Rhinoviruses significantly affect day-to-day respiratory symptoms of children with asthma

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Background: Viruses are frequently associated with acute exacerbations of asthma, but the extent to which they contribute to the level of day-to-day symptom control is less clear.

Objective: We sought to explore the relationship between viral infections, host and environmental factors, and respiratory symptoms in children.

Methods: Sixty-seven asthmatic children collected samples twice weekly for an average of 10 weeks. These included nasal wash fluid and exhaled breath for PCR-based detection of viral RNA, lung function measurements, and records of medication use and asthma and respiratory symptoms in the previous 3 days. Atopy, mite allergen exposure, and vitamin D levels were also measured. Mixed-model regression analyses were performed.

Results: Human rhinoviruses (hRVs) were detected in 25.5% of 1232 nasal samples and 11.5% of breath samples. Non-hRV viruses were detected in less than 3% of samples. hRV in nasal samples was associated with asthma symptoms (cough and phlegm: odds ratio = 2.0; 95% CI = 1.4-2.86, $P = .0001$; wheeze and chest tightness: odds ratio = 2.34, 95% CI = 1.55-3.52, $P < .0001$) and with cold symptoms, as reported concurrently with sampling and 3 to 4 days later. No differences were found between the 3 hRV genotypes (hRV-A, hRV-B, and hRV-C) in symptom risk. A history of inhaled corticosteroid use, but not atopic status, mite allergen exposure, or vitamin D levels, modified the association between viruses and asthma symptoms.

Conclusion: The detection of nasal hRV was associated with a significantly increased risk of day-to-day asthma symptoms in children. Host, virus genotype, and environmental factors each had only a small or no effect on the relationship of viral infections to asthma symptoms. (J Allergy Clin Immunol 2015;135:663-9.)

Key words: *Virus, rhinovirus, asthma, asthma control, children, mixed-model analysis, respiratory*

Most acute exacerbations of asthma that are severe enough to require presentation to the hospital are associated with respiratory viral infections, particularly with human rhinovirus (hRV).[1-4] However, it is not known whether these viruses play an important role in the day-to-day symptoms of asthmatic children. Many children with asthma, particularly those with more severe disease, experience only partial control of their day-to-day symptoms despite regular pharmacotherapy.[5] Understanding the role of respiratory viruses, alone or in combination with other potentially modifiable environmental or host risk factors,[2] might lead to interventions that would improve asthma management.

The effects of viral infections on day-to-day asthma symptoms have been examined in only a few longitudinal studies, most of which were conducted during peak hRV seasons,[6] in winter,[7] during symptomatic periods,[8] or in a mixture of routine and symptomatic periods.[9] All of these restrictions might limit the generalizability of the findings. In addition, analyses of the relationship between asthma symptoms and viruses in these studies have relied on relatively simple descriptive approaches, which make only limited use of the available data.

There is considerable interest in factors that determine the occurrence and severity of asthma symptoms in association with respiratory tract infections. One hypothesis is that worsening asthma symptoms reflect the spread of infection from the upper to the lower airways.[10] In the past, sampling of the lower airway for virus required bronchoscopy or sputum induction, neither of which is feasible for long-term monitoring of large community-based cohorts. Exhaled breath contains particles that are generated mostly in the lower airways.[11] We have developed a simple method for self-sampling viral RNA in exhaled breath,[12,13] which is suitable for use by children, and used this as a proxy for lower respiratory tract infection. There is also considerable evidence that the severity of asthma symptoms that occur in association with hRV infection differs according to the genotype of the infecting organism. In particular,

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Abbreviations used
c-ACT: Childhood Asthma Control Test
CCQ: Common Cold Questionnaire
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
HDM: House dust mite
hRV: Human rhinovirus
ICS: Inhaled corticosteroid
PEF: Peak expiratory flow
SPT: Skin prick test

infection with hRV-C is more likely to be associated with acute exacerbations of asthma than infection with hRV-A or hRV-B.[14-17] However, the effect of virus genotype on the risk of day-to-day asthma symptoms is not established.[18-20]

In addition, several host and environmental factors can interact with viral infections to influence asthma outcomes. These include atopy,[6,21,22] the combination of sensitization with allergen exposure,[23] and vitamin D concentrations.[24] It is not known whether these are also important modulators of day-to-day symptoms.

We sought to further explore the effect of viral infections in asthmatic children. Our 3 aims were as follows: (1) to determine the contribution of respiratory viruses in samples of nasal mucus and exhaled breath to asthma and cold symptoms; (2) to examine the effect of different rhinovirus genotypes on day-to-day symptoms; and (3) to explore the interactions of atopy, house dust mite (HDM) allergen exposure, vitamin D status, and medication use on the relationship between viral infections and asthma symptoms.

METHODS

We conducted a 10 week-cohort study among children with asthma. Virus samples were collected and clinical outcomes were recorded twice weekly (3-4 days apart) throughout the study period. Full details of the methods used in thus study are provided in the Methods section in this article's Online Repository at www.jacionline.org.

Study participants

Eligible children aged 5 to 12 years attended an asthma clinic or had an asthma-related emergency department attendance at Sydney Children's Hospital, Randwick, Australia, in the previous 12 months and had 1 or more asthma exacerbations in the previous 12 months. This study was approved by the Sydney Children's Hospitals Network Human Research Ethics Committee (HREC Approval: 11/CHW/90, SSA Approval: SSA/11/SCHN/168).

Before starting the study, respiratory function was measured in the hospital clinic. Skin prick test (SPT) results were obtained from the child's medical records. Most children had been tested to a panel of locally relevant allergens (see the Methods section in this article's Online Repository). If SPT responses to HDM were negative or if they were performed more than 2 years earlier, skin testing was performed to HDM *(Dermatophagoides pteronyssinus)* and ryegrass (*Lolium perenne*; Hollister-Stier Laboratories, Spokane, Wash). An average wheal size of 3 mm or greater and greater than that elicited by the negative control (glycerin) was regarded as positive.

Because this was an observational study, each child's asthma treatment was determined by their regular physician.

Home visits and sample collection

After enrollment, 2 research staff visited each child's home to administer questionnaires, collect dust and blood samples, supply materials, and teach the sampling and storage methods. This included written materials and a video (www.youtube.com/SAVEStudy2012). Additional home visits were made at 5 and 10 weeks to collect stored samples. Asthma symptom control was assessed with the Childhood Asthma Control Test (c-ACT), which was used with permission of GlaxoSmithKline (Research Triangle Park, NC). Sampling was conducted from October 2011 to December 2012, with rolling recruitment to minimize seasonal differences.

