Human enteroviruses and parechoviruses: disease spectrum and need for treatment in young children
Wildenbeest, J.G.

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Rhinovirus C is not associated with wheezing or severe disease in an unselected birth cohort from the Netherlands


* # Both authors contributed equally to this manuscript

Submitted
Abstract

Background
Human rhinovirus (HRV) is a frequent pathogen in young children, eliciting symptoms ranging from common colds to wheezing illnesses and lower respiratory tract infections. The recently identified HRV-C seems to be associated with asthma exacerbations and more severe disease, but results vary. We studied the prevalence and severity of infection with HRV in an unselected birth cohort.

Methods
Children with respiratory symptoms entered the symptomatic arm of the cohort and were compared to asymptomatic children. Severity of wheezing and other respiratory symptoms were registered and respiratory viruses were evaluated using throat and nasopharyngeal swabs on first presentation and after recovery (wheezing children). HRV genotyping was performed on HRV-PCR positive samples.

Results
HRV was the most prevalent respiratory virus and was found in 58 of 140 (41%) symptomatic children, in 24 of 96 (26%) control children and in 19 of 74 (25%) symptomatic wheezing children after recovery (p<0.05). HRV-A was the most commonly detected type (59%) followed by HRV-C (32%) and HRV-B (9%). Children with HRV mono-infection had more severe symptoms, but HRV infections were not associated with occurrence of wheezing. There was no association between the different HRV species and occurrence of wheezing or severity of disease. Symptomatic HRV-PCR positive children, in particular wheezing children, had a significant higher viral load than asymptomatic children.

Conclusions
In an unselected birth cohort from the Netherlands, HRV was the most prevalent respiratory virus. Our results suggest that HRV-C is not associated with more severe disease or wheezing in young children in the general population.
Introduction

Human rhinovirus (HRV) infections account for most respiratory infections in early life, being a major contributing factor to childhood morbidity (reviewed in Kieninger et al.). Furthermore, episodes of HRV-induced wheezing are strongly associated with the subsequent development of asthma in high risk children. HRV belongs to the genus Enterovirus in the family of Picornaviridae. There are over 100 serotypes which are classified into 3 species; A, B and C. HRV infections in childhood cause a wide variety in clinical presentations ranging from very mild ‘common cold’ symptoms to severe life-threatening lower respiratory tract infections (LRTI). Using novel molecular detection techniques, HRVs were identified as a common cause of bronchiolitis, wheezing and pneumonia. This spectrum of variation in clinical presentation is subject of ongoing research. Firstly, evidence suggests that symptomatic HRV infections reveal an underlying predisposition to develop asthma which may be modulated by genetic host factors. On the other hand, HRV may also play a causal role in the development of asthma through promoting exaggerated inflammation and airway hyper-responsiveness. Associations between varying clinical severity and different HRV serotypes favor evidence in support of a causal role for HRV in the onset of asthma. More specifically, the recently identified HRV-C was found to be present in the majority of children admitted to the hospital with wheezing or acute asthma exacerbations and HRV-C infections were associated with increased severity of those exacerbations. Furthermore, HRV-C was reported as the only species more frequently associated with lower respiratory tract infections in children as compared to the adult patient population. By contrast, asymptomatic HRV-C infections have also been reported in healthy controls. As suggested by a recent review from Kieninger et al., longitudinal studies on occurrence of both symptomatic and asymptomatic HRV infections in an unselected population will increase our knowledge on whether clinical manifestations of HRV infections are related to a predisposed host immune response or related directly to viral pathogenicity. In this study, we hypothesized that the presence of HRV-C is associated with more severe acute respiratory infections in pre-school children from an unselected birth cohort. We studied this by comparing prevalence, clinical symptoms (specifically wheezing) and viral loads of different HRV types in HRV positive and negative symptomatic children. These prevalences were compared to the prevalence of HRV infections in asymptomatic controls and of the symptomatic children after recovery from wheezing respiratory illnesses. This recovered group of wheezing children is of specific interest because they have a high-risk phenotype for the development of asthma later in life.
Methods

Subjects
This study is part of the EUROPA-trial (Early Unbiased Risk Assessment of Pediatric Asthma), a prospective cohort study in the Netherlands, focusing on prediction of early signs of asthma. Participants were recruited by targeted mailing from an unselected birth cohort of 12,033 infants born in greater Amsterdam and aged between 0 and 12 months old at inclusion. Exclusion criteria were a gestational age of less than 31 weeks or the presence of any manifest illness at inclusion, specifically any pulmonary disorder. A total of 1216 infants were included into the trial after both parents provided consent (Figure 1). At inclusion a structured baseline questionnaire was obtained.

