Letter to the Editor

Nasopharyngeal Proteobacteria are associated with viral etiology and acute wheezing in children with severe bronchiolitis

To the Editor:

Bronchiolitis is the leading cause of hospitalization for US infants[1] and is usually caused by respiratory syncytial virus (RSV) or human rhinovirus (HRV).[2] These early-life viral infections are associated with the development of recurrent wheezing and asthma.[3] While the primary focus of research on the development of wheezing and asthma has been on viruses, there is increasing evidence that bacteria also play a role in asthma pathogenesis.[4]

Bisgaard et al,[4] in a prospective study of 321 healthy neonates, found an increased risk of recurrent wheezing and asthma in infants who had hypopharyngeal bacterial colonization by *Streptococcus pneumoniae*, *Haemophilus influenzae*, or *Moraxella catarrhalis*. Moreover, Hilty et al[5] found that the pathogenic Proteobacteria phylum (eg, *Haemophilus* and *Moraxella* species) was significantly more common in children with asthma than in controls. It is unknown, however, whether specific bacteria colonizing the respiratory tract (ie, respiratory microbiome) contribute to recurrent wheezing and the development of asthma in children with severe bronchiolitis (eg, children hospitalized with bronchiolitis).

Because children with severe bronchiolitis are at high risk for later recurrent wheezing and asthma,[3] we examined whether these children would have similar microbial perturbations as those observed in older children with asthma.[5] As part of a prospective, multicenter, multiyear study of more than 2000 children hospitalized with bronchiolitis, we used 16S rRNA gene pyrosequencing to analyze 100 nasopharyngeal aspirates (NPAs) from children younger than 2 years hospitalized with bronchiolitis at a participating hospital. Because we did not have healthy controls, we hypothesized that the Proteobacteria phylum would be associated with the viral etiology of the child's bronchiolitis (RSV, HRV, both) and with acute wheezing status (present/absent). Detailed methods, including study inclusion/exclusion criteria and patients' demographic characteristics, and Table E1 may be found in this article's Online Repository at www.jacionline.org.

Site teams gathered detailed clinical data, collected NPAs, and performed short-term patient follow-up.[2] As described previously,[2] every child in this cohort had an NPA tested for 16 viruses by real-time RT-PCR.[2] 16S rRNA gene (n = 100) and whole genome shotgun (n = 10) sequencing and analysis were performed on bacterial DNA isolated from each sample (see details in the Online Repository). Statistical analyses and supervised machine learning (see Online Repository for more details) were used to identify bacterial taxa associated with viral etiology and acute wheezing status. Covariates examined but not associated with microbiome differences were age, exposure to cigarette smoke, antibiotic treatment, and history of breast-feeding (data not shown). In addition, restricting the following analyses to children with a more stringent definition of bronchiolitis (eg, age <1 year and no history of wheezing) did not materially change the results (data not shown).

We found that an increase in *H influenzae* and *M catarrhalis* discriminated between children with RSV/HRV coinfection and those children with a single virus infection (Table I). The RSV/HRV coinfection finding is of interest given that in a separate multivariable analysis from the more than 2000 children in this cohort, those with RSV/HRV coinfection had a significantly longer length of stay than did children with RSV-only infections.[2] In whole genome shotgun analysis, *H influenzae* was also detected in most RSV-only infected and coinfected samples, but not in samples from children infected with HRV only. These results suggest that in the context of bronchiolitis, it is possible that specific viruses may promote the presence of specific bacterial species or vice versa. Indeed, respiratory viruses disturb the respiratory epithelium, allowing for greater bacterial adherence, and possibly increase the chances of a secondary bacterial infection.[6] Interestingly, the opposite (ie, colonizing bacteria predisposing to viral disease) may also be true because specific bacteria may increase the chance of viral infection.[7] Therefore, a virus-only or bacteria-only approach to respiratory conditions may be too simplistic. And more studies with a larger number of samples are needed to confirm these results and determine whether and how bacterial species interact with RSV and HRV or, alternatively, whether the presence of specific bacterial species is a sign of a more global susceptibility state.

