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 ≥ 2) and either a decrease in PEF of at least 20% or increased use of albuterol ≥ 2 days, in accordance with NHLBI and ATS definitions.(1, 2) 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 (5, 6). The primers contain the appropriate Illumina adapters and the reverse primer contains a 12-base error-correcting barcode unique to each sample (7). DNA was amplified in triplicate PCR reactions using TaKaRa ExTaq enzyme mixture (Clontech). The PCR protocol was: 1 cycle of 10 minutes at 95^{o} C followed by 30 cycles of 95^{o} C for 30 seconds, 55^{o} C for 1 minute, 72 ^{o}C for 1 minute and a final elongation at 72^{o} C for 10 minutes (8). 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.*

	Children with Asymptomatic RV Infection	Children with RV-Induced Exacerbation of Asthma	Other RhinoGen Participants with Asthma	P-value
Number of subjects	10	7	150	
Age (y)	8.0 [8.0, 8.7]	6.8 [5.8, 8.1]	8.4 [6.8, 9.6]	0.23
Gender	2 F, 8 M	1 F, 6 M	52 F, 98 M	0.45
Race/ethnicity:				
White	100%	100%	87%	0.58
Black	0%	14%	13%	0.70
Hispanic or Latino	10%	0%	7%	0.74
Asian	0%	0%	4%	1.00
American Indian or Alaskan native	0%	0%	1%	1.00
Other	0%	0%	2%	1.00
Pacific Islander or Hawaiian	0%	0%	1%	1.00
Aeroallergen sensitization	70%	57%	61%	0.85
Asthma	100%	100%	100%	NA
FeNO	8.3 [7.4, 30.7]	19.2 [8.5, 41.6]	13.8 [8.0, 26.2]	0.87
Total IgE	318 [32, 497]	146 [96, 262]	125 [37, 388]	0.91

Supplemental Table II. Definition of Cold and Asthma Scores

		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

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=7.62×10^{-6}; *Dolosigranulum q*-value=1.13×10^{-8}; *Moraxella q*-value=5.5×10^{-7}; and unclassified OTU #1 *q*-value=1×10^{-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.

