To the Editor:

Rhinoviruses (RV) infect up to 90% of school-age children with asthma during the month of September, and the severity of clinical illness varies from no symptoms to severe wheezing illnesses. (1) We previously reported that detection by PCR of S. pneumoniae or M. catarrhalis in the upper airway is associated with RV-induced asthma exacerbations. (2) Based on these findings, we hypothesized that RV infection alters the upper airway microbiota, and that microbial changes correspond with infection severity. To test this hypothesis, we prospectively monitored respiratory symptoms in children with asthma during the peak fall RV season, obtained weekly nasal secretions, and concurrently analyzed these samples for RVs and airway bacteria.

Children included in this analysis were enrolled in a larger study to determine genetic correlates with severe RV illnesses (“RhinoGen”). Subjects collected nasal mucus samples on a weekly basis for five consecutive weeks during September (peak RV season). All samples were analyzed for common respiratory viruses and RV abundance (qPCR), and RV typing was determined. (3) Cold and asthma symptoms recorded in daily diaries were linked with RV infection data to identify infections that were either asymptomatic or associated with an asthma exacerbation. RV-B types caused 70% of asymptomatic RV infections, while exacerbations were only associated with RV-A or RV-C types. This study included 10 RhinoGen participants with
asymptomatic infections and 7 participants with exacerbations of asthma (Table 1; Supplemental Figure 1; also see supplemental data for inclusion and exclusion criteria). Compared to the other children with asthma in the RhinoGen cohort, the children in the asymptomatic and the exacerbation groups had similar total IgE levels and rates of allergic sensitization (Supplemental Table 1). 16S rRNA gene sequencing was performed on bacterial DNA isolated from each sample and statistical analysis was performed to identify bacterial taxa associated with viral infection and RV-associated asthma exacerbations (see Online Repository).

Within the 34 samples before and after RV infection, the dominant phyla detected were Firmicutes (50.3%); Proteobacteria (24.9%); Actinobacteria (17%); Bacteroidetes (4.3%); Fusobacteria (1.5%); and unclassified (1.1%). The most abundant genera were Dolosigranulum (12.2% total abundance); Streptococcus (11.3%); Staphylococcus (10.1%); Corynebacterium (9.7%); Moraxella (7.2%); unclassified OTU #1 (5.6%); unclassified OTU #2 (3.1%); Neisseria (3%); Gemella (2.2%); Rothia (1.9%); Actinomyces (1.6%); Haemophilus (1.4%); Acinetobacter (1.4%) and unclassified OTU #3 (1.1%). We then compared the RV-negative and RV-positive samples, and found a similar number of overall sequences before and after RV infection (p=0.95); and similar evenness and diversity. Furthermore, using principal component analysis (PCoA) of the Unifrac and Bray-Curtis distance matrices, there were no distinct clustering patterns between the two groups, suggesting that the overall community composition of the RV-negative and RV-positive samples were similar.

In the combined asymptomatic and exacerbation groups, RV infection was associated with several significant changes in specific genera in airway secretions (Supplemental Figure 2).
RV infection was associated with increased abundance of *Dolosigranulum* (base mean=213; log$_2$ fold change=0.60) and *Moraxella* (base mean=116; log$_2$ fold change=0.79), and reduced abundance of unclassified OTU #1 (base mean=209; log$_2$ fold change=2.54). These findings support our previous report based on PCR detection that RV infection increases *Moraxella* detection,(2) and indicate that RV infection also influences microbial community composition.