Another means of confirming the potential importance of Proteobacteria in severe bronchiolitis is to examine whether perturbations in the microbiome are associated with acute wheezing at admission. Wheezing is not required to diagnose a child with bronchiolitis,[8] but infants who wheeze at certain times of the year are more likely to develop asthma.[3] In the present

TABLE I. Taxa that discriminate between viral etiology of children with severe bronchiolitis

Operational taxonomic unit ID no.	Taxonomic classification	Change
RSV only vs HRV only		
122	*Porphyromonas*	Increased in HRV only
350	*Prevotella*	Increased in HRV only
1337	*Prevotella*	Increased in HRV only
232	*Fusobacterium*	Increased in HRV only
297	*Leptotrichia*	Increased in HRV only
Single (RSV or HRV) vs coinfection		
1878	*Haemophilus*	Increased in coinfection
1558	*Haemophilus*	Increased in coinfection
1944	*Actinomyces*	Increased in coinfection
RSV only vs coinfection		
1878	*Haemophilus*	Increased in coinfection
1558	*Haemophilus*	Increased in coinfection
1337	*Prevotella*	Increased in coinfection
350	*Prevotella*	Increased in coinfection
HRV only vs coinfection		
1878	*Haemophilus*	Increased in coinfection
1558	*Haemophilus*	Increased in coinfection
213	*Propionibacterium*	Increased in HRV only
252	*Moraxella*	Increased in coinfection
Acute wheeze on admission vs absence of acute wheeze on admission		
122	*Porphyromonas*	Increased in acute wheeze
350	*Prevotella*	Increased in acute wheeze
1558	*Haemophilus*	Increased in acute wheeze
296	*Moraxella*	Increased in acute wheeze
834	*Staphylococcus*	Increased in no acute wheeze
240	*Staphylococcus*	Increased in no acute wheeze

Taxa classified to the genus level (operational taxonomic unit ID no. and taxonomic classification) that were identified with randomForest and confirmed with the Boruta for feature selection to discriminate between each comparison group of interest.

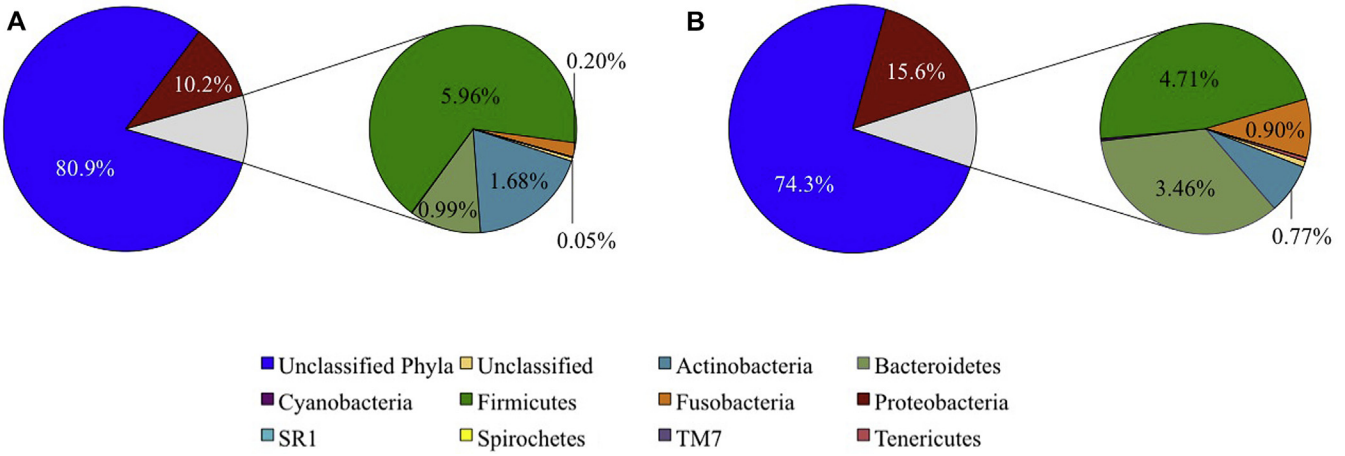

FIG 1. Average abundances of phyla present by patient wheeze status. Exploded pie charts illustrate the abundance (as a percentage) of phyla present across all samples in patients without wheeze (n = 36) **(A)** and patients with wheeze (n = 59) **(B)** on admission.

study, children who wheezed on admission had an insignificant increase in the mean relative abundance of Proteobacteria (15.6% vs 10.2%, $P = .58$) than did children without wheezing (Fig 1). Examining this phyla at the genus level demonstrated that *Moraxella* were significantly more common in children who were wheezing on admission than in those who were not wheezing (10.3% vs 3.64%, $P = .009$; Table I; also see Table E2 in Online Repository at www.jacionline.org).