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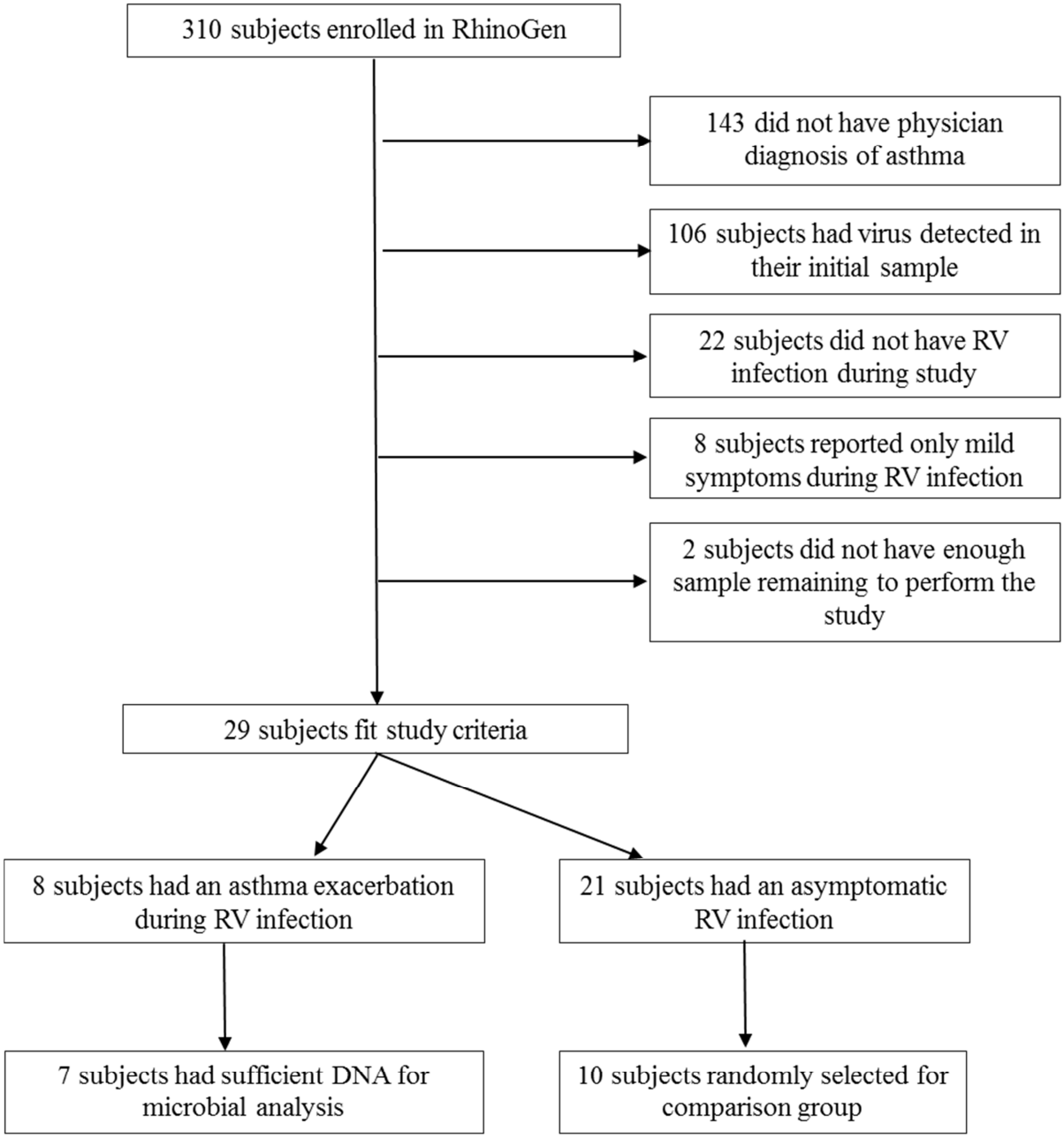
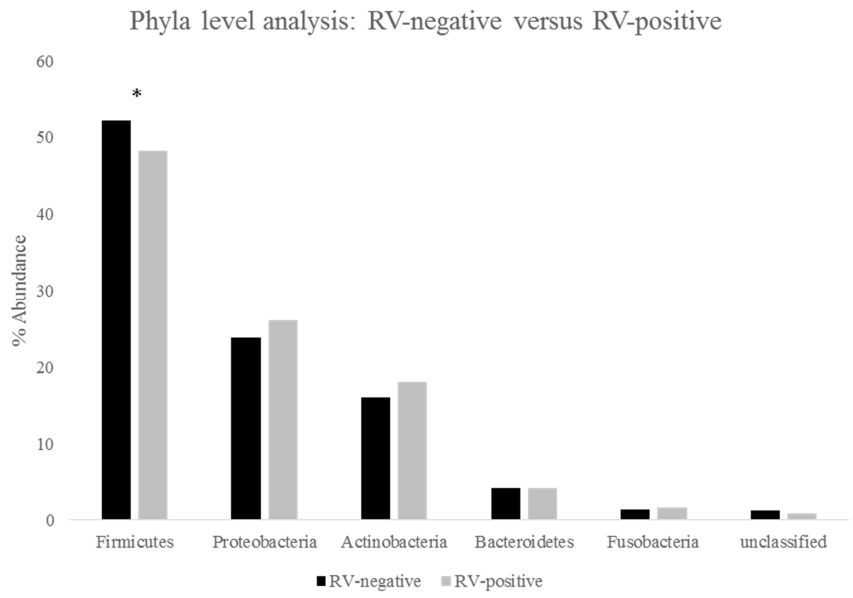
