

Rhinovirus Species and Clinical Characteristics in the First Wheezing Episode in Children

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The clinical data on the first wheezing episodes induced by different rhinovirus (RV) species are still limited. We aimed to investigate the prevalence of RV genotypes, sensitization status, and clinical characteristics of patients having a respiratory infection caused by either different RV species or other respiratory viruses. The study enrolled 111 patients (aged 3–23 months, 79% hospitalized, 76% with RV infection) with the first wheezing episode. RV-specific sequences were identified by partial sequencing of VP4/VP2 and 5' non-coding regions with 80% success rate. The investigated clinical and laboratory variables included atopic characteristics and illness severity, parental atopic illnesses, and parental smoking. Of the study children, 56% percent had >1 atopic characteristic (atopy, eczema and/or blood eosinophil count $>0.4 \times 10^9/L$) and 23% were sensitised to allergens. RV-C was detected in 58% of RV positive samples, followed by RV-A (20%) and RV-B (1.2%). Children with RV-A and RV-C induced wheezing were older ($P=0.014$) and had more atopic characteristics ($P=0.001$) than those with non-RV. RV-A and RV-C illnesses had shorter duration of preadmission symptoms and required more bronchodilator use at the ward than non-RV illnesses (both $P < 0.05$, respectively). RV-C is the most common cause of severe early wheezing. Atopic and illness severity features are associated with children having RV-A or RV-C induced first wheezing episode rather than with children having a non-RV induced wheezing. **J. Med. Virol. 88:2059–2068, 2016.**

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KEY WORDS: atopy; children; genotypes; rhinovirus; sequencing; wheezing

INTRODUCTION

Rhinovirus (RV)—induced early wheezing has been recognised as an important risk factor for subsequent asthma [Jackson et al., 2012; Lukkarinen et al., 2013]. It has also been associated with atopic characteristics and poor lung function both at acute phase and years later [Malmström et al., 2006; Guilbert et al., 2011; Jackson et al., 2012; Turunen et al., 2014]. The main explanations for these associations include pre-existing airway inflammation, bronchial epithelial damage, low interferon responses and genetic predisposition [Jakiela et al., 2008; Baraldo

Abbreviation: AdV, adenovirus; ANOVA, one-way analysis of variance; BLAST, basic local alignment search tool; BP, base pair; cDNA, complementary DNA; CI, confidence interval; CV, coronavirus; EV, enterovirus; Flu, influenza virus; HBoV, human bocavirus 1; IQR, interquartile range; MicroL, microlitre; MPV, metapneumovirus; NCR, non-coding region; OR, odds ratio; PBS, phosphate-buffered saline; PIV, parainfluenza virus; PCR, polymerase chain reaction; RT, reverse transcriptase; RV, rhinovirus; RSV, respiratory syncytial virus; SD, standard deviation; VP, viral protein

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et al., 2012; Carroll et al., 2012; Çalışkan et al., 2013; Bochkov et al., 2015; Contoli et al., 2015].

Rhinoviruses are genetically diverse RNA viruses belonging to the Enterovirus genus in the *Picornaviridae* family. Currently, over 160 different RV genotypes have been identified [Simmonds et al., 2010; McIntyre et al., 2013; Bochkov et al., 2014]. They are classified into A, B and C species. RV-A and RV-C seem to be the most common RV species in lower airways illness, wheezing and acute asthma, and cause more severe illnesses than RV-B in children [Arden et al., 2010; Bizzintino et al., 2011; Miller et al., 2011; Lee et al., 2012; Martin et al., 2015; Fawcner-Corbett et al., 2016]. Also, one study has reported that atopy could worsen the RV-C induced illness outcome when children have acute wheezing [Cox et al., 2013]. Previous studies on RV genotypes have not focused on the first wheezing episode.

Considering new prevention strategies for asthma, it is crucial to identify high-risk children early, preferably already at the time of their first wheezing episode. Currently, RV is considered as a noticeable risk marker for later asthma development in children with severe early wheezing. We focused on the first wheezing episode in these children and aimed to investigate the RV genotype prevalence, sensitisation status of patients and clinical characteristics of infections caused by different RV species and other respiratory viruses than RV.

METHODS

Study Population

The Vinku2 trial was carried out in the Department of Pediatrics, Turku University Hospital, Turku, Finland, from June 2007 to March 2009 (*Vinku* means “wheeze” in Finnish) [Turunen et al., 2014; Jartti et al., 2015]. The inclusion criteria consisted of patient’s age of 3–23 months, delivery at >36 gestational weeks, first wheezing episode (parental report and confirmation from medical records), and written informed consent from a parent or guardian. Main exclusion criteria consisted of chronic non-atopic illness and previous systemic or inhaled corticosteroid treatment. The study protocol was approved by the Ethics Committee of the Turku University Hospital. The study was commenced after obtaining written informed consent from a parent.

