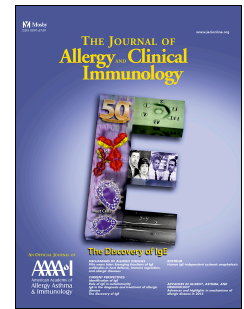


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**Association of Rhinovirus Species with Common Cold and Asthma Symptoms
and Bacterial Pathogens**

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Clinical Implications: Rhinovirus infections may cause clinical symptoms in part by promoting
the proliferation of bacterial pathogens in the airway, suggesting that preventive treatments
might target the bacterial pathogens.

Key words: Rhinovirus, virus, bacteria, asthma common cold, Streptococcus, Moraxella,
Haemophilus

Abbreviations: RV, rhinovirus

To the Editor:

Most exacerbations of asthma in children are initiated by viral infections, most commonly due to rhinoviruses (RV). RV-A and RV-C species are more likely to cause moderate to severe illnesses and wheezing in infants, and exacerbations of asthma leading to hospitalization.^{1, 2} Bacterial pathogens such as *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* have also been linked to acute wheezing illnesses and exacerbations of asthma in young children.^{3, 4} In a prospective study of school-aged children during peak RV season in September ("RhinoGen"), we previously reported that viral infections often precede increased detection of these bacteria.⁵ Furthermore, viral infections with concurrent or subsequent detection of bacterial pathogens were more likely to be associated with symptomatic illnesses and exacerbations of asthma.⁵ These studies suggest that RV-A and RV-C infections could lead to more severe wheezing illnesses by promoting the proliferation of bacterial pathogens.

To test this hypothesis, we obtained weekly nasal mucus samples during both September and April as part of the RhinoGen study, and analyzed them for RV species (RT-PCR and partial sequencing)⁶ and the bacterial pathogens *S. pneumoniae*, *M. catarrhalis* and *H. influenzae catarrhalis* (quantitative PCR; full methods in online supplement).⁵ Children with the help of their parents were instructed to record daily common cold and asthma symptoms, morning peak expiratory flow, and albuterol use on calendars during their enrollment in the study. This study was approved by the University of Wisconsin Human Subjects Committee, written informed consent was obtained from the parents, and written assent obtained from children ages 7 years and older.

The study population consisted of 167 outpatient children with asthma and 143 children without asthma ages 4-12 years (Supplemental Table I and online supplement). Children scored cold

and asthma symptom severity based on a 4-point scoring system (Supplemental Table II),⁵ and moderate asthma exacerbations were defined in accordance with ATS and NHLBI guidelines (see online supplement).

During the two-month monitoring period, there were 755 respiratory illnesses reported in the 310 children. In the 3028 nasal mucus samples, RV was the virus most commonly detected, and RV-A (n=566 solo, 674 total) was detected over twice as often as either RV-B (n=203 solo, 250 total) or RV-C (n=195 solo, 251 total). RV-B was detected more frequently in September, while RV-A and RV-C were detected more often in April (Supplemental Table III). For each RV species, we also determined the number of infections (one or more consecutive weeks with detection of the same viral type, Supplementary Table IV). The mean duration of viral infection varied by species ($p<0.001$); RV-B was longest (mean 2.09 weeks, 95% CI [1.92, 2.25]), followed by RV-A (1.77 weeks [1.68, 1.86]) and then RV-C (1.37 weeks [1.23, 1.50]).

Compared to weeks with no virus detected, weeks that were positive for any RV species had significantly greater cold symptom scores (Figure 1A). Both RV-A and C were associated with increased cold symptoms compared to RV-B ($p<0.02$). Similarly, RV-A and RV-C were associated with greater asthma symptoms (Figure 1B) and a greater probability of exacerbation (Figure 1C) compared to RV-B. Relative to no virus detection, RV-B detection was not associated with increased cold or asthma symptoms or exacerbations of asthma.

