Title: Anti-rhinovirus antibodies in children with asthma exacerbations and a known rhinovirus infection

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ABSTRACT

Background Asthma exacerbations are associated with human rhinovirus (HRV) infections and more severe exacerbations are associated with HRV-C. We have previously shown the HRV-C specific antibody response is low in healthy adult sera, and unexpectedly most of the antibody to HRV-C is cross-reactive with HRV-A.

Objectives To compare the antibody response to each HRV species in asthmatic and non-asthmatic children where infection with the type of HRV was known.

Methods Total and specific IgG1 binding to HRV viral capsid protein (VP1) antigens of HRV-A, -B and -C were tested in the plasma from non-asthmatic children (n=47) and children presenting to the emergency department (n=96) with asthma exacerbations. HRV, found in the majority of children at the time of their exacerbation (72%), was analysed using molecular typing.

Results Asthmatic children had higher total IgG1 antibody responses to HRV and the IgG1 titers specific to HRV-A, and to a lesser extent HRV-B, were higher than the non-asthmatic controls. The species-specific responses to HRV-C were significantly lower than titers to HRV-A and HRV-B in both asthmatic and non-asthmatic children ($p<0.001$) and were not associated with the susceptibility to asthma exacerbation or the detection of HRV-C at the time of recruitment.

Conclusions The higher antibody titers to HRV-A and HRV-B found in asthmatic children show that changes in T-cell responses described by others are not part of overall decreased anti-HRV immune responses. The low species-specific HRV-C titers found in all groups, even when virus was found, point to a different and possibly less efficacious immune response to this species.

Key Messages

- Children with asthma exacerbations have higher total IgG1 antibody titers to human rhinovirus compared to non-asthmatic controls showing they do not have deficient antibody responses to HRV.
- All children had low HRV-C specific responses, even when infected with HRV-C. The HRV-A and HRV-B specific IgG1 titers were higher for the asthmatic group.

Capsule Summary

The immune response to HRV-C, the species most frequently associated with more severe asthma exacerbations, is low and different to the common HRV-A found to be increased in asthmatics.

Keywords

Human rhinovirus; VP1; IgG1 antibody; Asthma exacerbation; Children

Abbreviations

HRV Human rhinovirus

70 ED Emergency department

71 VP1 Viral capsid protein 1

72

INTRODUCTION

Exacerbations of asthma in children are frequently associated with human rhinovirus (HRV) infections^{1,2} and HRV-induced wheeze in infancy is a predictor of the development of asthma.^{3,4} The newly recognised HRV-C species reportedly accounts for the majority of attacks in children presenting to hospital with asthma and is associated with more severe attacks than HRV-A and HRV-B.^{5,6}

To investigate anti-rhinovirus immunity, recent studies have been directed to the innate immune responses of epithelial cells and to adaptive immunity. For the latter, peripheral blood T cells from adult atopic asthmatics taken outside a period of exacerbation produce less IFN- γ in response to virion antigen compared to non-atopic subjects.^{7,8} Reduced production of immunoregulatory IL-10 was also reported although the antigen specificity of its induction was not ascertained.⁸ It was proposed^{7,8} that asthmatics could have a reduced Th1 component, leading to poor viral clearance and immunopathology, with both contributing to disease.

We have recently undertaken a detailed analysis of the species-specific and cross-reactive IgG1 antibody response to each HRV species in healthy adults using the viral capsid protein 1 (VP1).⁹ Our studies found high IgG1 antibody titers to the VP1 capsid antigens for all three species, but a large proportion of the antibody to HRV-C was cross-reactive to HRV-A. This was unexpected given the low amino acid sequence identity of VP1 between the HRV-C and HRV-A genotypes (~35%). The study here aims to identify whether the low HRV-C species-specific responses that were found for adults occurs for