We next tested whether microbial changes during RV infection differed between asymptomatic RV infections and RV-associated asthma exacerbations (Figure 1 and Supplemental Figure 3). RV infection was associated with increased abundance of *Moraxella* in both groups (asymptomatic group: base mean=175; log$_2$ fold change=1.04; and exacerbation group: base mean=158; log$_2$ fold change=0.9), and reduced abundance of unclassified OTU#1 (asymptomatic group: base mean=269; log$_2$ fold change=-3.6; and exacerbation group: base mean=123; log$_2$ fold change=-1.12). Interestingly, RV associations with the abundance of some bacterial OTUs depended on the symptom group. Namely, within the asymptomatic group, RV infection was associated with increased abundance of *Corynebacterium* (base mean=196; log$_2$ fold change=0.48), while in the exacerbation group the association was in the opposite direction (base mean=212; log$_2$ fold change=-0.45). RV infection was also associated with increased abundance of *Dolosigranulum* in the asymptomatic group (base mean=175; log$_2$ fold change=1.04).

An association network constructed to link RV quantity with the presence of specific OTUs of bacteria demonstrated that as the quantity of RV increased, the abundance of *Dolosigranulum* and *Corynebacterium* decreased while the abundance of *Haemophilus* increased.
(Supplemental figure 4). Furthermore, there were both increases and decreases of OTUs belonging to *Streptococcus* and *Moraxella*, indicating that the amount of RV replication is related to the magnitude of composition changes in the microbiome.(4)

Asymptomatic RV infections were associated with a significant increase in the abundance of *Dolosigranulum* and *Corynebacterium* compared to pre-infection samples, and the quantities of these bacteria were inversely correlated with viral shedding. *Dolosigranulum* and *Corynebacterium* are commensal bacteria within the respiratory tract in children and commonly co-occur.(5) They both are negatively associated with *S. pneumoniae* abundance, have been associated with reduced airway symptoms and a lower risk of otitis media during infancy,(6) and are inversely related to episodes of wheeze during infancy.(7) Our findings extend these findings and suggest that microbial communities featuring abundant *Corynebacterium* and possibly *Dolosigranulum* may confer protection against symptoms during RV infection.

This study has a number of advantages, and some limitations. The prospective study design allowed us to obtain samples from the same subject prior to and during RV infection. Samples were obtained during the same season, eliminating seasonal influences on microbial composition. Our findings are based on samples obtained from the upper airway for practical reasons. RV infections begin in the upper respiratory tract and thus the microbial environment in the upper airway is likely to influence initiation of RV infection and downstream events. Therefore investigations of the upper airway may identify new strategies for prevention and/or treatment of RV-induced exacerbations. Our results should be interpreted with caution due to the
small sample size, and the observational study design cannot distinguish causality among the observed associations between bacteria, viruses and symptoms.

In summary, RV infection is associated with changes in microbial composition of the upper airway. These changes differed between asymptomatic infection and exacerbation of asthma, and were related to RV quantity and possibly RV species. While RV infection was generally related to increased abundance of *Moraxella*, a well-known airway pathogen; RV was related to increased commensal bacteria (*Dolosigranulum* and *Corynebacterium*) during asymptomatic infection. Finally, while bacterial pathogens such as *Moraxella* can contribute to respiratory symptoms, our findings suggest that other microbial communities may help to maintain normal airway physiology during RV infection and thereby moderate or prevent respiratory symptoms. Addressing these gaps in knowledge may lead to new preventive strategies for RV illnesses and virus-induced exacerbations of asthma.

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Table I: Paired samples for analysis: 34 samples (17 pairs). Within each pair, the first sample was RV negative, and a second sample obtained 1-3 weeks later was RV positive.

<table>
<thead>
<tr>
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<th>Asymptomatic during RV infection</th>
<th>Moderate Asthma Exacerbation during RV infection</th>
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<tr>
<td>RV-negative</td>
<td>10 samples</td>
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</tr>
<tr>
<td>RV-positive</td>
<td>10 samples</td>
<td>7 samples</td>
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</table>
Figure 1: Relative abundance at the Genera level (OTUs >1%) between RV-negative and RV-positive samples in the asymptomatic group (A) and asthma exacerbation group (B).