Of the 310 participating children, 276 had at least one clinically defined respiratory illness (see online data supplement for criteria) during the study period, with a total of 755 illnesses.

Compared to periods with no virus detected, RV-A, RV-C, and multiple RV were each associated with increased risk for illness (OR = 2.35, 2.61, and 2.41, $p<0.0005$) while RV-B was not (OR = 0.99, $p=0.95$, Supplemental Figure 1).

79

80 We next tested whether detection of bacterial pathogens was differentially related to infection
 81 with specific RV species. *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were detected in
 82 45%, 21% and 19% of samples respectively, and were more often detected in younger children
 83 (Supplemental Figure 2). RV-C was associated with increased detection and quantity of *H.*
 84 *influenzae* (detection: OR 2.36 [1.65, 3.38]; quantity: ordinal OR 2.48 [1.64, 3.75]) and *M.*
 85 *catarrhalis* (OR 1.52 [1.04, 2.22]; ordinal OR 1.52 [1.01, 2.30]), RV-A was associated with
 86 increased *H. influenzae* (OR 2.43 [1.90, 3.10]; ordinal OR 2.60 [1.96, 3.45]) and *S. pneumoniae*
 87 (OR 1.52 [1.23, 1.89]; ordinal OR 1.97 [1.59, 2.43]), while RV-B was not associated with
 88 increased bacterial detection (Fig. 2).

89

90 We previously reported that during sampling of nasal mucus obtained in September, detection
 91 of both RVs and bacteria contributed to the probability of respiratory illness.⁵ In an analysis
 92 including samples from April and September (Supplemental Table V), detection of RV together
 93 with either *M. catarrhalis* (OR 1.79 [1.16, 2.78]) or *H. Influenzae* (OR 1.44 [1.01, 2.05]), but not
 94 *S. pneumoniae* (OR 1.22 [0.78, 1.62]), significantly increased the probability of respiratory
 95 illness compared to infection with RV alone. When detected in the absence of virus, none of the
 96 bacterial pathogens were significantly associated with respiratory illness.

97

98 The results of this study verify that infections with RV-A and RV-C species cause more severe
 99 illnesses and are more likely associated with exacerbations of asthma than infections with RV-
 100 B. We also demonstrated that RV-A and RV-C are more likely than RV-B to be associated with
 101 detection of bacterial respiratory pathogens. Strengths of this study include the large sample
 102 size, inclusion of children with and without asthma, and prospective collection of samples during
 103 periods of illness and health. Limitations include sample collection for only two months out of the
 104 year; however, these months were selected for the highest prevalence of RV infections and

asthma morbidity. In addition, there may be effects of other seasonal factors such as allergens that were not measured in this study.

These findings suggest that RV virulence could be partly related to secondary effects on the overgrowth of bacterial pathogens. This relationship is plausible, as these bacteria can stimulate inflammatory responses that might lead to airway narrowing and acute exacerbations.⁷ Accordingly, two randomized studies have demonstrated that treatment with azithromycin improves outcomes of wheezing illnesses in high-risk preschool children.^{8,9} Mechanisms for these effects are unclear, however, and use of antibiotics for respiratory illnesses could have unintended adverse effects including selecting for drug-resistant organisms, and killing beneficial microbes. Additional studies are needed to test whether other types of treatments (e.g. vaccines, probiotics) can prevent overgrowth of bacterial pathogens during viral respiratory infections and reduce the risk of more severe illnesses and exacerbations of asthma.

