allow the determination of whether HRVC strains infect asthmatics without leading to a severe asthma exacerbation. A further useful control group would be children from the same community with mild or moderate asthma that is not sufficiently severe to present to hospital. Finally, studies are needed to define the potential pathogenicity of HRVC in other respiratory conditions. Thus, there is an urgent need for a broad series of studies to define the epidemiology and pathogenicity of HRVC infection in children and to answer the many questions that arise from our data.

Interestingly, among the large number of HRVs that we identified in children with severe asthma exacerbations in Western Australia, 29 were new HRV strains and nine may be unique (W2, W3, W4, W16, W29, W30, W43, W44 and W46). New HRV strains have been found in Hong Kong (China) [12], Queensland (Australia) [14], New York State (USA) [6], California (USA) [5], Tennessee (USA) and New York City (NY, USA) [9], Amman (Jordan) [10], Seoul (South Korea) [7], and Turku (Finland) [22]. However, not all methods utilised the same HRV genome regions for identification, which would have facilitated interstudy comparison of all the new strains. Therefore, until the full genome sequences of all the new strains are available, their phylogenetic classification remains to be fully determined. Regardless of HRV group, 20 new HRV strains were common to children in Perth and infants in Wisconsin (USA) [13]. This suggests that a large number of new HRV strains circulate the globe irrespective of population differences and cause severe respiratory illness in childhood.

In conclusion, we used an optimal viral detection method to show that HRVC was present in the great majority of children with acute asthma and the prevalence was much higher than that of other respiratory viruses. The new HRVC strains were far more common than previously known HRV serotypes, and caused more severe asthma than both the previously known HRV serotypes and other viruses. These findings suggest that HRVC is by far the most important virus group in acute asthma. Further studies are needed to investigate the epidemiology and the pathogenicity of the different HRV strains and host susceptibility to these strains with a view to developing new therapeutic strategies.

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STATEMENT OF INTEREST
A statement of interest for J.E. Gern can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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