Study Protocol and Questionnaire Data

At study entry, the child was clinically examined, a nasopharyngeal aspirate (NPA) for viral diagnostics was taken using a standardized procedure and a baseline blood sample was collected [Jartti et al., 2004; Jartti et al., 2015]. The admission to the ward was decided independent of the study by a physician on duty. The guardian filled in a standard questionnaire on host and environmental risk factors for asthma (e.g., physician diagnosed eczema, parental

history of allergic rhinitis, asthma and smoking) [Appendix 1, Online Supplementary]. The respiratory symptom score, need for bronchodilators, respiratory frequency, heart rate, supplemental oxygen and fever was recorded at study entry and daily at the ward as described previously [Jartti et al., 2006; Turunen et al., 2014, Appendix 2, Online Supplementary]. After discharge, respiratory symptoms (i.e., those including rhinitis, cough, and wheezing) were recorded daily for 2 weeks in a 4-grade scale (0–3) [Turunen et al., 2014, Appendix 3, Online Supplementary].

Definitions and Laboratory Data

Wheezing was defined as a high-pitched whistling sound in expiration with breathing difficulty. Atopy was defined as allergen-specific immunoglobulin E (IgE) level of >0.35 kU/L or more to any of the following common allergens: codfish, cow’s milk, egg, peanut, soybean, wheat, cat, dog, horse, birch, mugwort, timothy, *Cladosporium herbarum* and *Dermatophagoides pteronyssinus* (Phadiatop Combi[®], Phadia, Uppsala, Sweden). Aeroallergen sensitisation was defined as positive IgE antibodies to any of the latter eight allergens. Perennial aeroallergen sensitisation was defined as positive IgE antibodies to the dog, cat or *Dermatophagoides pteronyssinus*. The levels of IgE, blood eosinophil count, CRP, and blood leukocyte count were analysed according to the routine diagnostics of the Central Laboratory, Turku University Hospital, Turku, Finland. The cut-off level of increased eosinophil count was $\geq 0.4 \times 10^9/L$. The diagnosis of eczema was based on typical symptoms including pruritus, typical morphology and chronicity of illness, and it was defined as atopic if any sensitisation was found. The variable any atopic characteristics included atopy, eczema, and/or elevated blood eosinophil count.

Virus Detection

Virus analyses were performed for all nasal samples taken. A sterile swab (nylon flocked dry swab, 520CS01, Copan, Brescia, Italy) was dipped into a nasopharyngeal aspirate and placed in a dry tube. The respiratory samples were refrigerated and analysed for RV, respiratory syncytial virus (RSV) and enteroviruses (EV) by an “in-house” real-time PCR test within 3 days (four times a week) [Jartti et al., 2015]. The samples were stored at -70°C until analysed for other respiratory viruses. Multiplex-PCR (Seeplex RV12 ACE Detection; Seegene, Seoul, Korea) was used to detect RV, RSV, EV, parainfluenza virus (PIV) types 1–3, metapneumovirus (MPV), adenovirus (AdV), coronavirus (CV) 229E, NL63, OC43, and HKU1 and influenza A or B virus (Flu A or B) using manufacturer’s instructions. Human bocavirus (HBoV) was analysed using PCR method and acute infections were serologically confirmed [Söderlund-Venermo et al., 2009].

Rhinovirus Typing and Genetic Characterization

The partial VP4/VP2 and 5' non-coding region (NCR) of RV genome from RV positive samples were amplified and sequenced using four different primer pairs shown in Table SI, Online Supplementary. The sequencing of 5' NCR was performed as previously described [Peltola et al., 2008]. The amplification and sequencing method for VP4/VP2 region is described in Online Supplementary [Linsuwanon et al., 2009; Wisdom et al., 2009; Bochkov et al., 2014].