children, and importantly if infection with HRV-C was able to elicit an increase in response. Since children who develop asthma,^{10,11} especially those susceptible to exacerbation,¹² have decreased antibody responses to the protein antigens of bacteria that colonise the respiratory mucosal, a secondary aim was to show if this would occur in response to viral infection. This would concur with the increased prevalence of bacterial and viral infections found in asthmatic children.¹³⁻¹⁵ To explore this, antibody binding to antigens of each HRV species was measured in children presenting to the emergency department for an asthma exacerbation, and again in convalescence. The majority of asthmatic children presenting to hospital were infected with HRV, and most had HRV-C. Contrary to the previously studied anti-bacterial immunity, children with asthma exacerbations had higher overall anti-rhinovirus antibody responses, and higher specific IgG1 titers to HRV-A and HRV-B than controls. Interestingly however, titers specific for HRV-C, the virus most associated with severe asthma, were low in both asthmatics and controls, and the titer did not change following infection with HRV-C.

METHODS

Study population

Paired acute and convalescent plasma from 96 children who presented to the Emergency Department (ED) of Princess Margaret Hospital for Children (PMH) with acute asthma exacerbation were examined. Peripheral blood samples were obtained within 24 hours of presentation and plasma were stored at -80°C. Further samples were obtained six to 26 weeks after the initial recruitment (short-term follow up), or more than 26 weeks after the initial recruitment (long-term follow up). Plasma from 47 non-asthmatic control children was also examined. The control children had no history of doctor-diagnosed asthma and were either siblings or friends of ED asthmatic children recruited for the study or community controls. The demographics for the 143 children are described in Table I. The study was approved by the Ethics Committee at PMH and parental/guardian written informed consent was obtained for each participant.

Table I Characteristics of the study population

	ED Asthmatics* (n = 96)	Non-asthmatic controls (n = 47)
Mean age at recruitment in years (SD)	6.4 (3.4)	4.5 (3.6)
Age range in years	0.8 – 15.6	0.8 – 15.5
Male, n (%)	60 (62.5)	23 (48.9)
Atopic*, n (%)	77 (80.2)	11 (23.4)
HRV positive, n (%)	69 (71.9)	18 (38.3)
HRV-A, n	19	12
HRV-B, n	1	4
HRV-C, n	47	2
Dual infection [§] , n	2	0

* Doctor-diagnosed asthma exacerbation. Three children under two years of age presented to hospital with viral-induced wheeze.

**Atopy was defined as at least one positive skin-prick test to 11 common allergens. A positive reaction was defined as a wheal size that was larger than the negative control and greater than or equal to 3 mm in diameter.

[§]Dual infection was HRV-A/HRV-C

HRV detection and typing

Nasal secretion specimens collected from each child were tested for HRV by direct fluorescent antibody testing and also analysed with an HRV molecular typing assay⁵ to determine which HRV strains were present at the time of recruitment. As shown in Table 1 and reported previously,⁵ most of the asthmatic children presenting to the ED had detectable HRV and the majority HRV-C (68%).

Antigens

HRV VP1 capsid proteins were produced as fusion polypeptides with a glutathione-S-transferase (GST) at the N-terminus and a hexa-histidine tag on the C-terminus as described.⁹ Briefly, DNA constructs representing VP1 antigens from each species were obtained by gene synthesis and expressed as recombinant proteins in *Escherichia coli* (*E.coli*). Recombinant VP1 antigens, assembled as discrete multimers, were purified by glutathione-agarose affinity chromatography (Sigma-Aldrich, St Louis, MO) and high resolution size exclusion chromatography (GE Healthcare, Uppsala, Sweden). The presence of secondary structures similar to the natural antigens was verified by circular dichroism.

The antigens produced for this study comprised six HRV representing two genetically disparate variants of each species (Online Repository Table E1). The VP1 proteins of the related enteroviruses, human poliovirus (HPV) Sabin VP1 and echovirus 30 (E30; AF128087.1) were similarly produced.

164

165 In addition to the representative VP1 antigens from the HRV species, two isolate-specific
166 HRV VP1 antigens were also produced. cDNA encoding the VP1 of two HRV isolates
167 infecting ED asthmatic subjects at the time of recruitment were made. Semi-nested PCR
168 amplification was conducted but using specifically designed primers flanking the VP1
169 region. PCR products were then sequenced by the Australian Genome Research Facility
170 to obtain the VP1 nucleotide sequences which were subsequently engineered for
171 expression as GST-fusion proteins.