Unselected birth cohort of 12,033 children born in greater Amsterdam
Age: 0-12 months

1216 children included and prospectively followed up
November 2009 till December 2012

Symptomatic:
Respiratory symptoms with dyspnea and/or wheezing, severe enough to be seen by a general practitioner
N=140 (56 F, 84 M)

Wheezing confirmed by researcher
N=90 (32 F, 58 M)

Recovered:
Visit minimum 6 weeks after wheezing episode and asymptomatic
N=74 (26 F, 48 M)

Control:
Asymptomatic; no respiratory symptoms at visit and no history of wheezing
N=96 (45 F, 51 M)

Wheezing not confirmed by researcher
N=50 (24 F, 26 M)

Figure 1. Selection process of children of the EUROPA birth cohort.
Design
This study was designed as a prospective case-control follow-up study. Participating parents were instructed to contact the study team whenever their infant experienced respiratory tract symptoms from November 2009 until December 2012. A standardized telephone interview was obtained to assess the presence of symptoms. Infants experiencing cough, wheezing, labored breathing and/or dyspnea sufficiently severe for parents to warrant a visit to their family physician entered the symptomatic arm of the study and were visited by the study team within 8 hours after establishing these symptoms.

During the visit the presence and severity of acute respiratory symptoms was assessed by both parents and the on-site researcher. The researchers were well trained to recognize wheezing. The intra-class correlation was validated by means of evaluation of tracheal sound recordings by 5 pediatric pulmonologists in the first 30 patients which reached a Cronbach’s alpha of 0.75. The study team assessed symptom severity (physician symptom score) by scoring the presence of suprasternal retractions, scalene muscle contraction, air entry and wheezing by auscultation, all part of the Pediatric Respiratory Assessment Measure. Values of the physician symptom score range from 0 to 10 with increasing values indicating increased severity. Parents self-assessed severity by Asthma Control Questionnaire (ACQ), modified for by proxy use (mACQ), although this questionnaire has not been validated for this application.

For our secondary aim we assessed the prevalence of HRV infection in asymptomatic children by recruiting controls from the same cohort who had never experienced lower respiratory tract symptoms severe enough to contact their family physician. If lower respiratory tract symptoms still occurred after being visited as a control a novel control was recruited. Furthermore, children who were evaluated for an episode of respiratory symptoms in this study and were found to have viral induced wheezing were re-assessed after a symptom free period of at least 1 week, minimally 6 weeks after their initial presentation. Symptom free was defined as a physician and parent (ACQ-based) severity score of 0.

This study was approved by the Medical Ethical Committee of the Academic Medical Center Amsterdam (09/066) and the parents gave written informed consent. The EUROPA study is registered in the Dutch Trial Register (NTR-1955).

Virological analysis
At each visit the study team obtained naso- and oro-pharyngeal swabs (Copan Swabs, Brescia, Italy). The collected naso- and oro-pharyngeal samples were assessed for the presence of respiratory associated viruses (HRV, human enterovirus (EV), human parechovirus (HPeV), influenza virus A and B, para-influenzavirus 1, 2, 3 and 4, human bocavirus (HBOV), human coronavirus, respiratory syncytial virus, adenovirus and human metapneumovirus), using a multiplex PCR as described previously by Jansen et al. A Ct-value of 40 or more was considered to be negative.
HRV typing

HRV RNA was extracted from 200 µl HRV-positive sample with the MagnaPure LC instrument® using the total nucleic acid isolation kit (Roche Diagnostics). Genotyping was performed by amplifying a 540-base pair fragment spanning part of the 5’-untranslated region (UTR), capsid protein VP4 and part of VP2 (VP4/VP2) of the HRV-genome using a two-step semi-nested protocol.¹⁹

First, 6 µl of RNA was reverse transcribed and amplified with the SuperScript III one-step RT/Platinum Taq polymerase kit (Invitrogen) according to the manufacturer’s instructions using primers adapted from Savolainen et al.²⁰ (shown in Table 1) and cycling conditions described by Harvala et al.²¹