The PCR products of VP4/VP2 were purified using Exonuclease I (Thermo Fisher Scientific, Waltham, MA) and the products of 5' NCR using the QIAquick PCR purification kit (Qiagen, Venlo, Netherlands). The sequencing of VP4/VP2 was carried out at GATC Biotech AG, Konstanz, Germany and of 5' NCR at the Turku Centre for Biotechnology's sequencing services, Turku, Finland. The sequences were edited from chromatograms using FinchTV[®] (FinchTV 1.5.0, Geospiza, Inc., Seattle, WA). The edited VP4/VP2 and 5' NCR sequences were analysed by multiple sequence alignment CLUSTALW2 method in SeaView editor [Gouy et al., 2010]. Phylogenetic trees were constructed and visualized using the neighbor-joining method and Kimura's two-parameter model with 1,000 bootstrapping value implemented in the MEGA5.5[®] version 5.0 program [Tamura et al., 2011]. Rhinovirus species were assigned by comparing each RV strains with all of the available RV reference sequences encoding either VP4/VP2 region or 5' NCR in BLAST program available in GenBank (<http://www.ncbi.nlm.gov/BLAST>). The type assignment was based on the $\geq 96\%$ identity of sequences when analysed from 5' NCR and $\geq 90\%$ from VP4/VP2 region in BLAST [Miller and Mackay 2013]. All the strains were further aligned in BLAST by pairwise method to determine their similarity. The sequence identity of $\geq 98\%$ was considered to represent the same RV type [Peltola et al., 2008].

Statistics

Statistical power calculation was carried out for the randomized trial but not specifically for the current analysis [Jartti et al., 2015]. As children having a RV infection were randomized to receive either prednisolone or placebo, prednisolone recipients were censored from the analysis at the time of the initiation of the drug [Jartti et al., 2015]. Because our type assignments based on 5' NCR were mainly equivalent to those based on VP4/VP2 region, genotyping results were merged and processed as equal for statistical analysis. The normality of the data distribution was tested using Kolmogorov–Smirnov test. Children infected with non-typeable RV species ($n=17$) and RV-B ($n=1$) were excluded from the statistical analysis. Children with other respiratory virus than RV caused infection were referred as non-RV. For comparisons of patient characteristics and

illness severity variables between RV-A, RV-C, and non-RV infections, one-way analysis of variance (ANOVA) or Kruskal–Wallis test were used for continuous variables and Pearson's chi square test or Fischer exact test (when count <5) were used for categorical variables. For continuous variables, pairwise comparisons were tested using Bonferroni corrections for skewed data or Tukey's test for normal distributed data. Normally distributed variables are expressed as mean and standard deviation (SD) and non-normally distributed variables as median and interquartile range (IQR). Results were considered statistically significant at the level of $P < 0.05$. Data was analysed using SPSS software (Version 23, SPSS Inc, Chicago, IL).

RESULTS

Study Population

Of 125 consecutive eligible children, 111 (89%) were enrolled in the study and included in the analysis (Fig. S1, Online Supplementary). Of them, 84 (76%) children had a RV infection and other respiratory viruses (non-RV) were detected from 27 (24%) patients. The total viral etiology at the study entry was previously described [Turunen et al., 2014]. One or more viruses were detected in the nasopharynx of all of the study children (111/111; 100%). The most common agent was rhinovirus (76%), followed by RSV (28%), HBoV (PCR or serodiagnosis, 18%), and other viruses ($<10\%$ each). Single virus infections were found in 62% of the samples ($n=69$), of which rhinovirus was the most common causative agent ($n=50$, 72%), followed by RSV ($n=11$, 16%).

Rhinovirus Species

In order to obtain the maximal sensitivity of viral genome detection and accuracy of RV typing, the PCR and sequencing were carried out from both partial 5' NCR and VP4/VP2 region of the RV genome. Of the 84 RV positive samples, 75% (63/84) were successfully genotyped using the 5' NCR specific primers and 40% (34/84) were successfully genotyped with VP4/VP2 specific primers. A total of 33 RV isolates (52%) genotyped by 5' NCR assay were not amplified with primers targeting VP4/VP2 region. RV-C was the most common species detected in 73% (46/63) of the samples using 5' NCR specific primers. RV-A isolates were detected in 25% (16/63) and RV-B in 1.6% (1/63) of the samples, respectively. In order to confirm 5' NCR based sequencing results and to verify RV-C species and type assignments, the sequencing of partial VP4/VP2 was also performed. Of those 34 RV positive samples, 88% (30/34) represented RV-C, 8.8% (3/34) RV-A, and 2.9% (1/34) RV-B. Most of the type assignments (93%) were congruent between the sequences obtained from both 5'NCR and VP4/VP2 region ($n=30$). Two sequences matching RV-A101 type

inVP4/VP2 region and RV-C23 or RV-C16 types in 5' NCR suggest either putative recombination or presence of two different RV types in the same sample. Of the 34 isolates genotyped from the VP4/VP2 region, sequencing of 5' NCR products was unsuccessful from four samples. Altogether, 58% (49/84) of the genotyped clinical isolates were identified as RV-C, 20% (17/84) as RV-A, and 1.2% (1/84) as RV-B while 20% (17/84) remained non-typeable. Of them, 11 could not be typed most probably due to low sensitivity of the primers used in sequencing reactions and six cases due to poor quality of the amplicons.