172

173 **IgG1 Binding Assay**

174 As described in detail,⁹ IgG1 antibody assays were performed with a dissociation-
175 enhanced immunofluorescence assay (DELFIA[®]) using microtiter plate wells coated with
176 GST-fusion antigens standardised by titrating the coating concentrations with monoclonal
177 anti-glutathione-S-transferase antibody (Sigma-Aldrich, St Louis, MO). Human myeloma
178 IgG1 (Sigma-Aldrich, St Louis, MO) was used as a negative control as well as
179 subsequent identification of negative control sera. Three negative controls and a titration
180 of reference sera for each antigen were included on every plate to construct a standard
181 curve. The reference sera were calibrated by interpolating the results from a parallel
182 titration curve of humanised anti-Der p 2 chimeric IgG1 (Indoor Biotechnologies,
183 Charlottesville, USA) of known concentration using the same molar coating of a
184 recombinant GST-Der p 2 as the VP1, as first determined with the monoclonal anti-GST.
185 Antibody values below the limit of detection (500 ng/ml) were assigned 50% of the lower
186 limit of detection (250 ng/ml).

187

188 As well as quantifying the total IgG1 binding to VP1 antigens, the species-specific titers
189 were determined by absorbing plasma with the VP1 antigens from the other HRV
190 species, HPV Sabin and E30. The absorption was conducted by first incubating the
191 plasma in lysate mixtures of *E.coli* producing the non-test VP1 antigens using
192 concentrations shown to be effective.⁹

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194 To measure isolate-specific IgG1 binding, the test sera were pre-incubated in a lysate
195 mixture of *E.coli* producing all HRV species, including the same species that the isolate
196 belongs to, as well as HPV Sabin and E30 VP1. They were then assayed for binding to
197 the VP1.

198

199 **Statistical analysis**

200 Differences in the level of specific antibody binding to different antigens were compared
201 by the related-samples Wilcoxon signed-rank test. Differences in antibody binding by
202 selected groups (ED asthmatics/non-asthmatic controls or male/female) were compared
203 by the Mann-Whitney test. Differences in prevalence between selected groups were
204 compared by the χ^2 test. After log-transformation, there was an approximately normal
205 distribution of antibody titers to HRV-A, thus the general linear model was employed to
206 compare differences between selected groups when accounting for possible confounding
207 factors. When subjects with titers below the assay limit of 500 ng/ml were excluded from
208 the analysis, differences in antibody binding were compared with an independent Student
209 *t*-test after log-transformation as there was an approximately normal distribution for all

antigens. Correlations were studied using Spearman's rho test. A p value <0.05 was considered significant. All analyses were performed using SPSS version 15.0 (Chicago, IL, USA) and GraphPad Prism Software (La Jolla, California, USA).

RESULTS

Anti-rhinovirus antibody specificities

As first determined for the 47 non-asthmatic controls, the total IgG1 binding to the VP1 antigens showed high titers for all species (Figure 1A). The species-specific titers obtained following immunoabsorption showed that a large proportion of the binding to each antigen was directed to epitopes shared with other HRV species or with other enteroviruses, with titers being 10-fold lower than the total antibody (Figure 1B). The species-specific titers to HRV-A were significantly higher than to HRV-B and HRV-C ($p < 0.001$), and remained significant after excluding subjects with titers below the assay limit ($p < 0.001$). The correlation of species-specific IgG1 binding between the two representative antigens for each HRV species was high for HRV-A and HRV-C being 0.977 and 0.991, respectively. The correlation of species-specific binding between the two HRV-B antigens was significant but lower (0.495).

Total and species-specific anti-rhinovirus antibody and asthma exacerbations

To compare how the responses to each species were associated with asthma, the total and species-specific titers to HRV-A, -B, -C and HPV Sabin from children with an asthma exacerbation recruited on presentation to the emergency department (ED asthmatics) were measured.