Details and modifications of the methods for twice-weekly sampling of nasal mucus[6] and exhaled breath,[13] recording of asthma symptoms and medication use,[25] and cold symptoms determined by using the Common Cold Questionnaire (CCQ)[26] are available in the Methods section in this article's Online Repository.

At the first home visit, reservoir dust was collected and analyzed for mite allergen, as previously described.[27] Settling airborne dust was also collected in the bedroom over the whole 10 weeks[28] and analyzed by means of ELISA.[29] A blood sample was collected by means of finger prick and analyzed for vitamin D, as previously described.[30]

Symptom scores

"Cough and phlegm" and "wheeze and chest tightness" were each scored from 0 to 3 (see the Methods section in this article's Online Repository). For each of these symptoms, a binary variable was treated as positive if the score was 1 or greater. In addition, an overall asthma score was calculated as the sum of the cough and phlegm and wheeze and chest tightness scores.

In this study population the CCQ was not sufficiently internally consistent to be summarized as a single score (Cronbach $\alpha = .74$[31]). Two separate factors (or domains) were identified by means of factor analysis: "febrile," being a composite of fever or chills, and "coryzal," comprising watery eyes, runny nose, sneezing, and/or cough. These were both coded as binary variables.

Extraction and identification of respiratory viruses

The processing and analysis of samples for respiratory viruses was performed by using PCR analysis, as described previously.[13] Details of the extraction, real-time PCR analysis for hRV, analysis of non-hRV with a multiplex PCR, and molecular typing of hRV are described in the Methods section in this article's Online Repository. The human housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to check the adequacy of sampling.

Sample size

Insufficient previous data were available to calculate sample size. The rationale for enrolling 67 participants is provided in the Methods section in this article's Online Repository.

Statistical analyses

Statistical analyses were performed with SAS software (version 9.3; SAS Institute, Cary, NC). Briefly, the effect of virus exposure on each outcome was estimated by using mixed-model regression, with a random intercept for each subject to account for correlation among repeated measures. Lags in symptom response to the presence of virus were evaluated by using symptom reports from subsequent diary entries; however, because some samples were not collected on schedule, only consecutive samples 3 or 4 days apart (lag 1) or 6 to 8 days apart (lag 2) were included in modeling of lags. Apart from this, all data of all participants were included in the analysis. Details of the analytic methods are available in the Methods section in this article's Online Repository.

RESULTS

A total of 67 children were enrolled: 5 weeks of data were collected for 62 (93%), 10 weeks of data were collected for 56 (84%), and 15 weeks of data were collected for 3. Details of participant recruitment and retention are shown in Fig E1 in this article's Online Repository at www.jacionline.org. The rate of sample collection varied, with 5.0% of total samples collected

TABLE I. Baseline characteristics of participants

	Enrolled (n = 67)
Age (y), mean ± SD	8.6 ± 2.0
Sex, male	64% (43/67)
SPT	
Any atopy	82.2% (53/64)
House dust mite	72.6% (45/62)
Mold	24.1% (14/58)
Pollen (ryegrass)	30.0% (18/60)
Cockroach	28.8% (17/59)
Cat	23.7% (14/59)
Dog hair	5.9% (2/34)
Alternaria species	21.1% (12/57)
c-ACT score	
Very poorly controlled (≤12)	1.5% (1/65)
Not well controlled (13-19)	30.8% (20/65)
Well controlled (≥20)	67.7% (44/65)
Respiratory function*	
FEV_1 % predicted, mean ± SD	99.8 ± 21.5
FVC, % predicted, mean ± SD	102.8 ± 16.5
FEV_1/FVC ratio, mean ± SD	0.82 ± 0.08
PEF % predicted, mean ± SD	104.7 ± 22.3
ED visits in previous 12 mo	
≥1 Attendance at ED for asthma	9.0% (6/67)
Medication use in previous 12 mo	
Any oral corticosteroids	46.3% (31/67)
ICS or ICS/LABA combination	88.1% (59/67)
ICS regular†	44.8% (30/67)
ICS intermittent‡	3.0% (2/67)
ICS/LABA regular†	28.4% (19/67)
ICS/LABA intermittent‡	11.9% (8/67)
Regular high-dose§ ICS or ICS/LABA†	62.7% (42/67)
Non-ICS controller‖ (no ICS or ICS/LABA)	3.0% (2/67)
SABA only	9.0% (6/67)
Exposure to domestic tobacco smoke¶	6% (4/67)

ED, Emergency department; *SABA*, short-acting β-agonist.
*There were 57 subjects with acceptable and reproducible spirometric records.
†Reported to have been taken for most of the previous 12 months.
‡Reported to have been taken intermittently or on an as-needed basis.
§High-dose ICS: greater than 200 μg/d fluticasone propionate or equivalent.
‖Montelukast (leukotriene receptor antagonist).
¶Based on parental responses to "My home is smoke free" versus "People occasionally/frequently smoke in my home."

in the last quarter of 2011 and 40.2%, 21.7%, 22.1%, and 10.9% collected in the 4 quarters of 2012.

Participant characteristics

Participants' characteristics are shown in Table I. Two thirds of the children were male, with an average age of 8.6 ± 2.0 years. Of the 64 undergoing skin tests, 82% were atopic, with the most common sensitization being to HDM. In the previous 12 months, 6 children required an emergency department visit for asthma, and most (88%) had used regular inhaled corticosteroid (ICS)–containing controllers, with 63% taking a regular high-dose ICS or ICS/long-acting β-agonist (LABA) fixed-dose combination. Asthma was well controlled (c-ACT score ≥20) in 68% of children and not well-controlled or very poorly controlled in the remainder. Only 6% were exposed to domestic tobacco smoke. Information on the ethnicity and socioeconomic status of participants is shown in Table E1 in this article's Online Repository at www.jacionline.org.