A total of 67 different clinical RV isolates representing 37 different genotypes were identified. Of them, 11 belonged to RV-A and one to RV-B. The remaining 25 types belonged to RV-C of which none was predominant (Fig. 1, Online Supplementary Fig. S2–S4). The detected clinical isolates used for type assignment are shown in Online Supplementary (Online Supplementary Table SII). For statistical reasons the single RV-B isolate was excluded from the clinical analyses.

Coinfections and Seasonality

Simultaneously with RVs, another respiratory virus was detected in 38% of the samples (34/84) (Table I). The prevalence of co-infections including respiratory viruses other than RV has previously been described [Turunen et al., 2014]. A total of 44% of patients with RV-C (15/34) had co-infections. Co-infections also occurred in 21% (7/34) of samples with RV-A, and in 1.2% (1/34) with RV-B. Another virus was also found in 32% (n=11) of non-typeable samples. RV-C in combination with HBoV was the most commonly detected (5/15, 33%). Coinfection status had no influence on clinical analyses (Online Supplementary, Table SIII). RV-C species circulated throughout the year, mostly in fall. RV-A species were evenly present year around and non-typeable RVs peaked during winter season (Fig. 2). RV-B was present in fall.

Patients Characteristics, Atopy, and RV Species

Children with RV-A (13 months [SD 6.1]) and RV-C (13 months [SD 6.3]) induced wheezing were older than those with non-RV wheezing (9.0 months [SD 5.1]) ($P=0.014$, Table II). The prevalence of atopic characteristics was differentially distributed between children with RV-A, RV-C, and non-RV infections ($P=0.001$). Food allergen sensitization was most significantly distributed ($P=0.029$): RV-A 47%, RV-C 23%, and non-RV 12%. Aeroallergen sensitization was only seen in RV-C and RV-A (18% and 17%; $P=0.049$). Parental allergic rhinitis also differed significantly between RV-A, RV-C and non-RV: RV-A 82%, RV-C 63%, and non-RV 41% ($P=0.019$). Furthermore, maternal allergic rhinitis differed significantly, respectively: RV-A 53%, RV-C 45%, and non-RV 19% ($P=0.032$). Paternal smoking was more

common in children with RV-A or RV-C infection than with non-RV infection ($P=0.037$) (Table II). Also, the seasonality and day care were differentially distributed between RV-A, RV-C, and non-RV ($P=0.008$ and 0.036) (Table II). In a subgroup analysis of only hospitalized patients, additionally male sex differed significantly between children with RV-A, RV-C and non-RV infection: RV-A 56%, RV-C 86% and non-RV 52% ($P=0.010$) (Table SIV, Online Supplementary). Moreover, there was no difference in distribution of food sensitization, however, perennial sensitization occurred only in RV-A and RV-C group ($P=0.042$). Also, maternal and parental asthma were differentially distributed occurring only in hospitalized children with RV-A and RV-C ($P<0.05$) (Table SIV, Online Supplementary).

Illness Severity and RV Species

Details between illness severity and RV-A, RV-C and non-RV are shown in Table III. The blood eosinophil count was significantly higher in children with RV-A and RV-C infection than in those with non-RV ($P<0.001$) (Table III). The median duration of wheezing before the recruitment was differentially distributed between RV species ($P=0.040$). The median duration of wheezing were 2 days (IQR 1, 3) for non-RV, 1 day (IQR 1, 2) for RV-A and 1 day (IQR 1, 2) for RV-C (Table III). Also, the median duration of fever was longest for non-RV (1 day [IQR 1, 3]), followed by RV-A (1 day [IQR 0, 2]) and RV-C (1 day [IQR 1, 0]) ($P=0.030$). The use of bronchodilator was differentially distributed between RV-A, RV-C and non-RV: RV-A 94%, RV-C 87% and non-RV 63% ($P=0.012$). The duration of cough before the recruitment tended to differ between RV-A, RV-C, and non-RV ($P=0.064$) (Table III). In a subgroup analysis of hospitalized children, no other significant differences were found in illness severity between children with RV-A, RV-C, and non-RV infection (Table SIV, Online Supplementary). The presence of atopy showed a tendency for prolonged wheezing (all $P=0.073$), especially in RV-A infected children ($P=0.053$). Otherwise, atopy showed no influence on illness severity variable (Table SVI, Online Supplementary). There was neither difference in illness severity between only RV-A and RV-C (data not shown).

DISCUSSION

Our study showed that RV-C was the most common pathogen in the first wheezing episodes most of which necessitated hospitalization, followed by RV-A. RV-B seems to remain rare in severe wheezing episodes. Interestingly, children with RV-A and RV-C induced first wheezing episodes were associated with atopy, more rapid onset of illness and greater illness severity than those with wheezing induced by other respiratory viruses.