One µl of the combined RT-PCR product was then used as input for the second semi-nested PCR amplification. The reaction mix contained 1x PCR buffer, 2.5 mM MgCl₂, 0.5 µM of each primer, 200 µM of each dNTP, 0.1 µg/ml BSA, and 0.05 U of FastStart Taq polymerase (Roche) in a 20 µl-reaction volume. Cycling conditions were as follows: 94°C for 2 min and 30 cycles each consisting of 94°C (18 sec), 55°C (21 sec) and 72°C (90 sec). Amplicons were sequenced using primers used for the second step of the semi-nested protocol with the BigDye Terminator reaction kit (Applied Biosystems). Species were determined by phylogenetically comparing sequences with published reference sequences as proposed and provided by McIntyre et al.¹⁹

Cross-reactivity of EV with HRV was suspected when both EV and HRV PCR were positive and typing resulted in an EV type (9 samples) or when only HRV PCR was positive and typing resulted in an EV type (2 samples). These samples were considered to be EV positive and HRV negative.

Table 1. Genotyping primers used in this study.

<table>
<thead>
<tr>
<th>Orientation</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>HRV-VP4-1</td>
<td>GGG ACC AAC TAC TTT GGG TGT</td>
</tr>
<tr>
<td>Antisense</td>
<td>9565-reverse</td>
<td>GCA TCI GGY ARY TTC CAC CAC CAN CC</td>
</tr>
<tr>
<td>Step 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>HRV-VP4-2-forward</td>
<td>GGG GAC CAA CTA CTT TGG GTG GCC TGG TGC T</td>
</tr>
</tbody>
</table>

Bacterial co-infection

At each visit a throat swab was collected which was cultured for respiratory bacterial pathogens according to standard care procedures.

Data analysis

Data were analyzed using SPSS for windows, version 20. Categorical variables were compared by means of chi-square test. Differences between continuous variables were analyzed using student-t test and one way ANOVA test (if normally distributed) or Mann-Whitney U test
and Kruskall-Wallis test and for paired continuous variables Wilcoxon signed rank test. A two-sided p-value <0.05 was considered to be significant.

Results

Subject characteristics
A total of 140 symptomatic and 96 control children were included in the study (Figure 1). Baseline characteristics of all included children are described in Table 2. Of the 140 symptomatic children, wheezing was confirmed by the study team in 90 children. Of these wheezing children 74 were visited again by the study team when they were asymptomatic after a minimum of 6 weeks (the recovered group). The median age of the control group (28 months) was significantly higher than of the symptomatic group both during symptoms (15 months, p=0.000) and after recovery (22 months, p=0.000).

Table 2. Characteristics of included children.

<table>
<thead>
<tr>
<th></th>
<th>Symptomatic RTI with confirmed wheezing</th>
<th>Symptomatic RTI without wheezing</th>
<th>Control</th>
<th>Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of children</td>
<td>90</td>
<td>50</td>
<td>96</td>
<td>74</td>
</tr>
<tr>
<td>Median age (months, IQR)</td>
<td>15 (10-25)</td>
<td>15 (10-24)</td>
<td>28 (26-31)</td>
<td>22 (17-27)</td>
</tr>
<tr>
<td>Sex (male:female)</td>
<td>1.8:1</td>
<td>1.1:1</td>
<td>1.1:1</td>
<td>1.8:1</td>
</tr>
<tr>
<td>Bacterial co-infection</td>
<td>2 (2%)</td>
<td>1 (2%)</td>
<td>2 (2%)</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>Use of inhaled corticosteroids</td>
<td>18 (20%)</td>
<td>10 (20%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Use of inhaled β2-mimetica</td>
<td>55 (61%)</td>
<td>15 (30%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Use of antibiotics</td>
<td>9 (11%)</td>
<td>1 (2%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Physician symptom score (median (IQR))</td>
<td>2 (1-4)</td>
<td>0 (0-0)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mACQ parents (median (IQR))</td>
<td>15.5 (10-21)</td>
<td>11.5 (8-14)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Significant (p<0.05) for symptomatic versus control and control versus visit after recovery (recovered).