TABLE II. Occurrence of hRV, each hRV genotype, and non-hRV viruses in exhaled breath and nasal wash samples from the 67 participants

	Nasal wash samples	Exhaled breath samples
Total no. tested	1232	1231
Positive by means of RT-PCR for any hRV	314 (25.5%)	142 (11.5%)
hRV-A sequenced by using nested PCR	129 (10.5%)	19 (1.5%)
hRV-B sequenced by using nested PCR	56 (4.6%)	2 (0.2%)
hRV-C sequenced by using nested PCR	46 (3.7%)	3 (0.2%)
RT-PCR hRV positive that could not be genotyped by using nested PCR	83 (6.7%)	118 (9.6%)
Positive for any non-hRV viruses*	28 (2.3%)	19 (1.5%)
Samples coinfected with an hRV and non-hRV virus	7 (0.6%)	1 (0.08%)

*Influenza A; influenza B; respiratory syncytial virus; parainfluenza viruses 1, 2, and 3; and human metapneumovirus. There were 8 samples that contained hRV and another virus, 5 of which were respiratory syncytial virus, and there were single occurrences of parainfluenza 1, parainfluenza 3, and metapneumovirus.

Allergen exposure and vitamin D levels

Wide variation was found in the concentration of HDM allergens in participants' bed dust and settling dust (see Fig E2, *A* and *B*, respectively, in this article's Online Repository at www.jacionline.org). The serum vitamin D concentration ranged from 10.2 to 91.2 nmol/L (mean, 46.9 nmol/L; SD, 19.1 nmol/L; see Fig E3 in this article's Online Repository at www.jacionline.org).

Asthma and cold symptoms

In the 1206 diary entries of the 67 children, the presence of cough and phlegm since the last diary entry was reported in 47.1% of records, and the presence of wheeze and chest tightness since the last diary entry was reported in 26.4% of records. Overall, 66% of the children with 10 weeks of data reported moderate or severe cough and phlegm at least once. The frequency distributions of the asthma symptom scores in the study and the distribution of the maximum scores among children with data collected over at least 10 weeks are shown in Table E2 in this article's Online Repository at www.jacionline.org.

Five children reported seeking urgent medical attention for symptoms during the study, although details could be confirmed against hospital records for only 3; none were admitted to the hospital. Two children had 3 attendances each. In 6 of 7 cases, attendances occurred close to diary reports of severe asthma and cold symptoms.

Detection of viruses

RNA for hRV was detected in 314 (25.5%) of 1232 nasal samples and in 142 (11.5%) of 1231 exhaled breath samples (Table II). At least 1 virus was detected in either a nasal or exhaled breath sample on 410 (33.3%) sampling occasions. In 54 (96%) of 56 participants who completed 10 weeks of sampling, hRV was detected at least once in either a breath or a nasal sample. Of the 142 hRV-positive exhaled breath samples, 46 (32.4%) were concordant with an hRV-positive nasal sample collected at the same time.

Of the 456 hRV-positive samples, 255 (55.9%) were able to be sequenced in the VP4-VP2 region by using a nested PCR; 231

TABLE III. ORs for the different outcomes at the time of sampling and with lags associated with a rhinovirus-positive nasal wash sample

	No lag			Lag of 3-4 d			Lag of 6-8 d		
Outcome	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**
Cough/phlegm	2.00	1.40 to 2.86	**.0001**	2.12	1.36 to 3.31	**.001**	1.34	0.87 to 2.08	.183
Wheeze/chest tightness	2.34	1.55 to 3.52	**<.0001**	2.43	1.48 to 3.99	**.0004**	1.53	0.94 to 2.49	.085
Reliever use	2.30	1.58 to 3.34	**<.0001**	2.03	1.29 to 3.19	**.0023**	1.75	1.14 to 2.69	**.011**
Composite febrile	1.66	1.01 to 2.71	**.045**	2.84	1.54 to 5.26	**.0009**	1.77	0.95 to 3.29	.072
Composite coryzal	1.89	1.23 to 2.89	**.004**	2.92	1.61 to 5.30	**.0004**	2.03	1.18 to 3.52	**.011**
	Difference								
PEF *z* score	−0.106	−0.235 to 0.024	.110	0.049	−0.106 to 0.20	.537	−0.019	−0.16 to 0.129	.797
FEV_1 *z* score	0.063	−0.192 to 0.066	.339	0.040	−0.118 to 0.197	.621	−0.053	−0.203 to 0.096	.482

Only those samples recorded in the period of 3 and 4 days and the period of 6, 7, and 8 days after the initial sample were used in the analysis of lags.
OR, Odds ratio.
Values in boldface are $P < .05$.

TABLE IV. Association of clinical outcomes with each of the 3 genotypes of hRV (A, B, and C) in nasal samples: each species is compared with the other 2 species combined

	hRV-A vs hRV-B + hRV-C			hRV-B vs hRV-A + hRV-C			hRV-C vs hRV-A + hRV-B		
Outcome	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**
Cough/phlegm	2.57	0.9 to 7.35	.08	0.48	0.14 to 1.72	.26	0.56	0.18 to 1.77	.32
Wheeze/chest tightness	0.63	0.18 to 2.17	.46	0.69	0.12 to 3.96	.68	2.15	0.56 to 8.25	.26
Reliever used	1.28	0.52 to 3.13	.58	1.02	0.33 to 3.13	.98	0.73	0.27 to 1.97	.53
Febrile	1.24	0.38 to 4.00	.72	0.75	0.15 to 3.89	.74	0.91	0.24 to 3.45	.89
Coryzal	2.26	0.79 to 6.45	.13	0.59	0.18 to 1.93	.38	0.58	0.17 to 1.92	.37

OR, Odds ratio.

(50.6%) of 456 were nasal samples, and 24 (5.3%) of 456 were breath samples. Among these, 148 (58.0%) of 255 were hRV-A, 58 (22.7%) of 255 were hRV-B, and 49 (19.2%) of 255 were hRV-C (Table II). hRV titers for nasal and breath samples that could or could not be sequenced are shown in Fig E4 in this article's Online Repository at www.jacionline.org. Respiratory viruses other than hRV were detected in 28 (2.3%) of 1232 of the nasal wash samples and 19 (1.5%) of 1231 of the exhaled samples. The prevalence of non-hRV viruses is shown in Table E3 in this article's Online Repository at www.jacionline.org.

GAPDH was detected in more than 99% of nasal samples irrespective of whether they were virus positive. Among the breath samples, 36.6% of the hRV-positive samples and 23.4% of the hRV-negative samples were GAPDH positive.

Association between virus detection and respiratory symptoms

The presence of hRV in nasal samples was associated with an approximately 2-fold increase in the odds of both asthma symptoms (cough and phlegm and wheeze and chest tightness) and cold symptoms (febrile or coryzal) at the time of virus sampling and at 3 to 4 days after sampling (Table III). Reliever use was also significantly associated with the presence of hRV from the time of sampling to 6 to 8 days later. However, there was no association between the presence of hRV in exhaled breath samples and the occurrence of either asthma or cold symptoms (see Table E4 in this article's Online Repository at www.jacionline.org). There was no association between the presence of hRV in either nasal wash or exhaled breath samples and changes in FEV_1 or peak expiratory flow (PEF; see Table III and Table E4 in this article's Online Repository).