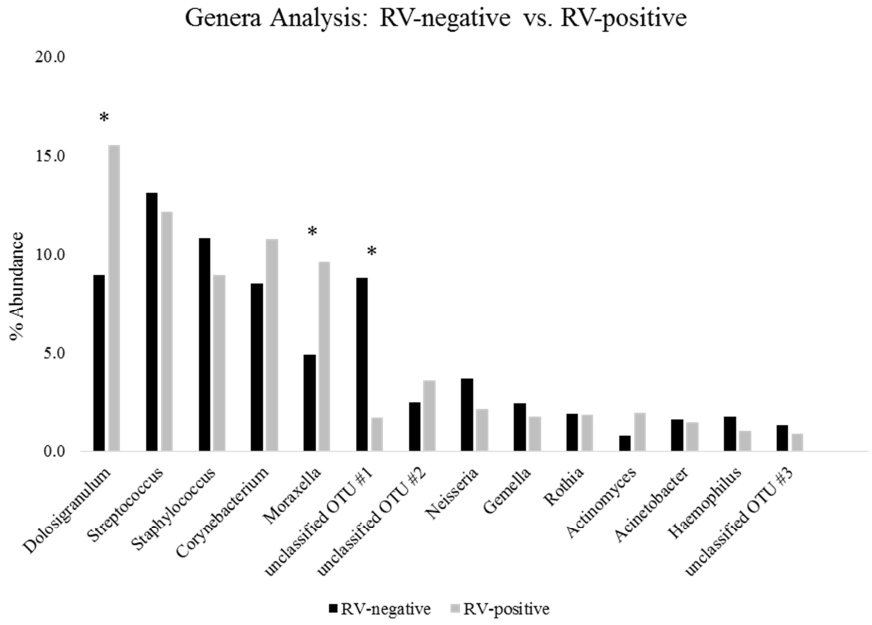
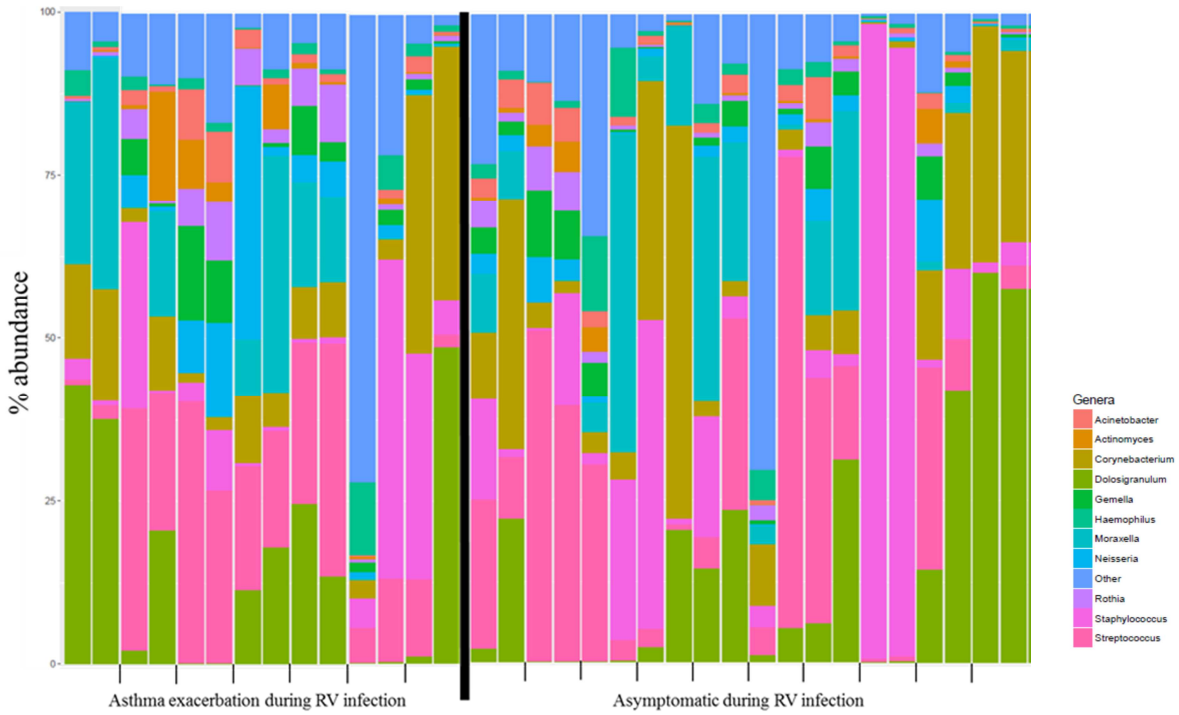
Supplemental Figure 1

