




Association of nasopharyngeal microbiota profiles with bronchiolitis severity in infants hospitalised for bronchiolitis

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ABSTRACT Little is known about the relationship between the specific airway microbiota composition and severity of bronchiolitis. We aimed to identify nasopharyngeal microbiota profiles and link these profiles to acute severity in infants hospitalised for bronchiolitis.

We conducted a multicentre prospective cohort study of 1005 infants (age <1 year) hospitalised for bronchiolitis over three winters, 2011–2014. By applying a 16S rRNA gene sequence and clustering approach to the nasopharyngeal aspirates collected within 24 h of hospitalisation, we determined nasopharyngeal microbiota profiles and their association with bronchiolitis severity. The primary outcome was intensive care use, *i.e.* admission to an intensive care unit or use of mechanical ventilation.

We identified four nasopharyngeal microbiota profiles: three profiles were dominated by one of *Haemophilus*, *Moraxella* or *Streptococcus*, while the fourth profile had the highest bacterial richness. The rate of intensive care use was highest in infants with a *Haemophilus*-dominant profile and lowest in those with a *Moraxella*-dominant profile (20.2% *versus* 12.3%; unadjusted OR 1.81, 95% CI 1.07–3.11, *p*=0.03). After adjusting for 11 patient-level confounders, the rate remained significantly higher in infants with *Haemophilus*-dominant profiles (OR 1.98, 95% CI 1.08–3.62, *p*=0.03). These findings were externally validated in a separate cohort of 307 children hospitalised for bronchiolitis.



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Introduction

Bronchiolitis is a significant public health problem worldwide [1–3]. Indeed, bronchiolitis is the leading cause of hospitalisations for US infants, accounting for 18% of all infant hospitalisations [3]. The severity of bronchiolitis can range from a minor nuisance to fatal infection; however, the reasons for this severity difference are not fully explained by traditional risk factors, such as age, prematurity and viral aetiology [4, 5].

While common viruses and bacteria are identifiable using conventional techniques (*e.g.* cultures), these pathogens represent only a small fraction of the microbes living within any one individual [6, 7]. The recent advances of genome sequencing have revealed the presence of highly functional bacterial communities inhabiting humans, *i.e.* the microbiota. Emerging evidence shows that airway microbiota can influence immune responses [8–13], suggesting a role for airway microbiota in the development and morbidity of acute respiratory infections (ARIs), including bronchiolitis [14]. However, the limited literature about the association of airway microbiota with ARI incidence during infancy is conflicting. Specifically, ARI incidence has been found to be lower [15] and higher [16] among infants with more abundant *Moraxella* genus in their nasopharynx. None of the aforementioned studies of airway microbiota have externally validated their findings. Moreover, to the best of our knowledge, no studies have investigated the airway microbiota and its relationship with the severity of illness in infants hospitalised for bronchiolitis; a vulnerable population with high morbidity.

In this context, we conducted a multicentre prospective cohort study of infants hospitalised for bronchiolitis to identify airway microbiota profiles and to link these profiles to acute severity (*i.e.* intensive care use and hospital length of stay). We also externally validated the findings in a separate multicentre study of children hospitalised for bronchiolitis.

Materials and methods

Study design, setting and participants

We conducted a multicentre prospective cohort study of infants (age <1 year) hospitalised for bronchiolitis (severe bronchiolitis). This study, called the 35th Multicenter Airway Research Collaboration (MARC-35) [17], was coordinated by the Emergency Medicine Network (EMNet, <http://www.emnet-usa.org/>), a collaboration of 235 participating hospitals.

Using a standardised protocol, site investigators at 17 sites across 14 US states (supplementary table E1) enrolled infants hospitalised with an attending physician diagnosis of bronchiolitis during three consecutive bronchiolitis seasons from November 1, to April 30, 2011–2014. Bronchiolitis was defined by the American Academy of Pediatrics guidelines as acute respiratory illness with some combination of rhinitis, cough, tachypnoea, wheezing, crackles and retractions [18]. We excluded infants with previous enrolment; those who were transferred to a participating hospital >24 h after the original hospitalisation; those who were consented >24 h after hospitalisation; or those with known heart-lung disease, immunodeficiency, immunosuppression or gestational age <32 weeks. All patients were treated at the discretion of the treating physician. The institutional review board at each of the participating hospitals approved the study. Written informed consent was obtained from the parent or guardian.