Prevalence and seasonality of HRV infections
Overall, in 86% of the symptomatic children a respiratory virus could be detected, compared to 40% in the control group (p=0.000) and 53% in the recovered group (p=0.000, Table 3). HRV was the most prevalent virus in symptomatic (41%) as well as control (26%) children, and was found significantly more often in symptomatic children (p=0.009, Figure 2). In the recovered group HBOV (35%) was the most prevalent virus, followed by HRV (25%).
Symptomatic children with HRV infection were significantly younger (median 13.5 months, IQR 8-20) than symptomatic children who were negative for HRV (median 17 months, IQR 13-27, p=0.005). This was also significant in the recovered group (HRV positive; median 19 months, IQR 16-20, versus HRV negative; median 24 months, IQR 18-30, p=0.003).

HRV was seen all year round (Figure 3A) with a peak during fall and winter. In the summer over 80% of symptomatic children were HRV positive contrasted by only 17% during the winter. This was seen in every year of the study. The percentage of asymptomatic children (control and recovered) who were HRV positive was relatively constant between seasons ranging from 17% to 31%.

### Table 3. Prevalence of respiratory viruses in symptomatic and asymptomatic (control and recovered) children.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Symptomatic</th>
<th>Control*</th>
<th>Recovered*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any virus</td>
<td>120 (86%)</td>
<td>38 (40%)*</td>
<td>39 (53%)*</td>
</tr>
<tr>
<td>Human rhinovirus</td>
<td>58 (41%)</td>
<td>24 (26%)*</td>
<td>19 (25%)*</td>
</tr>
<tr>
<td>Human bocavirus</td>
<td>41 (29%)</td>
<td>7 (7%)*</td>
<td>26 (35%)*</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>33 (24%)</td>
<td>0*</td>
<td>2 (3%)*</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>18 (13%)</td>
<td>0*</td>
<td>1 (1%)*</td>
</tr>
<tr>
<td>Para-influenzavirus type 3</td>
<td>15 (11%)</td>
<td>1 (1%)*</td>
<td>2 (3%)*</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>11 (9%)</td>
<td>3 (3%)</td>
<td>4 (5%)</td>
</tr>
<tr>
<td>Human coronavirus</td>
<td>9 (6%)</td>
<td>2 (2%)*</td>
<td>8 (11%)*</td>
</tr>
<tr>
<td>Human parechovirus</td>
<td>7 (5%)</td>
<td>0*</td>
<td>3 (4%)*</td>
</tr>
<tr>
<td>Humaan metapneumovirus</td>
<td>6 (4%)</td>
<td>1 (1%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Para-influenzavirus type 4</td>
<td>5 (4%)</td>
<td>1 (1%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Para-influenzavirus type 2</td>
<td>3 (2%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Para-influenzavirus type 1</td>
<td>2 (2%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Influenza A</td>
<td>2 (1%)</td>
<td>1 (1%)</td>
<td>0</td>
</tr>
<tr>
<td>Influenza B</td>
<td>1 (1%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Significant (p<0.05) symptomatic versus control or symptomatic versus visit after recovery (recovered).

$Significant (p<0.05) control versus visit after recovery (recovered).
Figure 2. Prevalence of HRV and non-HRV viruses in symptomatic and asymptomatic children (controls and recovered).

Co-infection
In the symptomatic group 44% of the children were infected with 2 or more viruses, significantly more than children in the control group (2%) and recovered children (26%, p=0.000). In addition, the number of recovered children with 2 or more viruses was significantly higher than in the control group (p=0.000), which was also reflected by a significantly higher total number of viruses detected (mean number of viruses 0.89) in recovered children compared to children in the control group (mean number of viruses 0.42, p=0.000). This is mainly caused by a higher frequency of HBOV, coronavirus and HPeV (Table 3). Co-infection of HRV with other viruses was found in 36 of 58 (62%) symptomatic children compared to 1 out of 24 controls (4%, p=0.00, Table S1). The most frequent co-infecting viruses were HBOV and adenovirus. There was no significant difference between the co-infection rate in wheezing (55%) and non-wheezing children (75%, p=0.141). For wheezing infants who were recovered from their symptoms, the rate of HRV co-infections was similar to that of symptomatic infants (63%).