Comparisons of total IgG1 titers to HRV VP1 between ED asthmatics and non-asthmatic controls (Figure 2A) showed that ED asthmatics had higher titers to HRV antigens which

were significant for HRV-A34 ($p=0.013$), HRV-A1B ($p=0.002$), HRV-B14 ($p=0.021$) and HRV-C5 ($p=0.007$), and there was a trend for HRV-C3 ($p=0.084$).

As determined after immunoabsorption, the ED asthmatics also had higher HRV-A specific titers than non-asthmatic controls (Figure 2B; $p=0.007$ for HRV-A34, $p=0.001$ for HRV-A1B). The prevalence of IgG1 binding was 88/96 (92%) for HRV-A34 and 91/96 (95%) for HRV-A1B in ED asthmatics compared with 36/47 (77%) for both HRV-A genotypes in non-asthmatic controls ($p=0.013$ for HRV-A34 and $p=0.001$ for HRV-A1B). ED asthmatics also had higher HRV-B specific titers, which was significant for HRV-B14 ($p=0.002$) and a trend for HRV-B69 ($p=0.086$). The prevalence of binding to HRV-B14 was also higher ($p=0.002$) for ED asthmatics (41/96, 43%) compared with non-asthmatic controls (8/47, 17%). The correlation of the titers to the two genotypes of HRV-A and -B remained high in the ED asthmatics with an increase to 0.779 for HRV-B. Excluding the three children under two years of age presenting to hospital with virus-induced wheeze from the analysis did not affect the significant difference in the titers of HRV-A34 ($p=0.004$), HRV-1B ($p<0.001$) and HRV-B14 ($p<0.001$) between the two groups. Comparisons of antibody binding between ED asthmatics and non-asthmatic controls to VP1 antigens excluding subjects with titers below the assay limit showed the differences were significant for HRV-A1B ($p=0.018$), HRV-B14 ($p=0.018$) and HRV-B69 ($p=0.001$) and a trend for HRV-A34 ($p=0.059$). The increased response to HRV-A in ED asthmatics was not associated with a current infection because the ED asthmatics who had no detectable HRV-A at asthma exacerbation still had significantly higher anti-HRV-A titers than non-asthmatics (Figure 3). There was no association between the severity of

respiratory symptoms at the time of recruitment and antibody titers to the different antigens.

The IgG1 titers specific to HRV-C were low in both non-asthmatic controls and ED asthmatic children (Figure 2B), including those known to have HRV-C at the time of admission (Figure 4A). ED asthmatics with detectable HRV-C still had high titers to HRV-A antigens (Figure 4A, $p<0.001$). The anti-HRV-C titers were not significantly different in the ED asthmatics (Figure 4B) compared with the non-asthmatic controls (Figure 4C) who had a virus other than HRV-C detected at recruitment.

There were no consistent changes found when the antibody binding was measured in convalescence. As illustrated for HRV-C in asthmatics with a known HRV-C infection (Figure 5), the anti-HRV-C titers of different subjects could either decrease or increase or remain the same and were low. The titers measured with the HRV-A antigen (HRV-A34) remained high with little variation. The same results were found for ED asthmatics with a known HRV-A infection at the time of hospital admission (data not shown).

High anti-HRV IgG1 titers were found in children from an early age (Figure 6) with both ED asthmatic and non-asthmatic control children below the age of three having high titers to HRV-A. Age did, however, have a significant, positive correlation with IgG1 titers for HRV-A ($\rho=0.250$, $p=0.014$ for HRV-A34; $\rho=0.252$, $p=0.013$ for HRV-1B) and HRV-B ($\rho=0.523$, $p<0.0001$ for HRV-B14; $\rho=0.487$, $p<0.0001$ for HRVB69), but not

for HRV-C, in ED asthmatics. No significant correlation between age and anti-HRV IgG1 titers was found in non-asthmatic controls.

There were more male admissions to the ED with asthma exacerbations than females (Table I). To determine if this affected the results, the subjects were stratified by gender which showed gender differences in ED asthmatics but not in non-asthmatic controls (data not shown). Despite the differences with age and gender, antibody titers to HRV-A in ED asthmatics remained significantly different from the non-asthmatic group, after adjusting for age and gender ($p=0.019$ for HRV-A34; $p=0.002$ for HRV-A1B).