Data collection

Investigators conducted a structured interview that assessed patients' demographic characteristics, medical and family history, and details of the acute illness. Emergency department and hospital chart reviews provided further clinical data, such as vital signs, physical examination, medical management and disposition. All data were reviewed at the EMNet Coordinating Center and site investigators were queried about missing data and discrepancies identified by manual data checks.

Nasopharyngeal aspirates were collected by trained site investigators using the same standardised protocol utilised in a previous cohort study of children with bronchiolitis [4, 19]. All sites used the same collection equipment (Medline Industries, Mundelein, IL, USA) and collected the samples within 24 h of hospitalisation. The nasopharyngeal sample was added to transport medium, immediately placed on ice and then stored at -80°C . Frozen samples were shipped in batches on dry ice to Baylor College of Medicine, where they were tested for 1) 17 respiratory viruses (*e.g.* respiratory syncytial virus (RSV), rhinovirus) using real-time PCR assays [4, 19, 20], and 2) microbiota using 16S rRNA gene sequencing.

16S rRNA gene sequencing and compositional analysis

16S rRNA gene sequencing methods were adapted from the methods developed for the National Institutes of Health (NIH) Human Microbiome Project [6, 7]. The details of the methods are described in the supplemental methods. Briefly, bacterial genomic DNA was extracted using a MO BIO PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). The 16S rDNA V4 region was amplified by PCR and sequenced in the MiSeq platform (Illumina, San Diego, CA, USA) using a 2×250 bp paired-end

protocol that yields paired-end reads which overlap almost completely. The primers used for amplification contained adapters for MiSeq sequencing and single-end barcodes, allowing pooling and direct sequencing of PCR products [21, 22].

Sequencing read pairs were demultiplexed based on the unique molecular barcodes, and reads were merged using USEARCH v7.0.1090 [23]. Rarefaction curves of bacterial operational taxonomic units (OTUs) were constructed using sequence data for each sample to ensure coverage of the bacterial diversity present. Samples with suboptimal amounts of sequencing reads were re-sequenced to ensure that the majority of bacterial taxa were encompassed in our analyses.

16S rRNA gene sequences were clustered into OTUs at a similarity cut-off value of 97% using the UPARSE algorithm [24]. OTUs were determined by mapping the centroids to the SILVA database [25] containing only the 16S V4 region to determine taxonomies. A custom script constructed a rarefied OTU table (rarefaction was performed at only one sequence depth) from the output files generated in the previous two steps for downstream analyses of alpha-diversity (*i.e.* Shannon index) and beta-diversity (*i.e.* weighted UniFrac) [26, 27]. We utilised multiple quality control measures, including the use of non-template controls in the microbial DNA extraction, 16S rRNA gene amplification, and amplicon sequencing processes. The details of the quality control measures are described in the supplemental methods.

Outcome measures

The primary outcome was intensive care use, defined as admission to the intensive care unit and/or use of mechanical ventilation (continuous positive airway pressure and/or intubation during inpatient stay, regardless of location) at any time during the index hospitalisation [19, 28]. The secondary outcome was hospital length of stay of ≥ 3 days, defined using the median length of stay of 2 days and similar to the approach used in prior studies [4, 19].

Statistical analyses

For each nasopharyngeal sample, the relative abundance of each OTU was calculated. Analyses were conducted at the genus level; because each genus was dominated by one OTU (*e.g.* the detected *Haemophilus* genus consisted solely of one OTU), all OTUs assigned to the same genus were collapsed into a single group for reporting [16]. To identify nasopharyngeal microbiota profiles, we performed unbiased clustering by partitioning around medoids (PAM) [29] using weighted UniFrac distance. Each PAM cluster is defined by a point designated as the centre (the ‘medoid’) and minimises the distance between samples in a cluster. The number of clusters to choose for the data was determined using the average Silhouette score (supplementary figure E1) [30].