The rate of bacterial (co-)infection was low in symptomatic (2%) as well as asymptomatic (control (2%) and recovered (4%)) children (Table 2).
**Figure 3.** HRV and HRV types in different seasons and years. **A.** Number of HRV positive children in different seasons and years in symptomatic, control and recovered children. **B.** Distribution of HRV-A, -B and -C across seasons and years.
Prevalence of HRV species

In total, 79 samples (71%) could be genotyped (48 samples (76%) of symptomatic children, 19 (70%) of control children and 12 (55%) of recovered children (Table 4). HRV-A was evenly detected across symptomatic (58%), control (50%) and recovered children (78%), while HRV-B was significantly more often detected in control children (31%) compared to symptomatic children (2%, p=0.001). HRV-C was detected in 40% of HRV positive symptomatic children compared to 19% of HRV positive control children and 22% of HRV positive children recovered from symptoms (p=0.25).

In the subgroup of symptomatic wheezing children, children with HRV-C infection were significantly older (median 20 months, IQR 15-27) than wheezing children with HRV-A infection (median 12 months (IQR 8-19, p=0.01).

HRV-A was most frequently seen in the summer and fall, while HRV-C was the most frequently found species in winter. HRV-A, B and C were evenly distributed across the years studied (Figure 3B).

Table 4. Results of molecular typing of HRV PCR positive samples.

<table>
<thead>
<tr>
<th>Rhinovirus species</th>
<th>HRV-A</th>
<th>HRV-B</th>
<th>HRV-C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptomatic</td>
<td>25 (58%)</td>
<td>1 (2%)*</td>
<td>17 (40%)</td>
<td>43</td>
</tr>
<tr>
<td>Wheeze</td>
<td>17 (63%)</td>
<td>0</td>
<td>10 (37%)</td>
<td>27</td>
</tr>
<tr>
<td>No wheeze</td>
<td>8 (50%)</td>
<td>1 (6%)</td>
<td>7 (44%)</td>
<td>16</td>
</tr>
<tr>
<td>Control</td>
<td>8 (50%)</td>
<td>5 (31%)*</td>
<td>3 (19%)</td>
<td>16</td>
</tr>
<tr>
<td>Recovered</td>
<td>7 (78%)</td>
<td>0</td>
<td>2 (22%)</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>40 (59%)</td>
<td>6 (9%)</td>
<td>22 (32%)</td>
<td>68</td>
</tr>
</tbody>
</table>

*Significant (p<0.05) symptomatic versus control.
% Indicates percentage of typable HRVs.

Symptoms

HRV and severity of symptoms

Overall, physician symptom scores were low (median 1, IQR 0-3), indicating symptoms were generally mild in this unselected cohort. There were no differences in the scores between HRV positive and HRV negative children. Remarkably, within the group of HRV positive children a significantly higher score was seen in children with HRV mono-infection compared to HRV positive children co-infected with other viruses (median 2 (IQR 0.75-5) versus median 1 (IQR 0-2), p=0.033). The physician symptom scores of children infected with different HRV species were also compared and showed no significant differences.

In addition, the individual symptoms were compared and showed that children with HRV mono-infection and/or HRV-C significantly more often experienced retractions (p=0.016). Parental assessment of symptoms (mACQ) did not correlate with the physician symptom score and showed a significant lower score in HRV positive children compared to HRV negative children (Table 5, p=0.02), however, the mACQ also assesses general symptoms.
of illness (like fever and intake) and upper respiratory tract symptoms while the physician symptom score assesses only severity of symptoms of the lower respiratory tract.

Table 5. Severity scores and symptoms of symptomatic children.

<table>
<thead>
<tr>
<th></th>
<th>Total HRV (n=58)</th>
<th>HRV-A (n=25)</th>
<th>HRV-B (n=1)</th>
<th>HRV-C (n=17)</th>
<th>HRV mono-infection (n=22)</th>
<th>non-HRV virus only (n=62)</th>
<th>No virus (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physician symptom score (median (IQR))</td>
<td>1 (0-2.25)</td>
<td>1 (0-2)</td>
<td>0</td>
<td>1 (0-2.5)</td>
<td>2 (0.75-5)</td>
<td>1 (0-3)</td>
<td>2 (0-3)</td>
</tr>
<tr>
<td>Retractions</td>
<td>19%</td>
<td>8%</td>
<td>0</td>
<td>35%</td>
<td>36%</td>
<td>18%</td>
<td>30%</td>
</tr>
<tr>
<td>Use of accessory muscles</td>
<td>17%</td>
<td>16%</td>
<td>0</td>
<td>12%</td>
<td>27%</td>
<td>16%</td>
<td>20%</td>
</tr>
<tr>
<td>Wheezing</td>
<td>60%</td>
<td>64%</td>
<td>0</td>
<td>59%</td>
<td>77%</td>
<td>58%</td>
<td>55%</td>
</tr>
<tr>
<td>Decreased ventilation</td>
<td>19%</td>
<td>24%</td>
<td>0</td>
<td>12%</td>
<td>18%</td>
<td>19%</td>
<td>10%</td>
</tr>
<tr>
<td>mACQ parents (median (IQR))</td>
<td>12 (8.75-16.25)</td>
<td>12 (9-15)</td>
<td>10</td>
<td>12 (7.5-19)</td>
<td>13 (9-22.25)</td>
<td>16 (11-21)</td>
<td>13 (8-16)</td>
</tr>
</tbody>
</table>