Isolate-specific response to HRV VP1

The VP1 proteins of the exact infecting HRV-A genotype of two ED asthmatic patients were produced by amplifying the VP1 regions from cDNA made from their nasal swabs. Immunoabsorption used to compare the species and isolate-specific responses for each subject demonstrated that the isolate-specific response was 8.4% of the species-specific response for one subject and negligible for the other subject (Figure 7). At convalescence, the isolate-specific response increased marginally to 9.3% (Subject 1) and 0.2% (Subject 2) of the species-specific response.

DISCUSSION

Two observations reported here provide information that could be of particular importance for elucidating the mechanisms of HRV-induced asthma exacerbation. The first is that the IgG1 antibody titers to the VP1 antigens of HRV-A, and to a lesser extent HRV-B, were higher in the asthma exacerbation group. This was evident at the time of exacerbation and, probably due to the high existing titers, did not reveal an increase following convalescence. The higher titers were not associated with the presence of, or type of virus infection found when presenting to the ED. The second is that although the plasma from asthmatic and non-asthmatic subjects contained high IgG1 titers to the HRV-C antigens, they were mostly antibodies cross-reactive with the other HRV species and not specific for HRV-C. The species-specific titers to HRV-C were not even elevated in the plasma of subjects presenting with a HRV-C infection and remained low without a consistent increase in convalescence. In contrast, antibodies specific for HRV-A and HRV-B could be readily measured and both the species-specific and total binding was higher in the ED asthmatics.

Neutralising antibodies and the antigenic variation of their targets have historically explained the repeated infections with different genotypes of HRV and the clustered escape mutations in coat protein sequences.¹⁶ Their exact function, however, needs to be put in context since at least for experimental infections, neutralising antibodies have been shown to develop well after the resolution of symptoms and virus shedding, and given that they are not detected until 2-3 weeks after infection,¹⁷⁻¹⁹ their usefulness in curtailing spread within the local environment could even be limited. Indeed, the presence or

absence of neutralising antibodies in experimentally induced infection was shown to have incomplete or no influence on the virus infection.^{20, 21} A key consideration from this and previous data⁹ is that even though different genotypes can evade neutralising antibodies, the viral proteins will still bind the high titers of antibody shown to persist from previous infections. The results here with matched virus isolates and plasma from infected hosts show that only a tiny percent of the antibody-binding to the VP1 can be attributed to the development of isolate-specific antibodies and the neutralising antibodies will be a subset of these. This might be expected from the small number of mutations in capsid protein amino acids required to escape neutralization.¹⁶ The high titers of capsid binding antibodies presumably accumulated from responses to repeated infection highlight the pertinence of non-neutralising antibodies, acting in concert with inflammatory cells, mediating the resolution of infections^{22,23} and producing immunopathology.²⁴ Indeed, this has been found for other viruses such as influenza. The high anti-HRV titers and the higher titers of ED asthmatics were found at a very early age with HRV-B showing the highest increase with age, which is possibly a reflection of its low prevalence. Adult data showed that HRV-B titers eventually reach levels similar to HRV-A.⁹ There was a strong concordance of titers between the genotypes of HRV-A and -C and was higher for HRV-B in asthmatics than non-asthmatics, perhaps due to increased infection with this less prevalent species.