Asymptomatic group: *Dolosigranulum*qt-value=1.4x10^{-8}; *Corynebacterium*qt-value=1.5x10^{-111}; *Moraxella*qt-value=1.4x10^{-8}; unclassified OTU#1qt-value=0.

Exacerbation group: *Corynebacterium*qt-value=7.8x10^{-25}; *Moraxella*qt-value=9.8x10^{-47}; unclassified OTU#1qt-value=5.9x10^{-50}.

Capsule summary: In school-age children with asthma, RV infection changes the upper airway microbiome and these changes are associated with symptom severity and viral load.

Key Words: Rhinovirus; microbiome; asthma; pediatric; bacteria.
References


Figure 1

A

Asymptomatic Group

B

Asthma Exacerbation Group

% Abundance

* RV-negative  RV-positive

% Abundance

* RV-negative  RV-positive

Bacteria:
- Dohertyella
- Stephanococcus
- Syphyllocecarus
- Crevibacterium
- Moraxella
- unclassified
- Neisseria
- Gemella
- Rothia
- Actinomycetes
- Actinobacter
- Hemophilus
- unclassified

Legend:
- RV-negative
- RV-positive
Community Acquired Rhinovirus Infection Is Associated With Changes in the Airway Microbiome

Online Data Supplement

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**Supplemental Methods**

### Recruitment and Inclusion/Exclusion Criteria

The study population was recruited from the general population in Madison, Wisconsin and surrounding areas via primary care physicians, allergy and asthma specialists and advertisements in the community. The study was designed to be as inclusive as possible to reflect the general population. 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, complications at birth, respiratory problems at birth or any other significant medical illness.

A subset of RhinoGen subjects were included in this pilot study based on the following criteria: 1) physician diagnosis of asthma per NHLBI and ATS criteria (1, 2); 2) the initial specimen tested negative for virus with an absence of cold and/or asthma symptoms for seven days prior to and four days following specimen collection; 3) the follow-up specimen tested positive for rhinovirus (and no other virus) and was the first viral infection since the initial specimen was collected; 4) the follow-up specimen was associated with either an absence of cold and asthma symptoms, or with an asthma exacerbation; and 4) enough sample remained for microbial analysis. Of the 310 eligible subjects, 29 met the above criteria, including 8 subjects who experienced an RV-associated asthma exacerbation (Supplemental Figure 1). From the 21 subjects who experienced asymptomatic infections, we randomly selected 10 subjects for analysis. Of note, one subject in the asthma exacerbation group was eliminated during analysis due to insufficient DNA detection during sample processing.

### Symptom scoring and asthma diagnosis

Children scored cold and asthma symptom severity based on a 4-point scoring system (supplemental Table 2) (3, 4). Moderate asthma exacerbations were defined as at least moderate
asthma symptoms (score $\geq 2$) and either a decrease in PEF of at least 20% or increased use of albuterol $\geq 2$ days, in accordance with NHLBI and ATS definitions. Current asthma was diagnosed at study completion based on the above criteria.

The asthma status of each participant was reported by their parent upon enrollment. Then, in the main RhinoGen study, we followed asthma symptoms and treatment over one year to confirm asthma status. Current asthma was diagnosed at the end of the study period based on the documented presence of one or more of the following characteristics in the previous year: (1) use of albuterol for coughing or wheezing episodes (prescribed by physician), (2) use of a daily controller medication, (3) step-up plan including use of albuterol or short-term use of inhaled corticosteroids during illness, (4) use of prednisone for asthma exacerbation, and (5) reversibility of pulmonary function tests after administration of a short-acting beta-agonist. Two separate investigators, blinded to any antecedent histories concerning viral illnesses or patterns of aeroallergen sensitization, independently evaluated each subject for the presence or absence of asthma based on the above criteria.