ACKNOWLEDGMENTS

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Figure Legends

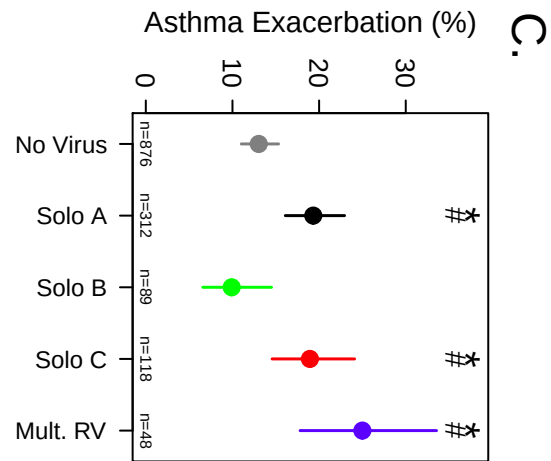
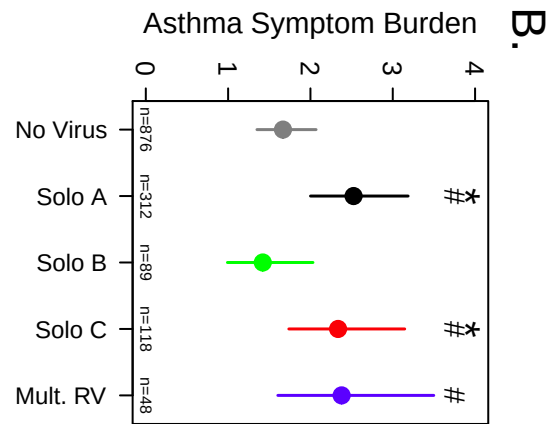
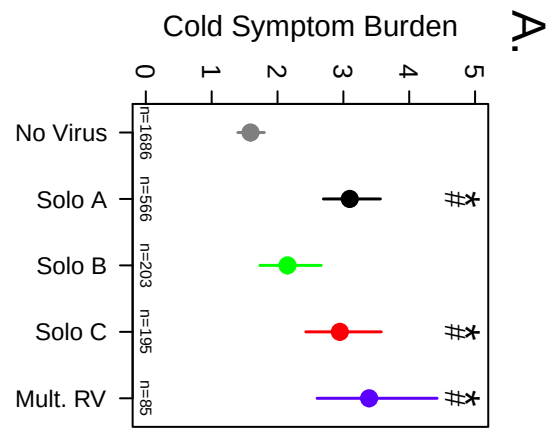
Figure 1. Association of viral detection with clinical outcomes. Detection of RV species in weekly samples were compared to contemporaneous reports of cold symptom scores (A), asthma symptom scores (B), and risk for exacerbation of asthma (C). Points and lines represent means and 95% confidence intervals. * $p < 0.05$ vs no virus; # $p < 0.05$ vs RV-B.