T-cell responses have provided the principal estimates of overall immune responses to HRV and have demonstrated that isolates of HRV-A with disparate amino acid sequences show considerable cross-reactivity.^{25,26} No overall increase in the T-cell proliferative

response however was found after experimental HRV infection, and although there was a positive relationship between virus shedding and *in vitro* T-cell proliferation, the same occurred for tetanus toxoid.²⁰ The resolution of infection has been linked to IFN- γ rather than the overall response. An increase in RV-induced IFN- γ release for example was found after experimental HRV-16 infection and the size of the IFN- γ release that could be induced in T cells before infection correlated inversely with virus shedding, as did the T-cell proliferative response.⁷ The more recent experimental infections studied by Message et al.⁸ reported similar observations, along with eosinophilic infiltrates in the lungs of asthmatics. Evidence that the severity of asthma is related to a reduced ability of HRV-stimulated PBMC to release IFN- γ has been found in two studies.^{7,27} Evidence for increased HRV-induced IL-10 has also been reported.⁷ Since inactivated HRV did not induce high IFN- γ responses⁷ and the studies of asthmatics have used live virus^{7,8,27} it needs to be determined if the phenomena reported were due to the known ability of live rhinovirus to modify innate immune functions and antigen presentation *in vitro*.²⁸

The IgG1 antibody isotype examined is the predominant antibody subclass usually found in responses of humans to bacteria and viruses and is the main subclass associated with protection from viruses.²⁹ The follicular central memory T-cells that are critical for isotype switching and B-cell expansion produce a broad range of cytokines.³⁰ Since the cytokines most prominent for helping IgG1 production include IL-21, IL-10 and IL-4,³¹ the heightened responses here are not inconsistent with reduced responses by IFN- γ -releasing cells but it does show that they are not part of an overall reduction in adaptive anti-HRV immunity. The higher IgG1 titers could result from higher viral loads produced

during infection or a heightened immune responsiveness of asthmatics. They also contrast markedly with the low IgG1 antibody responses found to the protein antigens of the colonising bacteria *H. influenzae* and *S. pneumoniae*^{10,11} that are further reduced in children recruited from the emergency department.¹² This is in the face of the known increased bacterial colonisation found in asthmatics¹³ and the frequent bacterial detection during viral infection, including with HRV.³² The controls were not stratified for a formal analysis of atopy as it is a difficult undertaking for this study, given the high association of asthma and atopy,³³ and that nearly all the ED children were highly atopic and furthermore, recruitment was based on asthma exacerbation. There was nevertheless a significant correlation of total IgE and anti-HRV-A titers for the whole study population (data not shown).

The low species-specific antibody titers to HRV-C have previously been described in healthy adults⁹ and they occur despite the fact that HRV-C infections are as prevalent as HRV-A and more prevalent than HRV-B.^{34,35} The advance here is to show they are not associated with the susceptibility to asthma exacerbation or linked to the detection of HRV-C in the nasopharynx. We propose an “original antigenic sin” phenomenon whereby the exceptionally high cross-reactivity with HRV-A antibodies might direct responses to the cross-reactive determinants and these might not be optimal to prevent the lower respiratory tract infections frequently found for HRV-C. Susceptibility to HRV-A associated with an epitope binding profile has been described by others.³⁶ The low specific responses and the proposed dominance of the cross-reactive responses could

result from the different biology of HRV-C including its interaction with different cell receptors and *a priori* different activation of innate immunity pathways.

This study provides the first description of antibody binding by VP1 antigens representing the three species of HRV in a paediatric population, including children presenting to hospital with asthma exacerbations. Importantly, this includes the first information for the newly described HRV-C species. The paucity of species-specific antibodies to HRV-C in both asthmatic and non-asthmatic children, in contrast to the readily detectable antibodies specific to HRV-A and HRV-B provides an insight into the mechanisms and prevalence of infection to different HRV species. The major finding that HRV-C specific antibody responses are low in children, even in children with a known HRV-C infection, might underlie the propensity to produce serious respiratory infections frequently described for HRV-C. Secondly, the aberrant antibody response to HRV in children with asthma exacerbations show they do not have overall decreased adaptive immune responses to HRV and provides evidence for the importance of HRV infection in asthma.

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FIGURE LEGENDS

Figure 1 IgG1 antibody binding to HRV and HPV Sabin VP1 antigens in non-asthmatic children ($n = 47$). (A) Total IgG1 (ng/ml) to VP1 antigens (B) Species-specific IgG1 binding (ng/ml). The median and interquartile range are indicated. Comparisons between antigens were performed using the Mann-Whitney paired test. $**p < 0.001$ between species-specific binding to HRV-A (HRV-A34 and HRV-A1B) and other antigens. The prevalence (%) of subjects with titers below the assay limit of 500 ng/ml is indicated for each antigen.