Data regarding the association of Proteobacteria with wheezing and asthma seem to be accumulating. Directly building on the data from Bisgaard et al[4] and Hilty et al,[5] Marri et al[9] found that Proteobacteria were present in higher proportions of adults with mild asthma than in controls (37% vs 15%, $P < .001$). Our data also support the hypothesis that Proteobacteria may play a role in wheezing respiratory illnesses.

We believe that this study is the first to examine the nasopharyngeal microbiome in children with severe bronchiolitis. Our data suggest that the microbial perturbations previously reported in children with asthma extend to younger children with severe bronchiolitis. Of particular interest are the Proteobacteria, specifically *H influenzae* and *M catarrhalis*, because our data show that Proteobacteria were associated with RSV and RSV/HRV infections and acute wheezing. However, studies following children with severe bronchiolitis until the development of recurrent wheezing or asthma are necessary to fully understand the relationship between the microbiome, the viral causes of bronchiolitis, and the risk of developing wheezing respiratory illnesses. As future respiratory microbiome studies are conducted, researchers will need to address the challenges of sampling the lung microbiome by including both upper and lower airway samples.[10] Further studies in this area may support and inform the development of new therapeutic strategies, including probiotics, for children with severe bronchiolitis to prevent the development of recurrent wheezing and childhood asthma.

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METHODS

Study design

One hundred NPAs were analyzed from a hospital participating in a 3-year, prospective cohort study that was part of the Multicenter Airway Research Collaboration, a program of the Emergency Medicine Network (www.emnet-usa.org). Inclusion criteria were an attending physician's diagnosis of bronchiolitis among hospitalized children younger than 2 years and the ability of the parent/guardian to give informed consent. The exclusion criterion was previous enrollment. All patients were treated at the discretion of the treating physician. The institutional review board at each participating hospital approved the study.

Sample processing and 16S pyrosequencing

Consistent with protocols benchmarked as part of the National Institutes of Health Human Microbiome Project, bacterial genomic DNA was extracted from nasopharyngeal samples by using the PowerSoil DNA Isolation Kit (MoBio, Carlsbad, Calif). The V3-V5 hypervariable regions of the 16S rRNA gene were amplified from genomic DNA by using primer 357F (5′-CCTACGGGAGGCAGCAG-3′) and barcoded primer 926R (5′-CCGTCAATTCMTTTRAGT-3′). The forward primer also contained 454 B Adaptor sequence (5′- CCTATCCCCTGTGTGCCTTGGCAGTCT CAG-3′), and the reverse primer contained 454 A Adaptor sequence (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′) for 454 Titanium pyrosequencing, which was performed in a multiplexed run at the Human Genome Sequencing Center at Baylor College of Medicine (Houston, Tex).

Sequence processing and analysis with QIIME

Sequence processing and analysis was performed with QIIME version 1.5. Sequences were demultiplexed and associated with their sample of origin on the basis of individual barcodes. Quality trimming was performed according to the following parameters: minimum/maximum sequence length of 200/1000bp, minimum average quality score of 25 over a sliding 50bp window, no ambiguous bases allowed, only 2 primer mismatches allowed, only 1 barcode mismatch allowed, and a maximum homopolymer length of 8bp. After quality trimming, 3 samples had less than 1000 sequences associated with them; thus, these samples were not included in downstream analyses. Quality trimming was followed by *de novo* and reference-based chimera checking and removal and binning of sequences into operational taxonomic units (OTUs) based on 97% identity (equivalent of species) using USEARCH. Taxonomic classification was performed by using the RDP classifier retrained with the GreenGenes database. Before alpha and beta diversity analyses, singletons were removed and the number of sequences per sample was normalized to 1007 (the smallest number of sequences associated with any one sample).