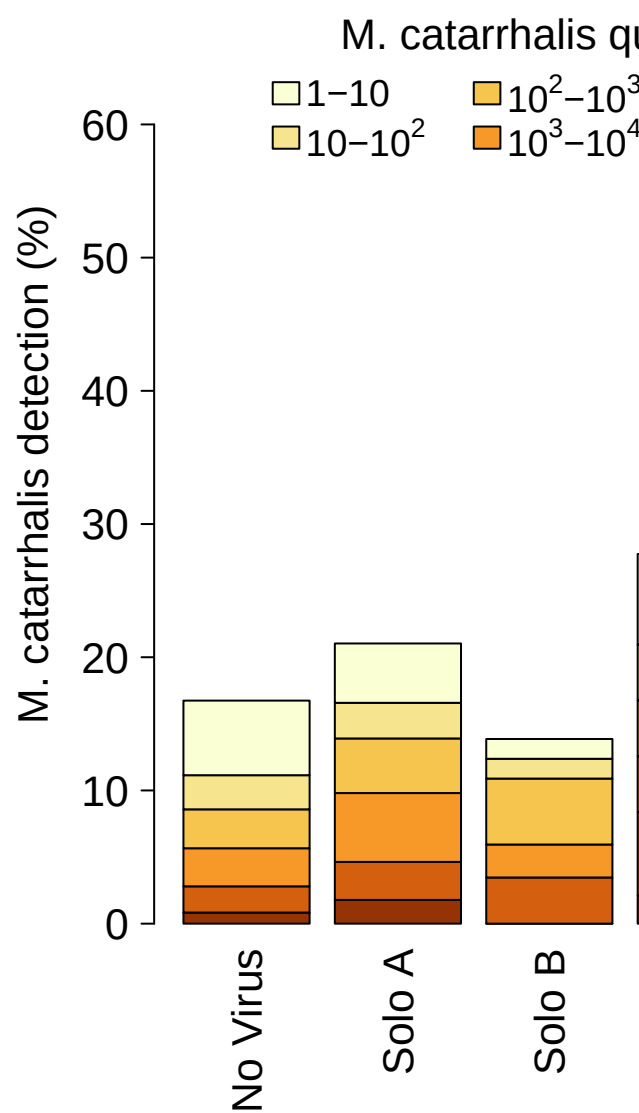
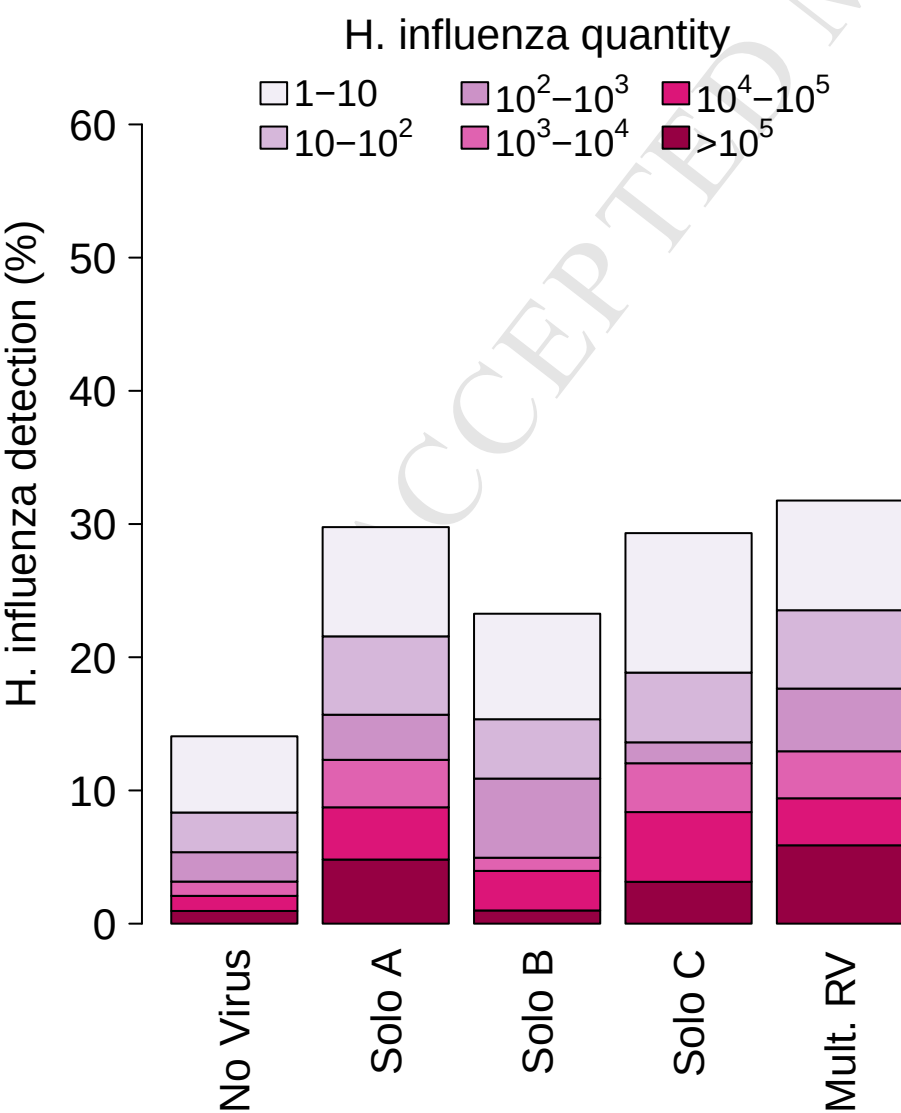
Figure 2. Association of RV species with quantity of bacterial pathogens.