Additionally, given that the presence and/or abundance of an individual genus likely influences other genera in the microbial community, we displayed the microbiota association network on the basis of the approach of FAUST *et al.* [31]. The details of the method are described in the supplemental methods.

Next, across the identified nasopharyngeal microbiota profiles, we compared patient characteristics and hospital course using the Chi-squared test or Kruskal–Wallis test as appropriate. To examine the association of microbiota profiles with the severity outcomes (*i.e.* intensive care use and hospital length of stay of ≥ 3 days), we constructed two logistic regression models for each outcome with the *Moraxella*-dominant profile as the reference [15]. First, we fitted an unadjusted model that included only microbiota profiles as the independent variable. Second, we constructed a two-level mixed-effects model to account for patient clustering at the hospital level. We also adjusted for 11 patient-level variables (*i.e.* age, sex, race/ethnicity, gestational age, history of breathing problems, nursery attendance, siblings at home, lifetime history of antibiotic use, history of corticosteroid use, use of antibiotics during the pre-hospitalisation visit and respiratory viruses detected by PCR). We chose these potential confounders on the basis of clinical plausibility and *a priori* knowledge [4, 5, 19, 28, 32]. We did not adjust for markers of severity (*e.g.* vital signs) because these were considered intermediate factors in the association of interest.

We performed sensitivity analyses to assess the robustness of our findings. First, we repeated the analysis stratifying by viral pathogen, similar to the approach used in prior studies [4, 33, 34]. Second, we modelled length of stay as a count variable by fitting a mixed-effects Poisson regression model. Analyses used R v3.2 with the *lme4* package for the mixed-effects models and the *phyloseq* package [35]. All p-values were two-tailed, with $p < 0.05$ considered statistically significant.

Validation of microbiota profiles and outcome models

Next, to validate our findings in MARC-35, we analysed data from another 16-centre prospective cohort of children aged < 2 years with severe bronchiolitis (MARC-30) [4, 19], in which nasopharyngeal samples were collected using the same methods as in MARC-35. The technical methods (*i.e.* microbial DNA

extraction, 16S rRNA gene amplification and amplicon sequencing) used for MARC-30 were also identical to those for MARC-35. Using nasopharyngeal samples from 156 children with intensive care use and 156 children with no intensive care use and a length of stay ≤ 1 day (*i.e.* nested case-control sample with a greater severity contrast), we tested for the nasopharyngeal microbiota, identified microbiota profiles, and determined the associations of these profiles with the severity outcomes.

Results

Population and sequence

We enrolled 1016 infants with severe bronchiolitis from 17 participating hospitals in MARC-35. We analysed nasopharyngeal samples from all of the enrolled infants by 16S rRNA gene sequencing and obtained 17399260 high-quality merged sequences, of which 16685637 (95.9%) were mapped to the database. Of 1016 infant samples, 1005 (98.9%) had sufficient sequence depth (rarefaction cut-off: 2128 reads per sample) and were eligible for the current analysis. Among the analytic cohort, the median age at hospitalisation was 3 months (interquartile range (IQR), 2–6 months), 60.0% were male and 42.6% were non-Hispanic white. The sequencing identified 24 phyla and 379 genera. The nasopharyngeal microbiota was dominated by three genera: *Streptococcus* (30.9%), *Moraxella* (29.8%) and *Haemophilus* (20.2%); followed by *Prevotella* (2.3%) and *Staphylococcus* (2.2%).

Nasopharyngeal microbiota profiles and network

PAM clustering of nasopharyngeal microbiota identified four distinct microbiota profiles: 1) *Haemophilus*-dominant profile (19.2%), 2) *Moraxella*-dominant profile (21.9%), 3) *Streptococcus*-dominant profile (28.2%) and 4) a mixed profile (30.7%) (figure 1).