Statistics

All statistics for 16S data were performed by using Metastats (http://metastats.cbcb.umd.edu), which uses a nonparametric 2-sided t test to effectively handle nonparametric data sets. In addition, Metastats uses the false-discovery rate to improve specificity in complex environments and uses the Fisher exact test to handle sparsely sampled or "rare" features separately. We set the significance threshold to $P = .05$ and the number of permutations to 1000.

Supervised machine learning

To identify bacterial taxa that were predictive of disease severity or viral agent of bronchiolitis, we performed supervised machine learning by using the Genboree machine learning pipeline, implemented as part of the Genboree Microbiome Toolset (www.genboree.org). The pipeline runs the randomForest algorithm and the Boruta package to identify discriminatory OTUs that can act as predictors of specific groups (ie, biomarkers for specific disease or health states). Together, the randomForest algorithm, which determines the robustness of group clustering, and the Boruta package for feature selection are implemented to identify the most important OTUs involved in discriminating each group of samples from other groups. If 1 OTU is removed from the data set and classification of the sample into the correct group becomes impossible, that OTU is identified as discriminatory. The Boruta package calculates the amount of "background noise"; if the decrease in classification accuracy is not above this background, that OTU is not confirmed as discriminatory. We compared groups of samples on the basis of the virus present and on the basis of patient's acute wheeze status.

Whole genome shotgun sequencing and analysis

On the basis of the results of the 16S rRNA gene pyrosequencing and analysis, we chose 10 representative samples (10% of the cohort; 3 RSV-only infected samples, 4 HRV-only infected samples, and 3 RSV-HRV coinfected samples) and performed whole genome shotgun sequencing and analysis. Bacterial genomic DNA isolated from each sample as described above was sequenced on 1 lane of the Illumina HiSeq 2000 platform at the Human Genome Sequencing Center at Baylor College of Medicine. FASTQ sequencing files (read one and read two) were then quality trimmed and aligned against the human genome (hg19) and PhiX to filter out known contaminants. The trimmed, filtered FASTQ files were interleaved into 1 FASTQ file, which was converted to the FASTA format by using a perl script. To obtain species-level identification of the bacterial taxa present in each sample, we passed the whole genome shotgun FASTA file for each sample through MetaPhlAn, a computational tool that relies on clade-specific marker genes for taxonomic assignment. The output table, which lists taxonomic assignment and percent abundance, identified the species present and their abundance in each sample.

TABLE E1. Characteristics of children hospitalized with bronchiolitis (n = 100)

Characteristic	n	Percent
Age (mo), median (IQR)	55	3.4 (1.2-7.9)
Sex: male	64	64
Race		
White	58	58
Black	22	22
Other or missing	20	20
Hispanic	36	36
Gestational age (wk)		
<32	6	6
32-36.9	16	16
≥37 or "full term"	78	78
Either parent with history of asthma	39	39
Maternal smoking during pregnancy	10	10
Child exposed to environmental tobacco smoke	4	4
Breast-fed	70	70
History of wheeze	18	18
Taken antibiotic during week preceding the preadmission visit	22	22
Wheeze during preadmission visit*	59	61
Virology		
RSV only	71	71
HRV only	16	16
RSV and HRV	5	5
Inpatient		
ICU stay during inpatient visit	35	35
LOS (d), median (IQR)	100	2 (2-4)

ICU, Intensive care unit; *IQR*, interquartile range; *LOS*, length of stay.
*Three subjects excluded from count because of missing data regarding the presence of wheeze during the preadmission visit.

TABLE E2. Mean relative abundance of genera present in children with severe bronchiolitis with acute wheezing on admission compared with those who did not have acute wheezing on admission

	Percent abundance			
Genus	**Wheeze**	**No wheeze**	***P* value**	***Q***
Moraxella	10.30	3.64	.01	0.16
Prevotella	2.93	0.85	.04	0.35
Porphyromonas	1.09	0.27	.03	0.35
Staphylococcus	0.18	0.62	.01	0.16
Leptotrichia	0.22	<0.005	.001	0.02