The prevalence of RV-C genotypes was 58%, which is in line with previous studies on prevalence of RV

TABLE I. Rhinovirus Species in Coinfections (n = 34)

Coinfections with RV-A	7 (21)
RV-A + HBoV	2 (29)
RV-A + RSV	1 (14)
RV-A + CV	1 (14)
RV-A + MPV	1 (14)
RV-A + PIV	1 (14)
RV-A + RSV + HBoV	1 (14)
Coinfections with RV-B	1 (2.9)
RV-B + HBoV	1 (100)
Coinfections with RV-C	15 (44)
RV-C + HBoV	3 (20)
RV-C + RSV	2 (13)
RV-C + CV	1 (6.7)
RV-C + EV	1 (6.7)
RV-C + MPV	1 (6.7)
RV-C + PIV	1 (6.7)
RV-C + RSV + EV	2 (13)
RV-C + RSV + Flu	1 (6.7)
RV-C + HBoV + CV	1 (6.7)
RV-C + MPV + PIV	1 (6.7)
RV-C + RSV + EV + AdV	1 (6.7)

RSV, respiratory syncytial virus; EV, enterovirus; AdV, adenovirus; MPV, metapneumovirus; PIV, parainfluenza virus; Flu, influenza virus; HBoV, human bocavirus 1; CV, coronavirus. Values are presented as number of cases (%).

genomes in the samples may represent an antecedent infection. However, the rate of non-typeable RVs is in agreement with previous studies, in which their prevalence has varied from <2% to 30% when using partial sequencing of 5' NCR and VP4/VP2 region [Linder et al., 2013; Bochkov et al., 2014; Principi et al., 2015; Müller et al., 2015]. Since non-typeable RVs could represent a heterogeneous group that included all three RV species, they were not used for statistical comparisons.

During our study period, 33 different RV genotypes were identified over a 21 months period of time indicating circulation of multiple RV types (Table SII, Online Supplementary). Earlier studies have shown that various RV types (from 20 to 30) can circulate simultaneously [Iwane et al., 2011; Lee et al., 2012]. However, the overall data on the genotype distribution in the community during the study period is not known. It is also known that children have frequent RV infections caused by different virus strains in a year [Lee et al., 2012; Principi et al., 2015]. The diversity observed among RV-C (n=21 types) was greater than that seen among RV-A (n=11), which is consistent with other studies focusing on children <5 years of age with acute respiratory illnesses (Fig. 1, Online Supplementary Fig. S2–S4) [Miller et al., 2009b, 2011]. In agreement with previous studies, RV-C peaked especially in fall and spring whereas RV-A was present equally throughout the year [Miller et al., 2011; Lee et al., 2012].

We showed RV co-infections with other respiratory viruses in 40% of the first-time wheezing children which is in line with earlier reports (10–50%) [Bizzintino et al., 2011; Cox et al., 2013; Fawkner-Corbett et al., 2016]. In contrast to previous studies showing more co-infections in RV-A cases [Miller et al., 2009a; Fawkner-Corbett et al., 2016], we found more of them in RV-C cases, which is in agreement with the study of Bizzintino et al. [2011]. The exact mechanism of higher rates of co-infection associated with RV-A versus RV-C species is currently unknown. Perhaps different viral pathogenesis, for example, lower interferon responses and increased epithelial permeability, in RV-C versus RV-A infection might predispose to co-infections with other viruses and bacteria