Among participants with hRV detected in nasal samples, the 3 hRV genotypes did not differ in their association with asthma or cold symptoms (Table IV).

The increase in asthma scores attributable to the presence of virus in nasal and breath samples is shown in Table E5 in this article's Online Repository at www.jacionline.org.

The association of hRV with symptoms did not differ from the association of other non-hRV viruses with symptoms, except that febrile symptoms were less likely (odds ratio, 0.17; 95% CI, 0.05-0.57; $P = .004$) to be associated with hRV than with other viruses.

Estimation of population attributable risk

The overall prevalence of nasal hRV detection was 25.5%. The relative risks of cough and phlegm and wheeze and chest tightness in subjects with nasal samples positive for hRV compared with those without hRV were 1.27 and 1.46, respectively. Hence the population attributable risk of hRV detection for these symptoms was 6.3% and 10.4%, respectively.

Interactions with other factors

The factors that significantly modified the relationship between hRV detection in nasal samples and asthma symptoms are shown in Table V. Cough symptoms were more likely to occur in the 3 to 4 days (interaction $P = .023$) and 6 to 8 days (interaction $P = .035$) after an hRV-positive sample in those participants with low or no use of ICSs in the previous 12 months. In participants with high (>50 nmol/L) vitamin D levels, wheeze was more likely with a viral infection ($P = .039$), and in atopic subjects with low HDM exposure in reservoir dust, cough was more likely 6 to 8 days after virus detection ($P = .036$). Atopic status did not modify the effect of hRV detection on asthma in any of the other models examined.

TABLE V. Significant results for interaction terms that modified the relationship between nasal virus detection and asthma outcomes

Outcome	Virus	Lag (d)	Effect modifier	Level	OR	95% CI	*P* value (interaction)
Wheeze/chest tightness	hRV nasal	0	Low vitamin D	≤50	1.78	0.89-3.58	**.039**
				>50	4.93	2.50-9.71	
Cough/phlegm	hRV nasal	3-4	ICS in previous 12 mo	High use	1.29	0.70-2.39	**.023**
				No or low use	3.64	1.88-7.04	
Cough/phlegm	hRV nasal	6-8	ICS in previous 12 mo	High use	0.85	0.46-1.57	**.035**
				No or low use	2.18	1.16-4.12	
Cough/phlegm	hRV nasal	6-8	HDM high reservoir	HDM atopy + high reservoir quartiles	0.86	0.36-2.05	**.036**
				HDM atopy + low reservoir quartiles	2.97	1.36-6.46	

OR, Odds ratio.
Values in boldface are $P < .05$.

DISCUSSION

This is the first longitudinal, community-based study to use frequent routine sampling and a mixed-model regression analysis to examine the effect of respiratory viruses on day-to-day respiratory symptoms in children.

Our data provide a perspective that differs from many common perceptions about the role of viruses in respiratory symptoms, which have largely been derived from studies of acute exacerbations. Our study shows that virus detection is common and associated with a significantly increased risk of day-to-day symptoms. This relationship occurred irrespective of the genotype of the virus or the presence of atopy or allergen exposure, which is contrary to findings in studies of asthmatic children with more severe exacerbations.

The high prevalence and proportion of rhinovirus infections are similar to those in several other longitudinal cohort studies in children. Compared with our finding of 25.5% hRV and 2.3% non-hRV in nasal specimens, others have found 33.7% hRV and 8.6 non-hRV,[6] 48% hRV and 8.5% non-hRV,[9] and 22.6% hRV and 13.9% non-hRV.[32] The lower proportions of hRV and non-hRV infections in our study might reflect differences generated by the timing of sampling relative to the seasonal prevalence of different viruses, population age, effects of geography, and differences in the methods of sampling or analysis used by us compared with others. It is noteworthy that we found a prevalence of hRV of 33.3% if both nasal and breath sample results at each time point were considered, suggesting nasal sampling alone might underestimate community prevalence. It is also recognized that other microorganisms,[33] including coronaviruses and bocavirus[34,35] and some transient bacterial infections,[36] for which we did not test, might also play a role in wheezing. Any estimate of the role of viruses on asthma symptoms is sensitive to the nuances of the ability to detect these infections.

The detection of virus in nasal samples was significantly associated with asthma and respiratory symptoms both at the time of virus sampling and 3 to 4 days later; however, whether subsets of subjects differed in the timing of symptom onset within this time frame would have required daily diaries for the whole study. This finding of relatively weak but highly significant associations between virus nasal positivity and day-to-day asthma symptoms contrasts with previous reports of strong associations between the presence of respiratory viruses and the occurrence of acute exacerbations of asthma. For example, in an emergency department study, odds ratios of 31.5 (95% CI, 8.3-108; $P < .001$) have been reported for the risk of acute wheezing in highly mite atopic children with an hRV infection compared with those seen in virus-negative children[37] and 19.4 (95% CI, 3.7-101.5; $P = .001$) for the combination of atopy, allergen exposure, and viral infection for hospital admission compared with those in patients with stable asthma. In this latter study the odds ratio for rhinovirus alone was 7.0 (95% CI, 2.5-19.9; $P < .001$).[23] The contrasting findings during day-to-day sampling in the present study are consistent with increasing awareness that in asthmatic patients symptom control can be dissociated from the risk of exacerbations,[38] and a recent task force report found that it is not possible to distinguish between transient loss of symptom control and a mild exacerbation.[39]

The finding that hRV-positive exhaled breath samples were not generally associated with asthma symptoms was surprising, given the hypothesis that increased asthma symptoms during the course of a cold might be associated with migration of the virus from the upper to the lower respiratory tract.[10] Exhaled aerosols carrying viral RNA are thought to be mainly generated by the bursting of the mucus film in the lower airways,[11,40] and we presume that such aerosols reflect viral infection at this site; however, we are not aware of any studies directly validating this. These aerosols of viral RNA have previously been detected by us and others using a variety of methods.[11,41-43] It is unknown whether differences in the detection of viral RNA in exhaled samples compared with nasal samples was due to the virus being present less frequently in the exhaled samples or because of the smaller volume of airway fluid in these samples. It can be estimated from other published data[44,45] that the exhaled breath samples would contain several orders of magnitude less airway fluid than nasal wash samples.