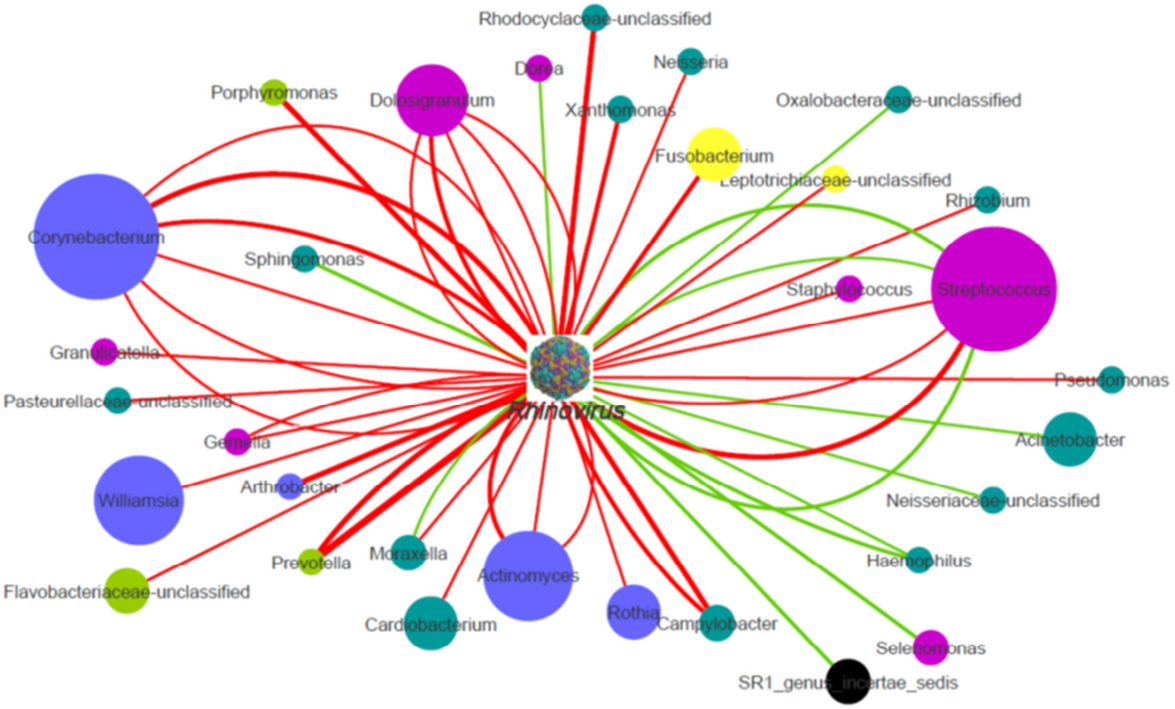



Phyla level analysis: RV-negative versus RV-positive
60
50
40
30
20
10
0
% Abundance
*
Firmicutes
Proteobacteria
Actinobacteria
Bacteroidetes
Fusobacteria
unclassified
RV-negative
RV-positive


Genera Analysis: RV-negative vs. RV-positive
20.0
15.0
10.0
5.0
0.0
% Abundance
*
*
*
Dolosigranulum
Streptococcus
Staphylococcus
Corynebacterium
Moraxella
unclassified OTU #1
unclassified OTU #2
Neisseria
Gemella
Rothia
Actinomyces
Acinetobacter
Haemophilus
unclassified OTU #3
RV-negative
RV-positive



Paired RV-negative and RV-positive sample from each subject


Rhodocyclaceae-unclassified
Dorea
Neisseria
Porphyromonas
Dolosigranulum
Xanthomonas
Oxalobacteraceae-unclassified
Fusobacterium
Leptotrichiaceae-unclassified
Rhizobium
Corynebacterium
Sphingomonas
Staphylococcus
Streptococcus
Granulicatella
Pseudomonas
Pasteurellaceae-unclassified
Rhinovirus
Gemella
Acinetobacter
Williamsia
Arthrobacter
Neisseriaceae-unclassified
Moraxella
Prevotella
Haemophilus
Actinomyces
Flavobacteriaceae-unclassified
Rothia
Campylobacter
Cardiobacterium
Selenomonas
SR1_genus_incertae_sedis

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