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176





Online Supplement

Association of Rhinovirus Species with Cold and Asthma Symptoms
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Study subjects and design. Children included in this analysis were enrolled to determine asthma-specific rates of RV infections and illness ("RhinoGen"). The study population was recruited from Madison, Wisconsin and surrounding areas via primary care physicians, allergy and asthma specialists and advertisements in the community. Any child with or without asthma, ages 4-12 years, was considered eligible for the study provided they did not have a history of prematurity, respiratory problems at birth or any other significant medical illness. Admission to RhinoGen occurred on a rolling basis between 2007 and 2010.

Nasal samples were collected weekly for a total of 5 weeks during the fall (beginning in September) and 5 weeks in the Spring (beginning in April). The samples were collected using a nasal blow technique, mixed with viral transport medium, and then kept in a home freezer pending pickup by the study team.¹ Children with the help of their parents were instructed to record daily URI and asthma symptoms, morning peak expiratory flow (PEF) and albuterol use on calendars during their enrollment in the study. Of the 478 children and families who expressed interest in the study, 422 subjects passed the initial screening process, provided consent and were enrolled in the study. Reasons for failing the initial screen included not being comfortable with procedures, family issues such as an illness or parental schedules, were outside the set age parameters, were born prematurely, or had a respiratory complication at birth. Children who collected 6 of 8 weekly nasal specimens and were missing less than 20% of diary card data were included in the analyses (n=310).

Detection of bacterial pathogens. Nasal samples from the September samples were analyzed for *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* by multiplexed quantitative real-time PCR as previously described.¹ DNA was extracted using the BiOstic Bacteremia DNA Isolation Kit (Mo Bio laboratories, Carlsbad, California). Samples that tested positive for bacterial DNA on multiplex PCR were retested with single-target qPCRs

specific for each bacteria to exclude false positives (primers and probes listed in Supplemental Table VI). The April samples were tested only with the three single-plex qPCR tests.

Outcomes definitions. Current asthma was defined at the end of the two monitoring periods based on previously reported criteria.¹ Children scored cold and asthma symptom severity based on a 4-point scoring system (Supplemental Table II).¹ Infection was defined as viral detection in the nasal secretions, whether or not symptoms were present. If the same virus type was detected in serial specimens, it was considered a single infection. Illnesses were defined as two or more consecutive days of cold or asthma symptoms of at least mild severity, whether or not a virus was detected. If an illness occurred within three days of a positive viral sample, the infection was designated a viral illness.¹ Moderate asthma exacerbations were defined in accordance with ATS and NHLBI guidelines^{2, 3} to consist of at least moderate asthma symptoms and either a decrease in PEF of at least 20% or use of albuterol ≥ 2 days per week.¹

Statistical analysis. Demographic characteristics were compared by asthma status using the Wilcoxon rank-sum test and chi-square test for association. Factors influencing viral detection rates were examined using multivariate logistic regression models with covariates for season, age, gender, race, atopy, and asthma. Similar models that also included covariates for virus detection were used to examine factors influencing bacterial detection rates. To compare weekly virology data to concurrent symptoms, cold and asthma symptoms from diaries were analyzed in 7 day increments (date of nasal specimen ± 3 days) and symptom scores were summed over this 7-day period. Generalized linear mixed effect models were used to compare cold symptom burdens and asthma symptom burdens (quasi-Poisson regression) and occurrence of asthma exacerbations (logistic regression) among viruses. Illness and exacerbation rates during solo RV infections were analyzed using logistic regression models with covariates for species and duration of infection. To examine the relationships between clinical illnesses and detection of viruses and bacteria, a matching algorithm was developed to

uniquely pair each participant with a second participant who was asymptomatic on the same days that the first subject exhibited respiratory symptoms. Illnesses and matched asymptomatic control periods of the same duration were then analyzed using a matched-pairs conditional logistic regression model. The presence and quantity of bacteria were compared among viruses using mixed effect logistic and ordinal logistic regression models. For all analyses a two-sided p-value < 0.05 was regarded as statistically significant.