Sample Analysis

DNA was extracted from nasal samples using the BiOstic Bacteremia DNA Isolation Kit (Mo BIO laboratories, Carlsbad, California). Specimens were multiplexed using the 515f/806r primer set that amplifies the V4–V5 region of the 16S rRNA gene. The primers contain the appropriate Illumina adapters and the reverse primer contains a 12-base error-correcting barcode unique to each sample. DNA was amplified in triplicate PCR reactions using TaKaRa ExTaq enzyme mixture (Clontech). The PCR protocol was: 1 cycle of 10 minutes at 95° C followed by 30 cycles of 95° C for 30 seconds, 55° C for 1 minute, 72 °C for 1 minute and a final elongation at 72° C for 10 minutes. The resulting amplicons were purified with
UltraClean PCR Clean-Up Kit (MO BIO) and the triplicate reactions were pooled together in equimolar concentrations (7).

Sequencing was performed on an Illumina MiSeq (5). The resulting sequence reads were de-multiplexed using CASAVA software installed on the MiSeq Illumina sequencer producing 6,042,668 sequencing tags. Separate pairs of fastq files were generated for each specimen. The splicing of forward and reverse fastq files produced an average of 100,710 ± 48,567 tags per specimen.

**Sequence Quality Analysis**

16S rRNA sequence processing and analysis was performed utilizing Mothur (v.1.33.3) software (9, 10). Raw paired-end fastq sequences of each sample were combined into contigs using make.contigs from the Mothur package which scans across the alignment and identifies any positions where the two reads disagree. To improve the quality of our data we excluded the following: 1) bases with quality score less than 25; 2) sequences with ambiguous bases; 3) sequences with a read length longer than 275 bp; and 4) duplicated sequences. SILVA-based bacterial reference alignment (release 119) was used to align the processed reads (11). Maximum homopolymer length was set to 8 and the gap characters in alignment were removed to improve the overall alignment quality. Within the Mothur package, we used the UCHIME algorithm to detect and remove chimera sequences.

**Operational Taxonomic Unit (OTU) clustering**

For fragment quality control, we trimmed off both the undesirable 18s fragments, and the 16s fragments from Archaea, chloroplasts, and mitochondria. Using the dist.seqs command, uncorrected pairwise distances between aligned DNA sequences were calculated and stored in the column formatted distance matrix. To assign sequences to respective OTUs, clustering was
performed using the average neighbor method at a 99% identity cut-off level. Finally, taxonomical classification for each OTU was obtained by using the classify.seqs command within the Mothur software package (10).

**Sequence Analysis**

Rarefaction curves describing the number of OTUs observed as a function of sampling effort were generated using the sobs calculator in Mothur. Random sub-sampling was performed to address concerns of different sequencing depths across samples, affecting the rarefaction curves. To calculate significance between pre and post infection, Pearson's Chi-squared test was used. Finally, Shannon diversity and evenness and Simpson diversity and evenness indices were calculated from the sub-sampled OTU abundance data.

To identify if the presence of OTUs differed significantly between the subject groups, Fisher’s exact test was performed. The Unifrac and Bray-Curtis distances were calculated between the community structures of the RV subjects for variation analysis. Principal coordinates (PCoA), which employs an eigenvector-based approach, was performed with the Mothur package to represent the multidimensional data of OTU abundance in three dimensions. Species-axes correlations were obtained by using the corr.axes command with the Mothur package.

**Rhinovirus abundance and microbial association analysis**

For association analysis, individual OTUs were assigned to the lowest available taxonomy of bacteria, and OTUs not present in at least 4 samples were not included. Next, both negative (Spearman's $\rho < -0.5$, P-value < 0.05) and positive (Spearman's $\rho > 0.5$, P-value < 0.05) Spearman rank-order correlations were calculated between OTU abundance and RV abundance.
Supplemental Table I: Demographics between subjects included in this study and the other RhinoGen participants with asthma. Race/ethnicity: subjects may select more than one category.