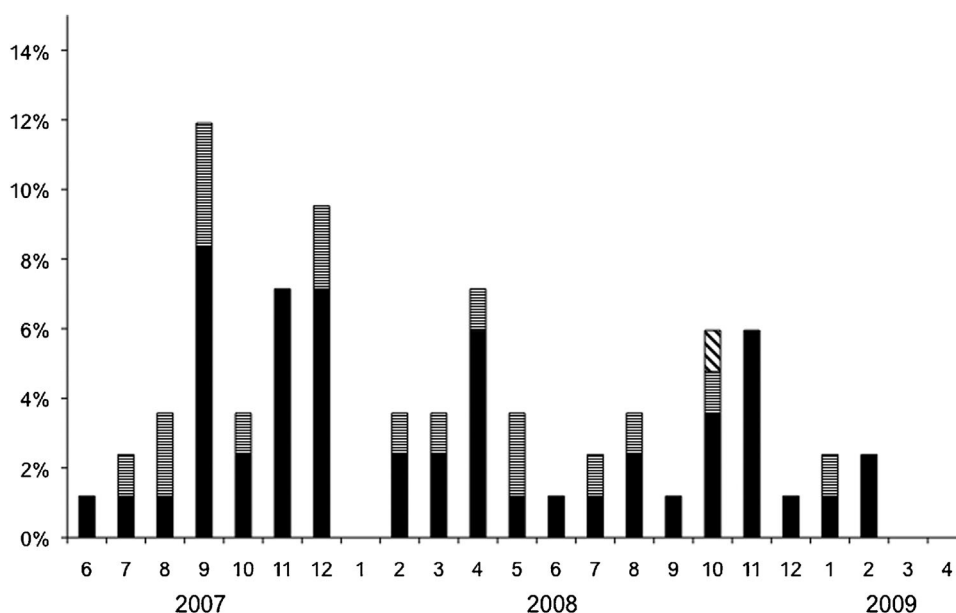


Fig. 2. The seasonality of rhinovirus (RV) species (gray, RV-A; diagonal line, RV-B; black, RV-C).

TABLE II. Patient Characteristics According to Infection With RV-A, RV-C, or Other Respiratory Virus (Non-RV)

Factor	RV-A (n = 17)	RV-C (n = 49)	non-RV (n = 27)	P-value
Age, months	13 (6.1)	13 (6.3)	9.0 (5.1)	0.014
Male sex	9 (53%)	37 (76%)	14 (52%)	0.065
Inpatients	16 (94%)	36 (74%)	23 (85%)	0.14
Atopic characteristics	10 (59%)	34 (69%)	7 (26%)	0.001
Eczema	4 (24%)	20 (41%)	3 (11%)	0.021
Atopic eczema*	4 (24%)	10 (21%)	1 (3.8%)	0.11
Any sensitisation*	7 (41%)	13 (28%)	3 (12%)	0.083
Food*	8 (47%)	11 (23%)	3 (12%)	0.029
Aeroallergen*	3 (18%)	8 (17%)	0 (0%)	0.049
Perennial*	3 (18%)	7 (15%)	0 (0%)	0.063
Coinfection	7 (41%)	15 (31%)	8 (30%)	0.70
Parental self-reported allergic rhinitis	14 (82%)	31 (63%)	11 (41%)	0.019
Maternal self-reported allergic rhinitis	9 (53%)	22 (45%)	5 (19%)	0.032
Paternal self-reported allergic rhinitis	6 (35%)	16 (33%)	8 (30%)	0.92
Parental physician-diagnosed asthma	4 (24%)	9 (18%)	3 (11%)	0.54
Maternal physician-diagnosed asthma	4 (24%)	6 (12%)	2 (7.4%)	0.29
Paternal physician-diagnosed asthma	0 (0%)	4 (8.2%)	2 (7.4%)	0.73
Parental smoking	7 (41%)	21 (43%)	6 (22%)	0.18
Maternal smoking	4 (24%)	8 (16%)	5 (19%)	0.80
Paternal smoking	6 (35%)	19 (39%)	3 (11%)	0.037
Indoor pets	3 (18%)	10 (21%)	0 (37%)	0.22
Duration of hospitalization, hours	24 (20, 41)	22 (16, 32)	27 (17, 42)	0.52
Duration of breast feeding, months	6 (3, 10)	5 (2, 8)	4 (2, 9)	0.78
Duration of sole breastfeeding, months	3 (1, 5)	4 (2, 5)	4 (1, 5)	0.71
Day care, no				
Home	11 (65%)	31 (63%)	23 (85%)	0.036
Small group	5 (29%)	5 (10%)	2 (7.4%)	
Kindergarten	1 (5.9%)	13 (27%)	2 (7.4%)	
Number of children in the family	2 (2, 3)	2 (1, 2)	2 (2, 3)	0.076
Season of recruitment				
Spring (March–May)	3 (18%)	8 (16%)	5 (19%)	0.008
Summer (June–August)	5 (29%)	5 (10%)	2 (7.4%)	
Fall (September–November)	5 (29%)	24 (49%)	4 (15%)	
Winter (December–February)	4 (24%)	12 (25%)	16 (59%)	

RV, rhinovirus.

Values are presented as number of cases (%) or median (IQR). Bold face indicates a significant result, $P < 0.05$. Data were analysed using Pearson's chi-square test, Fischer exact test or Kruskal–Wallis test. *Any sensitisation, $n = 90$; food sensitisation, $n = 90$; aeroallergen sensitisation, $n = 90$; perennial sensitisation, $n = 89$; atopic eczema, $n = 90$; indoor pets, $n = 92$; duration of breast feeding, $n = 79$; duration of sole breast feeding, $n = 86$; duration of hospitalization, $n = 74$.