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Legends for Supplemental Figures

Supplemental Figure 1. Risk of respiratory illnesses associated with detection of individual or multiple RV species. The OR was calculated as the risk of detecting RV-A, RV-B, RV-C or multiple ("Mult") RV species during a period of illness compared to a period of equal length during which no respiratory illness symptoms were reported.

Supplemental Figure 2. Effects of age on the prevalence of bacterial pathogen detection.

Supplemental Table I. Subject Demographics

	Asthma		P-value
	No (n=143)	Yes (n=167)	
Male	55%	67%	0.03
Age (Years)	8.2 [8.0, 9.2]	8.2 [6.8, 9.4]	0.25
Ethnicity*			
White	95%	88%	0.03
African American	2%	13%	0.0006
Asian	3%	4%	0.96
Native American	3%	1%	0.31
Pacific Islander	1%	1%	0.91
Hispanic	3%	7%	0.08
Maternal Asthma	28%	40%	0.04
Maternal Allergic Disease	54%	60%	0.27
Paternal Asthma	22%	33%	0.05
Paternal Allergic Disease	49%	66%	0.004
Total IgE	39 [18, 148]	129 [37, 384]	<0.0001
Any Aeroallergen Positive sIgE	43%	64%	0.0002
FeNO	8.7 [6.5, 13.5]	13.2 [8.0, 29.2]	<0.0001
PEF Pre-BD	3.45 [2.75, 4.02]	3.53 [2.84, 4.22]	0.87
FEV ₁ Pre-BD	1.67 [1.48, 1.99]	1.75 [1.41, 1.97]	0.28
FEV ₁ /FVC Pre-BD	0.84 [0.80, 0.88]	0.82 [0.77, 0.86]	0.10

*Study subjects were asked to choose one or more categories.
Continuous measures summarized as median [25th, 75th].

113 **Supplemental Table II.** Definition of Common Cold and Asthma Scores¹
 114

Cold Symptoms			Asthma Symptoms
0	Absent	None	None
1	Mild	Mild stuffy or runny nose but does not affect daily activity	Occasional cough or wheeze but does not affect daily activity
2	Moderate	Moderate stuffy or runny nose and reduced activity but does not affect sleep	Frequent cough or wheeze with some shortness of breath and reduced activity but not affecting sleep
3	Severe	Cannot breathe through the nose and not able to sleep well because of symptoms	Unable to sleep well because of symptoms

115

Supplemental Table III. Factors Influencing Detection of RV Species

Predictor	OR (95% CI)		
	RV-A	RV-B	RV-C
April vs. September	1.21 (1.03, 1.42)	0.12 (0.09, 0.15)	1.56 (1.26, 1.92)
Age (1 SD change)	0.84 (0.74, 0.97)	0.84 (0.65, 1.09)	0.72 (0.60, 0.87)
Male gender	0.87 (0.66, 1.15)	1.10 (0.66, 1.86)	1.14 (0.78, 1.66)
Non-Caucasian	1.13 (0.77, 1.65)	0.52 (0.24, 1.15)	1.28 (0.78, 2.12)
Atopy	1.05 (0.79, 1.39)	1.11 (0.65, 1.89)	1.05 (0.71, 1.54)
Asthma	1.05 (0.80, 1.39)	0.70 (0.42, 1.18)	1.28 (0.88, 1.88)

Supplemental Table IV. Numbers of Viral Infections (n, %)*

	Infections		
	Sept	April	Total
RV-A	199 (41%)	208 (46%)	407 (43%)
RV-B	107 (22%)	19 (4%)	126 (13%)
RV-C	80 (16%)	110 (24%)	190 (20%)
Other virus	100 (21%)	113 (25%)	213 (23%)
Total	486	450	936

*Same virus detected in consecutive weeks was considered one infection. Of the 723 total RV infections, 649 were non-overlapping infections with a single RV type.