This study used PCR detection of viral RNA at each point in time as a proxy for the presence of viruses, as is common to other studies of the relationships of viruses and symptoms.[6,9,32,46] The relationship of such RNA to the cytopathology of the infectious process is beyond the scope of this study.

A strength of this study is our use of mixed regression models, rather than simple descriptive approaches, for analysis of cohort data. This approach allows more precise estimates of the associations between the effect of virus detection in a subject at a single point in time and the occurrence of symptoms (eg, cough or wheeze) at that time or at various times afterward. A further refinement of our models was the inclusion of lag terms to account for delay effects of exposures on outcomes and the testing of effect modification by factors that might affect these associations. Similar approaches have analyzed asthma-related health effects associated with exposure to nitrogen dioxide,[25] particulates,[47] and pollen.[48]

Our estimation of the population attributable risk can be interpreted to mean that if respiratory virus exposure was eliminated from this population, the risk of day-to-day asthma symptoms would only decrease by 6% to 10%. This raises the issue of other factors that might be responsible for day-to-day variation in asthma and respiratory symptoms. Much of our understanding of this subject is derived from studies of acute exposure episodes,[49] patients' perceptions about triggers,[50] and studies of uncommon events within large community studies.[51] Recent reviews[52-54] cite multiple types of inhaled exposures, medication adherence, and emotional, dietary, and hormonal factors as risk factors for exacerbations, but population attributable risks for day-to-day asthma and respiratory symptoms have not been reported. Although clearly many factors can contribute to both asthma and respiratory symptoms, the challenge is to determine their relative contributions and how they interact with each other.

Our data suggest that the variation in asthma and respiratory symptoms could not be simply attributed to the different hRV genotypes circulating in the community. Although some,[14-17] but not all,[18-20] studies have shown a greater propensity of hRV-C to be more strongly associated with exacerbations, we did not detect any difference between genotypes in their association with day-to-day symptoms. This finding does not discount a role for specific pathogenic genotypes of hRV in causing acute exacerbations of asthma but rather argues that at a day-to-day community level, where both infections and respiratory symptoms are common, these distinctions might be less important.

There was no evidence from our analysis that several host or environmental factors known to be commonly associated with asthma exacerbations interacted with the relationship between viral infections and day-to-day asthma symptoms. With the possible exception of a history of low ICS use increasing cough after a viral infection, the other interactions shown in Table V were not repeated across the lagged times and are inconsistent with other studies. For example, the lowest quartile of mite exposure was associated with increased cough, whereas other data suggest mite exposure is positively associated with cough.[55,56] Similarly, our finding that a vitamin D level of greater than the cut point of 50 nmol/L was associated with increased wheeze is inconsistent with most data that higher vitamin D levels are associated with better respiratory outcomes.[24] Whether the enhanced immune response to viral infection associated with higher vitamin D levels is also associated with increased wheeze requires further study. The most surprising finding was the lack of interaction with atopy. This is in contrast to several cohort studies that show a synergy between asthma exacerbations and viruses, either alone[22,37,57] or in combination with allergen exposure.[23,58] However, in the longitudinal cohort study most comparable in structure to ours,[6] the effect of sensitization on symptoms was most evident with severe symptoms, and this would not be identified by using our analysis.

Part of the reason for the absence of effect modification in our study population might have been a lack of power to test these interactions. The ranges of values in which some interactions might occur are not well represented in our cohort: 82% of our population were atopic, 84% were above the cut point for high HDM exposure used in the studies demonstrating synergistic effects,[23,58] and 88% had used ICSs or ICS/LABA combinations in the previous 12 months. Another reason might be that our study routinely monitored all viruses and day-to-day symptoms and used a mixed-model analysis; such findings might differ from observations based on exacerbations in large populations. In a sample of this size, we were not able to investigate the potential contribution of different asthma phenotypes, which in this age group have largely been studied among children with severe asthma.[59]

In conclusion, this study identifies the clinical significance of hRVs in day-to-day asthma and respiratory symptoms in children but does not confirm the role any of the host, viral, or environmental factors thought to modify the role of hRV in patients with acute asthma exacerbations. However, given the complexity of clinical asthma, the differences in phenotypes, the multiplicity of factors that can modulate symptoms, the diverse clinical responses to viral infections, and the likely variation in the pathogenicity of individual strains of hRV, a better understanding of the role of viruses presents new challenges for longitudinally monitoring both the causal factors and the associated clinical outcomes.

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Key messages

- **Viral infections are common in children with asthma and significantly increase the risk of day-to-day symptoms.**
- **Host factors, environment, and virus genotype had little effect on this relationship.**

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METHODS

Inclusion criteria

Children aged 5 to 12 years (inclusive) were recruited from the Sydney Children's Hospital Randwick Respiratory Clinics (Sydney Children's Hospital) and associated clinical practices. The children were required to have a clinical diagnosis of asthma confirmed by the treating pediatrician with a history of 1 or more exacerbations of asthma in the preceding 12 months. For this purpose, an exacerbation of asthma was defined as an increase in asthma symptoms necessitating an increase in medical therapy (other than increased reliever medication) for asthma.

Exclusion criteria

This study excluded children receiving antiviral treatment or anti-IgE treatment, children with other chronic respiratory conditions (eg, bronchiectasis, cystic fibrosis, or bronchopulmonary dysplasia) or any immunodeficiency status or taking long-term oral immunosuppressive therapy (including long-term but not short-term oral corticosteroids), and children with severe or life-threatening comorbidity.

Patients' characteristics

Smoking in the home. Smoking in the home was assessed by using a questionnaire about home characteristics completed by the parent at the first home visit. It included the following item: "Which of the following best describes your home situation: My Home is Smoke Free (b) People Occasionally/Frequently Smoke in my Home."

Ethnicity and socioeconomic status. Ethnicity was documented at the time of spirometry and is as recorded in the hospital records. The Index of Relative Socioeconomic Disadvantage was based on residential postcode (http://www.abs.gov.au/websitedbs/censushome.nsf/home/seifa). Additional supporting material can be found at the Census of Population and Housing: Socio-Economic Indexes for Areas (SEIFA), Australia (http://www.abs.gov.au/ausstats/abs@.nsf/mf/2033.0.55.001/).