1Significant higher than HRV positive children with co-infection (p=0.033).
2Significant more than HRV-A and HRV-B positive children (p=0.023).
3Significant more than HRV positive children with co-infection and HRV negative children (p=0.037).
4Significant lower than HRV negative children (p=0.02).

Ct-value and severity of symptoms

Ct value was used as a semi-quantitative read-out for viral load. HRV PCR Ct-values were significantly lower in symptomatic children (throat median Ct-value 30.6, nose median Ct-value 28.6), versus asymptomatic children (throat median Ct-value 31.7 (p=0.009), nose median Ct-value 29.7 (p=0.006)), indicating a higher viral load in symptomatic children. Also, wheezing children had a significantly lower Ct-value than non-wheezing symptomatic children (only nose, median Ct-value 27.9 versus 29.1 p=0.024, throat median Ct-value 30.0 versus 30.9, p=0.28, Figure 4). However, a cut-off value for symptomatic disease could not be determined.

There was no correlation between Ct-value and severity of symptoms in HRV infected symptomatic children (Figure 5).
Discussion

Our study is the first to describe the prevalence of HRV in the first years of life in an unselected birth cohort with accurately objectified symptoms and to compare these results with an asymptomatic control group from the same birth cohort. We showed that HRV infection was very prevalent in symptomatic as well as asymptomatic young children in the general population. HRV was significantly more often detected in
respiratory swabs of symptomatic children compared to asymptomatic children. While HRV mono-infections but not co-infections were associated with a greater clinical severity, HRV infections were not associated with occurrence of wheezing. However, wheezing HRV infected children had a significantly higher HRV viral load than non-wheezing HRV infected children. In addition, there was no association between the different HRV species and occurrence of wheezing or severity of disease. In this unselected birth cohort no association between HRV-C and wheezing or more severe disease was found.

HRV-A and C were found to be the most prevalent species detected in our unselected population. This is in accordance with previous reports in both hospitalized patients and high-risk cohorts as well as unselected birth cohorts. HRV-B was the least prevalent species and seemed to be associated with asymptomatic infection in our study. This is in accordance with Lee et al. who showed that HRV-B is less virulent than HRV-A and HRV-C in an high risk cohort of young children. However, others found that HRV-B was associated with pneumonia in hospitalized children. The lack of association between wheezing or more severe disease and HRV-C infections in this study is in contrast with several other studies who reported an association between HRV-C and more severe disease and/or more asthma exacerbations in hospitalized patients. However, this was not consistently reproduced.

Studies in unselected cohorts or non-hospitalized symptomatic children also showed different results, ranging from no association of wheezing in children infected with HRV-C, to more (severe) lower respiratory tract infections in children infected with HRV-C. These conflicting results indicate a discrepancy between various studies with respect to associations of HRV-C infection with wheezing and severity of symptoms. It could be possible that specific HRV types of the HRV species C (or A) give more severe disease, including wheezing illness and hospitalization, as was suggested by Lee et al. Another explanation could be that this association is only seen in susceptible children above a certain age. This is supported by our observation and from others that wheezing children with HRV-C infection were significantly older than HRV-A infected wheezing children.