[Jakiela et al., 2008; Baraldo et al., 2012; Contoli et al., 2015]. Besides, the overall prevalence of RV-C isolates in our study samples could also affect the results of co-infection analysis. Our finding also strengthens the role of respiratory viruses, especially RV, in wheezing illnesses [Jartti et al., 2004; Jackson et al., 2008]. RV is shown to be a strong predictor for recurrent wheezing episodes, and together with allergic sensitization the risk for developing asthma is further increased [Jackson et al., 2008; Jackson et al., 2012; Lukkarinen et al., 2013].

Earlier publications have shown that children with RV-A or RV-C infection had increased risk for wheezing when being sensitised to dust mite, and that atopic sensitisation might increase the risk for hospitalization in children with RV-C induced lower airway illnesses [Soto-Quiros et al., 2012; Cox et al., 2013]. Our study adds to previous reports that atopy is especially common in young children with first wheezing episode caused by RV-A or RV-C rather than by other respiratory virus infection [Soto-Quiros et al., 2012; Cox et al., 2013; Lukkarinen et al., 2013; Turunen et al., 2014].

Furthermore, our finding may indicate that children who are sensitized to many environmental factors are more prone to be infected by RV-A or RV-C than by other respiratory viruses. Our results also add to previous findings, that blood eosinophilia is associated especially with RV-A or RV-C induced early wheezing [Midulla et al., 2010]. Maternal atopy has been associated with RV induced lower airway illnesses in young children; yet, no association with specific RV species has been found either in a one previous study or in our study [Miller et al., 2011]. However, in a subgroup analysis, hospitalized children with RV-A and RV-C infection were associated with parental and maternal asthma when compared to those with non-RV infection. An earlier study has shown that RV induced wheezing in children is associated with male sex [Turunen et al., 2014]. This finding is supported by our subgroup analysis of hospitalized patients when children with RV-A and RV-C infections were more associated with male sex than those with non-RV infections. This might also indicate that boys are having a more severe illness than girls.

TABLE III. Illness Severity and Rhinovirus Species or Other Respiratory Virus (Non-RV) Infection

Factor	RV-A (n = 17)	RV-C (n = 49)	non-RV (n = 27)	P-value
Duration of symptoms before recruitment, days				
Rhinitis	3 (2, 6)	3 (2, 5)	4 (2, 5)	0.81
Cough	2 (1, 4)	2 (2, 4)	4 (3, 6)	0.064
Wheezing	1 (1, 2)	1 (1, 2)	2 (1, 3)	0.040
Fever	1 (1, 2)	1 (0, 2)	1 (1, 3)	0.030
Total immunoglobulinE, kU /L*	26 (8, 210)	143 (6, 937)	10 (3, 36)	0.10
Blood eosinophil count, 10 ⁹ /L*	0.46 (0.18, 0.65)	0.44 (0.287, 0.75)	0.08 (0.06, 0.19)	<0.001
At study entry				
Heart rate, per minute	147 (16)	142 (19)	139 (15)	0.31
O ₂ -saturation, %	96 (95, 97)	97 (94, 98)	98 (96, 98)	0.26
Respiratory frequency, per minute	48 (42, 60)	50 (40, 60)	42 (40, 60)	0.41
Blood leukocyte level, ×10 ⁹ /L	12 (9, 14)	11 (10, 14)	10 (8, 11)	0.13
C-reactive protein, mg/L	14 (8, 26)	12 (6, 21)	4 (2, 26)	0.21
Temperature, °C	37 (37, 38)	38 (37, 38)	37 (37, 38)	0.51
Severity score, scale 0–12*	6 (5, 8)	5 (4, 8)	5 (4, 8)	0.49
Intravenous fluid therapy or nasogastric tube	2 (12%)	0 (0%)	2 (7.7%)	0.058
Use of supplementary oxygen	2 (13%)	6 (12%)	1 (3.8%)	0.56
Use of racemic epinephrine at the ward	6 (35%)	7 (15%)	4 (15%)	0.14
Use of bronchodilator at the ward	16 (94%)	41 (87%)	17 (63%)	0.012
Doses of inhaled bronchodilator racemic adrenalin	3 (1, 5)	3 (1, 5)	1 (0, 3)	0.12
Duration of cough after discharge for 2 weeks, days				
Cough	10 (5, 12)	8 (5, 11)	5 (4, 7)	0.15
Moderate cough	3 (0, 4)	2 (0, 5)	2 (0, 3)	0.58
Severe cough	0 (0, 1)	0 (0, 1)	0 (0, 1)	0.85
Duration of wheezing after discharge for 2 weeks, days				
Wheezing	2 (1, 6)	2 (0, 5)	2 (0, 4)	0.58
Moderate wheezing	1 (0, 2)	0 (0, 1)	0 (0, 1)	0.22
Severe wheezing	0 (0, 0)	0 (0, 0)	0 (0, 0)	0.53

RV, rhinovirus.