<table>
<thead>
<tr>
<th></th>
<th>Children with Asymptomatic RV Infection</th>
<th>Children with RV-Induced Exacerbation of Asthma</th>
<th>Other RhinoGen Participants with Asthma</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Number of subjects</td>
<td>10</td>
<td>7</td>
<td>150</td>
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</tr>
<tr>
<td>Age (y)</td>
<td>8.0 [8.0, 8.7]</td>
<td>6.8 [5.8, 8.1]</td>
<td>8.4 [6.8, 9.6]</td>
<td>0.23</td>
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<td>Gender</td>
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<td>1 F, 6 M</td>
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<td>0.45</td>
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<td>Aeroallergen sensitization</td>
<td>70%</td>
<td>57%</td>
<td>61%</td>
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<tr>
<td>Asthma</td>
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<td>Total IgE</td>
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<td>146 [96, 262]</td>
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<td>0.91</td>
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Supplemental Table II. Definition of Cold and Asthma Scores

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<td>Absent</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Mild</td>
<td>Occasional cough or wheeze but does not affect daily activity</td>
</tr>
<tr>
<td></td>
<td>Mild stuffy or runny nose but does not affect daily activity</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
<td>Frequent cough or wheeze with some shortness of breath and reduced activity but not affecting sleep</td>
</tr>
<tr>
<td></td>
<td>Moderate stuffy or runny nose and reduced activity but does not affect sleep</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Severe</td>
<td>Unable to sleep well because of symptoms</td>
</tr>
<tr>
<td></td>
<td>Cannot breathe through the nose and not able to sleep well because of symptoms</td>
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Supplemental Figure 1: Subject Inclusion

Supplemental Figure 2: Relative abundance at the Phylum and Genera level between RV-negative and RV-positive samples. Firmicutes $q$-value=$7.62 \times 10^{-6}$; *Dolosigranulum* $q$-value=$1.13 \times 10^{-8}$; *Moraxella* $q$-value=$5.5 \times 10^{-7}$; and unclassified OTU #1 $q$-value=$1 \times 10^{-24}$.

Supplemental Figure 3: Microbial composition of individual samples. First bar in each pair is uninfected, second bar is RV infected.

Supplemental Figure 4: Association networks to examine if a relationship exists between viral load and bacterial abundance. Each line represents an OTU. Green line = increase in bacterial abundance as viral load increases. Red line = decrease in abundance as viral load increases. Size of circle represents the number of sequences associated with that OTU. Node color represents the phyla associated with that OTU. Increasing viral load is associated with decreases in *Dolosigranulum*, *Corynebacterium*, *Prevotella*, *Actinomyces* and some OTUs of *Streptococcus* and *Moraxella*. However, increased viral shedding is also associated with increases in *Haemophilus* and other OTUs of *Streptococcus* and *Moraxella*. Readers should note the following: 1) the position of each node in the network is user-defined, and 2) the structure of the network does not represent any biological functions.
References


Supplemental Figure 1

310 subjects enrolled in RhinoGen

- 143 did not have physician diagnosis of asthma
- 106 subjects had virus detected in their initial sample
- 22 subjects did not have RV infection during study
- 8 subjects reported only mild symptoms during RV infection
- 2 subjects did not have enough sample remaining to perform the study

29 subjects fit study criteria

- 8 subjects had an asthma exacerbation during RV infection
  - 7 subjects had sufficient DNA for microbial analysis
- 21 subjects had an asymptomatic RV infection
  - 10 subjects randomly selected for comparison group
Phyla level analysis: RV-negative versus RV-positive

Genera Analysis: RV-negative vs. RV-positive
Paired RV-negative and RV-positive sample from each subject
### Table: Comparison of Children with Asymptomatic RV Infection, Children with RV-Induced Exacerbation of Asthma, and Other RhinoGen Participants with Asthma

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<td>Unable to sleep well because of symptoms</td>
<td></td>
</tr>
</tbody>
</table>