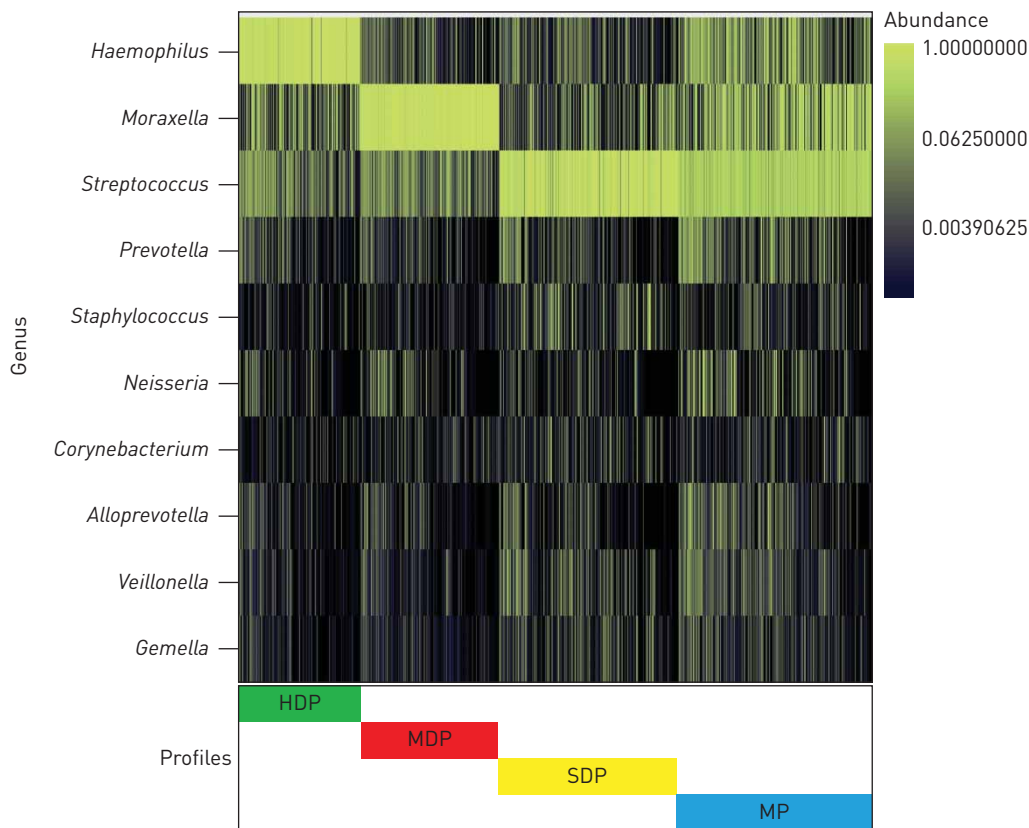


FIGURE 1 Clustering and composition in nasopharyngeal microbiota of 1005 infants hospitalised for bronchiolitis, MARC-35. All nasopharyngeal microbiota profiles of infants were clustered using the partitioning around medoids clustering method with weighted UniFrac distance. Coloured bars indicate four microbiota profiles: *Haemophilus*-dominant profile (HDP, green), *Moraxella*-dominant profile (MDP, red), *Streptococcus*-dominant profile (SDP, yellow), and mixed profile (MP, blue). The optimal number of clusters was identified by the Silhouette index. To obtain further information about the bacterial composition of samples within microbiota profiles, the 10 most abundant genera present are displayed in the adjacent heatmap. The taxonomy depicted is on the genus level because each genus was dominated by one operational taxonomic unit.

The first three profiles were dominated by one of *Haemophilus*, *Moraxella* or *Streptococcus* genera. By contrast, the mixed profile had the highest bacterial richness ($p < 0.001$) and alpha-diversity index (Shannon index, $p < 0.001$) with highest relative abundance of *Prevotella*, *Alloprevotella* and *Veillonella* (supplementary table E2). Sub-clustering of the mixed profile identified three sub-clusters: a sub-cluster co-dominated by *Streptococcus* and *Moraxella*, a sub-cluster co-dominated by *Streptococcus* and *Haemophilus*, and a sub-cluster with a relatively high abundance of *Streptococcus*, *Prevotella* and *Alloprevotella* (supplementary table E3).