Lung function testing in the laboratory

Lung function testing was conducted in the hospital clinic before the start of this study. Bronchodilator medications were withheld before lung function testing, according to standard laboratory protocols (inhaled bronchodilators: short-acting bronchodilator, 4 hours; long-acting bronchodilator, 12 hours; oral bronchodilator: short-acting bronchodilator, 8 hours; long-acting bronchodilator, 12 hours).

Skin testing

For most children, skin prick testing had been conducted in the hospital clinic before recruitment into the study, as part of normal clinical management. The allergens used were selected by the treating physicians and varied between participants. Almost all participants had SPTs to HDM, mold, ryegrass, cockroach, and *Alternaria* species (see Table I). If SPT responses to HDM were negative or if SPTs were not performed or performed more than 2 years earlier, testing to HDM and ryegrass, which together detects 89.5% of allergies detected by a large panel in local children, was performed as previously described. Before testing, parents were advised that oral antihistamines should be discontinued at least 4 days before testing.

Measurement of respiratory function, asthma, and cold symptoms

The participants recorded respiratory function and completed 2 diary cards twice each week. Bags for storage of nasal wash and exhaled breath samples were prelabeled (eg, "First week, Sunday/Monday" and "First week, Thursday/Friday"). Participants were instructed to write the date of collection on the sample bags. The wall chart was similarly labeled with both requested days and a space to record the actual collection date. PEF and FEV_1 were recorded with a Mini-Wright Digital (Clement Clarke International, Harlow, Essex, United Kingdom), which automatically recorded the date. An asthma diary card[E1] was used to report asthma symptoms by using the following question: "Did your child have any of the following since the last diary entry (for asthma symptoms)?" Briefly, this used a 4-point scale (0, no symptoms; 1 [mild], symptoms but did not disturb daily activities or sleep; 2 [moderate], symptoms disturbed part of daily activities or sleep; and 3 [severe], symptoms that disturbed whole or most of day or sleep) to separately measure cough and phlegm and wheeze and chest tightness symptoms. It also asked whether preventer and/or reliever medications had been used (yes/no) since the diary was previously used. Cold symptoms were reported based on the CCQ.[E2] Briefly, this used a 4-point scale (0 for no symptoms and a scale of 1 to 3 for mild, moderate, and severe symptoms, respectively, without further defining these) to measure 9 items typically associated with colds (fevers, chills, muscle pains, watery eyes, runny nose, sneezing, sore throat, cough, and chest pain) over the previous 2 days. Participants were instructed to write the date on both asthma and CCQ questionnaire cards.

Asthma symptom control was assessed at baseline and at weeks 5 and 10 with the c-ACT. The c-ACT is a trademark of QualityMetric Incorporated. GlaxoSmithKline has given permission for the c-ACT to be used in this study in accordance with the conditions of GlaxoSmithKline's license with QualityMetric.

Sampling of nasal mucus and exhaled breath

Nasal mucus was collected by using the method of Olenec et al,[E3] with slight modifications. The child delivered 100 μL of saline into each nostril 4 times by using a spray bottle (Children's Fess, Laboratories PharmaSter, Erstein, France) and then blew the nose gently into an 11 × 15–cm ziplock plastic bag. One milliliter of gelatin/saline preservative was added and mixed in by massaging the bag; this mixing was facilitated by having a small quantity of an inert food dye in the gelatin solution. The sample bag was labeled, double bagged to prevent any loss or leakage, and stored in a sealed container in a domestic freezer (typically −20°C).

The method for sampling exhaled breath was similar to our previous report,[E4] except that a modified mouthpiece (Vitalograph V20980; Vitalograph, Buckingham, United Kingdom) with the valves removed was used to hold two 25-mm-diameter disks of electret filter cloth (EWE50; Vilene, Tokyo, Japan). This mouthpiece was used in place of a mask because in a previous study supervision was required to ensure the children formed a good seal between the face and the mask. The children wore a nose clip, and the sampling time was 5 minutes. After sampling, the mouthpiece was placed in a ziplock plastic bag and stored as above.

A separate study of virus stability in mucus using a domestic freezer for 5 weeks showed no reduction in hRV titer by means of real-time PCR (data not shown). Samples were transported from the houses to the laboratory at −50°C to −80°C and stored in the laboratory at −80°C.

Extraction and identification of respiratory viruses

Briefly, the electret was extracted for 30 minutes in viral transport medium by using an in-house process. Total nucleic acid in the filter extract and nasal mucus was extracted on the Kingfisher Flex (Thermo Fisher Scientific Australia, Scoresby, Victoria Australia) by using the MagNA Pure Total Nucleic Acid Isolation Kit (Roche Australia, Castle Hill, Australia), according to the manufacturer's instructions. PolyA Carrier RNA (Roche Australia) was added to the Lysis Buffer to facilitate binding of RNA. Extracts were stored in 20-μL aliquots at −80°C in microtubes.

Real-time RT-PCR detection for hRV was performed for all samples by using a probe-based detection method,[E5] with slight modification to the forward primer.[E6] Amplification was performed with a Lightcycler 2.0 (Roche Australia) using the following cycling conditions: 45°C for 20 minutes and 94°C for 2 minutes, followed by 55 cycles of 94°C for 5 seconds and 60°C for 60 seconds (single acquisition). Viral load was quantified by using plasmid standards, as previously described.[E7] DNA products were visualized by using 2% agarose gel electrophoresis with SYBR1 Safe staining (Invitrogen Life Technologies, Mulgrave, Australia). A nested multiplex RT-PCR was used for detection of influenza A; influenza B; respiratory syncytial virus; parainfluenza viruses 1, 2, and 3; and human metapneumovirus and confirmed by using an enzyme-linked amplicon hybridization assay with specific oligonucleotide probes, as described previously.[E4,E8] All samples were tested for DNA of the human housekeeping gene *GAPDH*, as previously described.[E9]

Molecular typing of hRV

Molecular typing of all samples positive for hRV was performed, as previously described.[E10] Briefly, a 540-bp region of the variable VP4-VP2 region of the hRV genome was amplified by means of nested RT-PCR. Those samples unable to be amplified with the primers and protocol described by Wisdom et al[E10] were again run on a nested RT-PCR by using additional forward primers designed in this study (outer forward primer, 5′CTNWGCCNTGCGTGGC3′; inner forward primer, 5′TACTTTGGGTGTCCGTGTTTC3′); the reverse primers remained as described by Wisdom et al.[E10] The second-round 540-bp PCR product was purified by means of polyethylene glycol precipitation and sequenced with BigDye terminator mix (Applied Biosystems, Scoresby, Australia) at the Ramaciotti Centre for Gene Function Analysis at the University of New South Wales, Sydney.