Supplemental Table V. Effects of Virus, Bacteria, or Both on Risk of Respiratory Illness*

Pathogen	Sept. Only			April Only			Sept and April Combined		
	OR [†]	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value
RV & H flu									
V-B- vs. V-B+	0.99	0.54, 1.82	0.98	1.47	0.83, 2.60	0.19	1.21	0.80, 1.83	0.37
V-B- vs. V+B-	1.77	1.22, 2.56	0.003	2.46	1.58, 3.83	<0.0001	2.04	1.53, 2.70	<0.0001
V-B- vs. V+B+	2.76	1.69, 4.53	<0.0001	3.18	1.83, 5.52	<0.0001	2.93	2.03, 4.23	<0.0001
V+B- vs. V+B+	1.56	0.97, 2.53	0.07	1.29	0.77, 2.17	0.33	1.44	1.01, 2.05	0.04
RV & Morax									
V-B- vs. V-B+	0.90	0.43, 1.87	0.78	1.27	0.72, 2.23	0.41	1.09	0.71, 1.69	0.69
V-B- vs. V+B-	1.81	1.28, 2.56	0.0007	2.49	1.58, 3.91	<0.0001	2.03	1.54, 2.67	<0.0001
V-B- vs. V+B+	4.37	2.23, 8.56	<0.0001	3.28	1.72, 6.27	0.0003	3.64	2.30, 5.76	<0.0001
V+B- vs. V+B+	2.41	1.27, 4.58	0.007	1.32	0.72, 2.43	0.37	1.79	1.16, 2.78	0.009
RV & Strep									
V-B- vs. V-B+	0.69	0.42, 1.11	0.13	1.19	0.69, 2.07	0.53	0.86	0.60, 1.24	0.43
V-B- vs. V+B-	1.48	0.90, 2.46	0.12	2.67	1.54, 4.64	0.0005	1.93	1.34, 2.78	0.0004
V-B- vs. V+B+	1.91	1.25, 2.92	0.003	2.68	1.56, 4.61	0.0004	2.16	1.55, 3.01	<0.0001
V+B- vs. V+B+	1.29	0.76, 2.17	0.34	1.00	0.59, 1.70	0.99	1.12	0.78, 1.62	0.55

*Abbreviations: V, virus; B, bacteria.

[†]OR for A vs. B refers to (odds of B)/(odds of A). This analysis excludes illnesses associated with non-rhinoviruses.

Supplemental Table VI. Sources and Identities of Primers, Probe and Standards

Material	Sequence or Source
<i>Streptococcus pneumoniae</i>⁴	
LytA forward	ACGCAATCTAGCAGATGAAGCA
LytA reverse	TCGTGCGTTTTAATTCCAGCT
LytA TaqMan probe	FAM-TGCCGAAAACGCTTGATACAGGGAG-MGB- NFQ
<i>S. pneumoniae</i> standard	ATCC 49619
<i>Haemophilus influenzae</i>⁵	
hpdF822 forward	GGTTAAATATGCCGATGGTGTG
hpdR952 reverse	TGCATCTTTACGCACGGTGTA
hpdPb896 modified for MGB	VIC-TTACCAACGGAGTGTACACAA-MGB-NFQ
<i>H. Influenzae</i> standard	ATCC 49144
<i>Moraxella catarrhalis</i>⁶	
copB forward	GTGAGTGCCGCTTTTACAACC
copB reverse	TGTATCGCCTGCCAAGACAA
copB TaqMan probe	NED-TGCTTTTGCAGCTGTTAGCCAGCCTAA- MGB-NFQ
<i>M. catarrhalis</i> standard	Patient isolate