Our study showed that a higher viral load was associated with symptomatic disease, and within the symptomatic group, with wheezing. Unfortunately, a cut off value of viral load for symptomatic disease could not be established due to considerable overlap between groups. We also did not find a correlation between severity of disease and Ct-value. Few other studies described the relation between viral load and symptoms. Kennedy et al. found no difference in viral load between outpatient wheezing and non-wheezing children, while others found that a high viral load was associated with symptomatic disease and an increased risk of LRTI, but not with more severe symptoms. An explicit strength of our study is that children were visited and sampled both during the symptomatic period and after recovery from symptoms. Remarkably, a significantly higher
number of (co-infecting) viruses was found after recovery from symptoms compared to controls. It is possible that wheezing children are more prone to be (asymptomatic) carriers of viruses, compared to non-wheezing children. However, during the symptomatic visit no difference between wheezing and non-wheezing children and number of viruses detected was seen. In our study, control children were significantly older than recovered children, making it impossible to draw conclusions from this observation alone. In addition, in another large cross-sectional study in asymptomatic children a consistent high viral detection rate of 58-74% in 6, 12, 18 as well as 24-month old children was found with HRV as the most prevalent virus (31-50%) and multiple viruses in 18-38% of the children.\(^{36}\) No data on preceding wheezing illness were available.

This brings us to the first limitation of this study; the children in the control group were significantly older than symptomatic infants, thereby biasing the comparison of prevalence between symptomatic and asymptomatic children. This is an unfortunate secondary effect of the fact we chose to study asymptomatic controls that never met the criteria for a symptomatic episode from the same birth cohort. This means we would have required to recruit a novel control when a former control became symptomatic. We do however feel this is also an implicit strength as it means our control group is very strictly defined. In addition, it is important to notice that comparisons of severity of symptoms and wheezing between HRV species within the symptomatic group were not influenced by this bias.

Furthermore, 44% of the symptomatic infants were infected by multiple viruses. Although this is in accordance with previous studies, it is likely to have influence on the analysis of associations between HRV species and clinical severity.\(^{13,23,30,37,38}\) Remarkably, we found that children with a HRV mono-infection had more severe symptoms compared to children with HRV and a co-infection. Other hospital-based studies found that co-infection with RSV is associated with an increased severity and/or a longer duration of hospitalization.\(^{13,38}\) In our cohort co-infection with RSV was low and not associated with more severe symptoms.

Another limitation is the sample size. It is possible that the lack of association of HRV-C with more severe symptoms or wheezing is due to an insufficient sample size. We evaluated this with a post hoc power analysis, showing that our study had enough power to pick up large differences in wheezing frequency or symptom severity, however, small differences could have been missed (data not shown).

In conclusion, in an unselected birth cohort from the Netherlands with mild respiratory disease, we found a high prevalence of (multiple) respiratory viruses with HRV being the most prevalent. We found no association between HRV infection and wheezing illness, although a higher HRV viral load was seen in wheezing children. We did find that children infected with only HRV had more severe disease than HRV negative children and children with HRV and co-infections. We did not establish a correlation between HRV-C infection and wheezing or more severe disease, suggesting that HRV-C is not associated with more severe disease in young children in the general population.
References


Table S1. Frequency of HRV co-infections with different viruses.

<table>
<thead>
<tr>
<th></th>
<th>Symptomatic</th>
<th>Control</th>
<th>Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Wheeze</td>
<td>No wheeze</td>
</tr>
<tr>
<td>Any virus</td>
<td>36 (62%)</td>
<td>21 (55%)</td>
<td>15 (75%)</td>
</tr>
<tr>
<td>Human bocavirus</td>
<td>21 (36%)</td>
<td>15 (40%)</td>
<td>6 (30%)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>11 (19%)</td>
<td>8 (21%)</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>6 (10%)</td>
<td>3 (8%)</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>Human parechovirus</td>
<td>6 (10%)</td>
<td>4 (11%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>5 (9%)</td>
<td>4 (11%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Para-influenzavirus type 4</td>
<td>4 (7%)</td>
<td>2 (5%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Para-influenzavirus type 3</td>
<td>3 (5%)</td>
<td>3 (8%)</td>
<td>0</td>
</tr>
<tr>
<td>Human coronavirus</td>
<td>2 (3%)</td>
<td>1 (3%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Para-influenzavirus type 2</td>
<td>1 (2%)</td>
<td>1 (3%)</td>
<td>0</td>
</tr>
<tr>
<td>Human metapneumovirus</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Significant (p<0.05) symptomatic versus control and/or symptomatic versus visit after recovery (recovered).
$^5$Significant (p<0.05) control versus visit after recovery (recovered).