Statistical and phylogenetic analyses were performed with the MEGA version 5 DNA analysis package.[E11] The ClustalW program[E12] was used to determine nucleotide distances by using a Kimura 2-parameter distance matrix, and phylogenetic trees were constructed with the neighbor-joining method, including unrelated control sequences from the GenBank database made available through the National Center for Biotechnology Information's Web site (http://www.ncbi.nlm.nih.gov).

Sensitivity and specificity of PCR assays

The sensitivity of the RT-PCR was 1×10^2 copies of virus per reaction. The sensitivity of the nested PCR was 1×10^3 copies of virus per reaction.[E9] There was no inhibition seen with the exhaled breath condensate or the nasal wash, as we have published.[E4,E9] The RT-PCR did not detect nucleic acids from any other respiratory viruses tested.[E5] The specificity of the nested PCR is 100%.[E13]

STATISTICAL ANALYSES

Asthma symptoms (cough and phlegm and wheeze and chest tightness), cold symptoms (febrile and coryzal), and reliever medication use were treated as binary outcome variables in the mixed model (score 0 vs >0). The model was fitted by using Proc GLIMMIX with the Laplace estimation routine, a logistic link, and binary error structure. For normally distributed continuous outcomes (FEV_1 and PEF), we used Proc Mixed to fit the mixed model, assuming a normal error distribution. For the asthma score variable, we used Proc GLIMMIX with the Laplace estimation routine, a log link, and a Poisson error distribution. We assessed the relationship between viral exposure and outcomes for concurrent records and also with lags. In addition to concurrent associations, we tested associations with virus isolation both 3 to 4 days and 6 to 8 days before the relevant outcome. This lagged analysis was constrained to records that were 3 or 4 days or 6, 7, or 8 days apart in accordance with the intended data collection schedule. Children were asked to collect virus, symptom, and peak flow data on the same date; where there were discrepancies of only 1 day, virus and peak flow were linked to the symptom date because this had the greatest number of records.

The effect of all respiratory viruses, all hRV isolates, and the 3 hRV species in nasal samples was tested; breath samples were tested only for all viruses and all hRV viruses. The asthma and cold symptom scores were compared for each hRV species against the other 2 species to determine whether the clinical effect of hRV infections was a function of hRV species.

We assessed whether the association between virus exposure and outcome was modified by including interaction terms for the following variables: sensitization status (any atopy vs no atopy and HDM atopy vs non-HDM atopy), HDM atopy plus HDM exposure (highest quartile vs lowest quartile as for both reservoir and settling allergen), vitamin D level (≤25, ≤50, and >50, nmol/L), and use of ICSs in the previous 12 months (high vs low or no use).

The population attributable risk percentage was calculated as follows:

$$\frac{P_e(RR_e-1)}{1+P_e(RR_e-1)},$$

where P_e is the proportion of persons exposed (to the virus) and RR_e is the risk ratio associated with exposure (hRV detection in nasal sample), as previously described.[E14] RR_e was estimated by fitting a log-binomial model with generalized estimating equations. The unadjusted risk ratio was calculated.

Power calculation

We estimated, based on logistic and budgetary considerations, that it would be feasible to enroll 65 participants in this study. We were also influenced by 3 previous related studies of a similar size or smaller.[E3,E15,E16] In the absence of adequate preliminary or prior data, we calculated that this sample size would enable us to estimate the 95% CI for a proportion (incidence) of 10% as 3.9% to 16% and for a proportion (incidence) of 50% as 37.3% to 62.7%.

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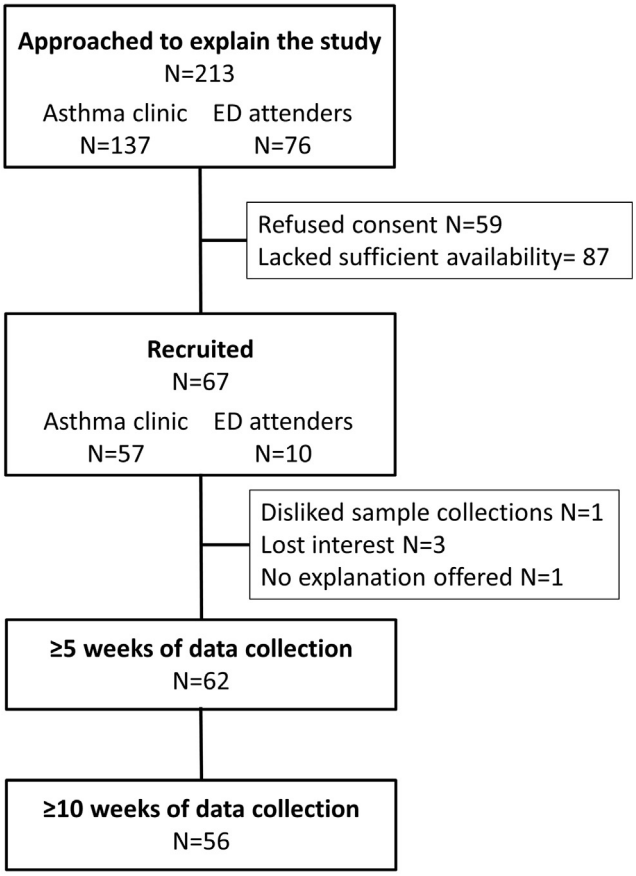

FIG E1. Recruitment and retention details.

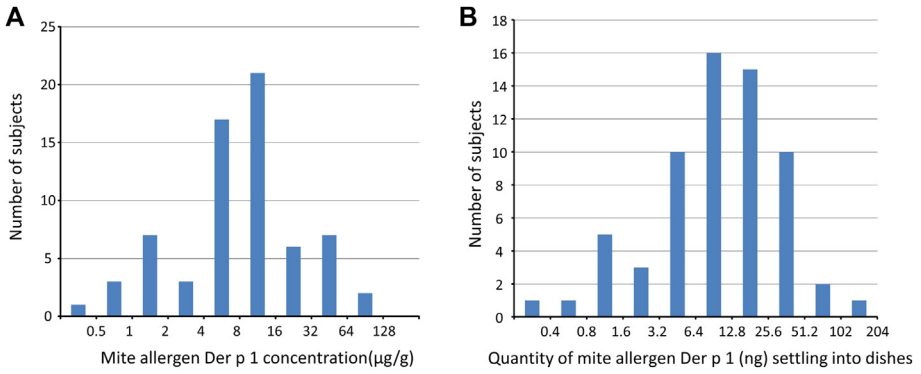

FIG E2. Distribution of the mite allergen Der p 1. **A,** Concentration of allergen in reservoir dust samples. **B,** Quantity of allergen settling in a Petri dish over 10 weeks.