Values are presented as number of cases (%), mean (SD) or median (IQR). Bold face indicates a significant result, $P < 0.05$. Data were analysed using Pearson's chi-square test, Fischer exact test, one-way ANOVA or Kruskal–Wallis test. Pairwise comparisons for skewed data were done using Mann–Whitney U-tests with Bonferroni corrections. For duration of wheezing before recruitment: $P = 0.78$ between RV-A and non-RV, $P = 0.028$ between RV-C versus non-RV, and $P = 1.0$ between RV-A and RV-C. For duration of fever before recruitment: $P = 0.32$ between RV-A versus non-RV, $P = 0.036$ between RV-C and non-RV, and $P = 1.0$ between RV-A and RV-C. For blood eosinophil count, $P = 0.007$ between RV-A versus non-RV, $P < 0.001$ between RV-A versus non-RV and $P = 1.0$ between RV-A versus RV-C.

*Data available: total immunoglobulin E, $n = 89$; blood eosinophil count, $n = 89$; heart rate, $n = 92$; O₂-saturation, $n = 88$; blood leukocyte level, $n = 90$; C-reactive protein, $n = 87$; temperature, $n = 89$; severity score, $n = 92$; intravenous fluid therapy or nasogastric tube, $n = 91$; supplementary oxygen, $n = 91$; rasemic epinephrine, $n = 92$; use of bronchodilator, $n = 91$; doses of bronchodilator, $n = 91$; duration of wheezing, $n = 88$; duration of cough, $n = 88$. Severity scores were assessed on a scale from 0 (none) to 12 (severe).

Earlier studies have found RV-A and RV-C to cause more clinically severe lower airway illnesses than RV-B species and in general RV to cause more aggressive illness than RSV [Arden et al., 2010; Bizzintino et al., 2011; Cox et al., 2013; Martin et al., 2015; Fawkner-Corbett et al., 2016]. This adds to the previous findings that rapid onset of RV illness is essentially prevailing in children with RV-A and RV-C induced wheezing [Jartti et al., 2014]. The association with the use of bronchodilator in our study also supports the more severe illness in RV-A and RV-C infected children than in non-RV. On the contrary, our findings are in agreement with three recent studies reported that there is no significant difference in the illness severity between only RV-A and RV-C infected children [Müller et al., 2015; Fawkner-Corbett et al., 2016]. Furthermore, neither in our study nor in one previous study did co-infection status influence the illness severity; however, the sample size was small in our study [Fawkner-Corbett et al., 2016]. Severe wheezing in association with RV-A and RV-C infections might be explained by impaired antiviral responses and

reduced epithelial barrier function [Jakiela et al., 2008; Baraldo et al., 2012; Contoli et al., 2015]. RV has shown to be a strong risk factor for recurrent wheezing regardless of whether being detected alone or with other viruses [Jackson et al., 2008; Hasegawa et al., 2014; Bergroth et al., 2016]. However, the role of RV coinfections causing recurrent wheezing needs further studies.

The strengths of the study include the focus on the first wheezing episode, consecutive patients, prospective design and detailed clinical data. For RV typing, we used specific primers from both partial 5' NCR and VP4/VP2 region of the viral genome. Typing of VP4/VP2 is known to be more definitive for RV species and type differentiation. However, it is generally known that 5' NCR primers are more sensitive for RV PCR amplification than primers targeting VP4/VP2 region [Wisdom et al., 2009; Simmonds et al., 2010; Bochkov et al., 2014]. In our study, the genotyping results from the VP4/VP2 region were similar in 93% of samples when compared to those from the 5' NCR; however, sequences from both regions were obtained only in 30 samples. RV-C was

the most common species based on both 5' NCR and VP4/VP2 region sequencing. RV-C types were also phylogenetically grouped with RV-C isolates derived from GenBank [Simmonds et al., 2010; McIntyre et al., 2013]. One of the limitations of our study is that the size of study population was relatively small and mostly represented hospitalized patients.

In conclusion, our study demonstrates the role of RV-C being the most common RV species in the first wheezing episodes in hospitalized children. Atopic and illness severity features are associated with children having RV-A or RV-C rather than non-RV induced first wheezing episode. Further studies are warranted on the pathogenesis and preventive strategies for RV-induced early wheezing. Understanding the connection between atopic status and wheezing induced by RV species is clinically important when evaluating subsequent risk of asthma.

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