Figure 2 IgG1 antibody binding to VP1 antigens representing HRV-A (HRV-A34 and HRV-A1B), HRV-B (HRV-B14 and HRV-B69) and HRV-C (HRV-C3 and HRV-C5) as well as HPV Sabin in 96 Emergency Department (ED) asthmatics (filled circles) and 47 non-asthmatic controls (open circles). (A) Total IgG1 binding (ng/ml) (B) Species-specific IgG1 binding (ng/ml). The median and interquartile range are indicated. Comparisons between ED asthmatics and non-asthmatic controls were analysed using the Mann-Whitney test. The prevalence (%) of subjects with titers below the assay limit of 500 ng/ml is indicated for each antigen. Comparison of prevalence between the two groups were analysed using the χ^2 test.

Figure 3 Non-asthmatic controls with no detectable HRV-A at the time of recruitment had significantly lower anti-HRV-A IgG1 response compared to ED asthmatics with no detectable HRV-A. The species-specific IgG1 titers to HRV-A antigens (HRV-A34 and

HRV-A1B) in 35 non-asthmatic controls with no detectable HRV-A (open circles) and 54 ED asthmatics with no detectable HRV-A (closed circles) are indicated. ED asthmatics with no detectable HRV-A did not have significantly different titers to HRV-A antigens to ED asthmatics infected with HRV-A at presentation to hospital ($n=19$, closed triangles). The median and interquartile range are indicated.

Figure 4 (A) Children with asthma exacerbations with detectable HRV-C ($n = 47$) have low species-specific IgG1 titers to HRV-C. The titers to HRV-A was significantly higher than HRV-B and HRV-C ($p<0.0001$) as indicated by **. (B) The IgG1 titers to HRV-C in ED asthmatics infected with HRV-C was not significantly different to ED asthmatics with no detectable HRV-C at the time of their acute asthma exacerbation ($n = 34$). (C) The anti-HRV-C response in ED asthmatic children was not significantly different to non-asthmatic controls with no detectable HRV-C at the time of recruitment ($n = 82$). The median and interquartile range are indicated.

Figure 5 Species-specific IgG1 binding to HRV-C (HRV-C3) and HRV-A (HRV-A34) antigens for plasma from 47 ED asthmatic children with a known HRV-C infection at the time of hospitalisation. The IgG1 titers for each individual immediately following exacerbation from asthma (acute) and at either short-term follow up (STFU) visit or long-term follow up (LTFU) visit are indicated.

Figure 6 Specific IgG1 binding to HRV-A (HRV-A34), HRV-B (HRV-B14) and HRV-C (HRV-C3) in relation to age in (A) ED asthmatics following acute asthma exacerbation (n

= 96) and (B) non-asthmatic controls (n=47). As determined by the Spearman's rho test, there was a significant positive correlation between age and IgG1 titers for HRV-A and HRV-B in ED asthmatics.

Figure 7 Isolate-specific IgG1 titers in two HRV infected subjects following asthma exacerbation and at convalescence. Results are represented as the percent of isolate-specific response to specific-specific response. Both HRV isolates were HRV-A. The species-specific titer of subject 1 to the isolate was 2687584 ng/ml at asthma exacerbation and 1976063 ng/ml at convalescence (short-term follow up). The species-specific titer of subject 2 to the isolate was 110739 ng/ml at asthma exacerbation and 1086187 ng/ml at convalescence (long-term follow up).

Figure No.2

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Online Repository Table E1 Human rhinovirus (HRV) and human poliovirus (HPV) genotypes used in this study

Species	Genotype	Prototype	Accession number
HRV-A	A34		FJ445189.1
HRV-A	A1B		D00239.1
HRV-B	B14		NC001490
HRV-B	B69		FJ445151
HRV-C	C3	QPM	EF186077
HRV-C	C5	025	EF582386
HEV-C	HPV Sabin		AY184219.1