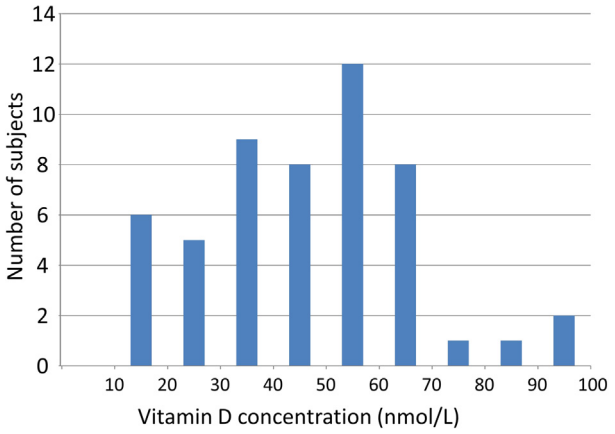

FIG E3. Distribution of serum vitamin D levels (n = 52). The serum concentration of 25-hydroxyvitamin D (25-OHD) recommended in a National position statement for optimal musculoskeletal health is ≥50 nmol/L.[E17]

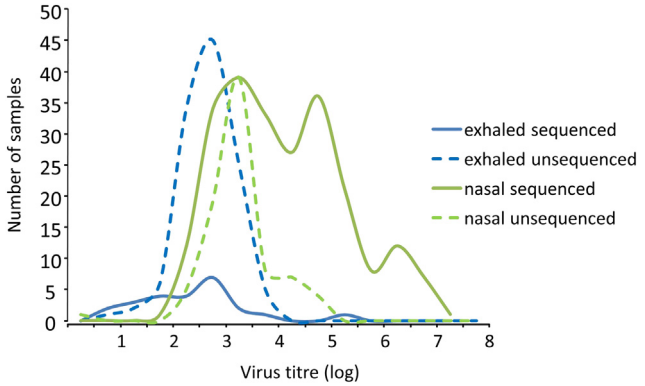

FIG E4. Distribution of hRV titers determined by means of quantitative RT-PCR in nasal and breath samples, which were subsequently able or not able to be genotyped in a nested PCR assay.

TABLE E1. Ethnicity and socioeconomic status of participants (n = 67)

Ethnicity	
White	45
Asian	4
Hispanic	3
Other	2
Missing	13
Socioeconomic status	
1 (most disadvantaged)	5.9%
2	22.4%
3	31.4%
4	19.4%
5 (least disadvantaged)	20.9%

TABLE E2. Distribution of asthma symptom scores among all records in the study and as maximum scores for children for whom 10 weeks of data were collected

	Distribution of all symptom scores in all participants (n = 1206 observations), % samples		Maximum symptom scores reported by children with 10 wk of data (n = 56 participants), % of children with maximum score at each score level	
Scores*	Cough and phlegm	Wheeze and chest tightness	Cough and phlegm	Wheeze and chest tightness
0 = No symptoms	52.9	73.6	13	25
1 = Mild	32.6	15.5	21	20
2 = Moderate	12.7	9.1	43	38
3 = Severe	1.8	1.8	23	18

**0*, No symptoms; *1*, symptoms that did not disturb daily activities or sleep; *2*, symptoms disturb part of daily activities or sleep; *3*, symptoms that disturbed whole or most of the day or sleep.

TABLE E3. Occurrence of non-hRV viruses detected by using multiplex PCR in nasal wash and exhaled breath samples and confirmed by means of enzyme-linked amplicon hybridization assay with specific oligonucleotide probes

Type of virus	Nasal wash samples (n = 1232)	Exhaled breath samples (n = 1231)
Influenza A	4 (0.32%)	0
Influenza B	4 (0.32%)	6 (0.49%)
Respiratory syncytial virus	7 (0.57%)	0
Parainfluenza virus 1	7 (0.57%)	11 (0.89%)
Parainfluenza virus 2	0	2 (0.16%)
Parainfluenza virus 3	5 (0.41%)	0
Human metapneumovirus	1 (0.08%)	0

TABLE E4. ORs for reported outcomes at the time of sampling and with time lags associated with an hRV-positive breath sample

	No lag			Lag of 3-4 d			Lag of 6-8 d		
Outcome	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**
Cough/phlegm	1.12	0.70 to 1.79	.62	1.65	0.92 to 2.98	.94	0.71	0.40 to 1.28	.26
Wheeze/chest tightness	0.79	0.43 to 1.44	.44	1.65	0.84 to 3.27	.15	1.61	0.83- to 3.12	.16
Reliever use	0.90	0.55 to 1.48	.67	1.15	0.63 to 2.13	.65	1.03	0.58 to 1.83	.92
Composite febrile	0.45	0.17 to 1.17	.11	1.84	0.75 to 4.51	.18	1.14	0.45 to 2.87	.78
Composite coryzal	0.75	0.44 to 1.29	.30	1.55	0.76 to 3.17	.23	1.21	0.61 to 2.39	.59
	Difference								
PEF z score	−0.040	−0.22 to 0.14	.66	0.14	−0.080 to 0.35	.22	0.20	0.0097 to 0.40	**.040**
FEV_1 z score	−0.0048	−0.18 to 0.17	.96	−0.04	−0.25 to 0.18	.75	0.22	0.03 to 0.42	**.030**

Only those samples recorded in the period of 3 and 4 days and the period of 6, 7, and 8 days after the initial sample were used in the analysis of lags.
OR, Odds ratio.
Values in boldface are $P < .05$.

TABLE E5. Virus detection as a predictor of asthma score (0-8) in the study population

Virus variables	Estimate ± SE	Probability
Nasal, any virus vs no virus	0.384 ± 0.064	**<.0001**
Nasal, hRV	0.338 ± 0.065	**<.0001**
Nasal, hRV-A vs hRV-B + hRV-C	0.110 ± 0.166	.511
Nasal, hRV-B vs hRV-A + hRV-C	−0.321 ± 0.220	.145
Nasal, hRV-C vs hRV-A + hRV-B	0.086 ± 0.177	.630
Breath, any virus	0.0131 ± 0.091	.886
Breath, hRV	−0.0021 ± 0.100	.983

Values in boldface are $P < .05$.