The presence or abundance of individual bacteria likely affects those of others due to ecological interactions. To examine the community structure of the microbiota, we created a microbiota association network (supplementary figure E2). *Haemophilus*, *Moraxella* and *Streptococcus* genera were negatively associated with each other, which is consistent with the observed *Haemophilus*-dominant, *Moraxella*-dominant and *Streptococcus*-dominant profiles. The *Streptococcus* genus was positively associated with *Veillonella*, which was also positively associated with *Prevotella* and *Alloprevotella*. This community structure was consistent with the mixed microbiota profile.

Microbiota profiles and patient characteristics

Patient characteristics differed across the four microbiota profiles (table 1). For example, infants with the *Haemophilus*-dominant profile were older, less likely to be non-Hispanic white and more likely to have used antibiotics before the index hospitalisation compared to infants with other profiles (all $p < 0.05$). At presentation to the hospital, infants with the *Haemophilus*-dominant profile also had a higher weight and were more likely to receive antibiotics during a pre-hospitalisation visit (e.g. to the emergency department before enrolment) and have sole rhinovirus infection (all $p < 0.001$), compared to infants with other profiles. By contrast, there was no significant difference in the vital signs across the profiles.

Microbiota profiles and bronchiolitis severity

Overall, 161 (16.0%) infants hospitalised for bronchiolitis required intensive care use. The rate of intensive care use was highest in infants with a *Haemophilus*-dominant profile and lowest in those with a *Moraxella*-dominant profile (20.2% versus 12.3%; unadjusted OR 1.81, 95% CI 1.07–3.11, $p = 0.03$; table 2). In the multivariable model adjusting for 11 patient characteristics (e.g., demographics, use of antibiotics during the pre-hospitalisation visit, virus) and clustering at the hospital level, the rate remained significantly higher in infants with a *Haemophilus*-dominant profile (OR for comparison with *Moraxella*-dominant profile 1.98, 95% CI 1.08–3.62, $p = 0.03$; table 2 and supplementary table E4). There was also a positive linear relationship between relative abundance of *Haemophilus* and rate of intensive care use after adjusting for the 11 patient characteristics ($p = 0.03$; figure 2a). By contrast, the rate of intensive care use in those with a *Streptococcus*-dominant or mixed profile was not significantly different from that of infants with the reference *Moraxella*-dominant profile. Stratified analyses across virology strata are shown in supplementary table E5. Although there was limited statistical power in most viral strata, infants with RSV infection and a *Haemophilus*-dominant profile had higher odds of intensive care use. Additionally, in another sensitivity analysis ($n = 978$) that excluded all infants with no viral pathogen detected, the results did not change materially, i.e. the rate of intensive care use remained significantly higher in infants with a *Haemophilus*-dominant profile (OR for comparison with *Moraxella*-dominant profile 2.15, 95% CI 1.16–3.99, $p = 0.01$).

Similar to results of the primary outcome, infants with a *Haemophilus*-dominant profile had a higher rate of length of stay of ≥ 3 days than did those with the *Moraxella*-dominant profile (51.3% versus 33.2%; unadjusted OR 2.12, 95% CI 1.43–3.17, $p < 0.001$; tables 1 and 2). In the multivariable model, the rate remained significantly higher in infants with a *Haemophilus*-dominant profile (OR 2.47, 95% CI 1.60–3.83, $p < 0.001$; table 2 and supplementary table E4). There was also a positive linear relationship between relative abundance of *Haemophilus* and rate of length-of-stay ≥ 3 days ($p < 0.001$; figure 2B). Likewise, in the sensitivity analysis modelling length of stay as a count variable (i.e. not as binary variable), the significant association between microbiota profiles and the outcome persisted (supplementary table E6).

Validation of profiles and association with severity

In 312 nasopharyngeal samples selected from children in the MARC-30 study, 307 (98.4%) had sufficient sequencing depth (rarefaction cut-off: 1064 reads per sample) and were used for validation. The use of the PAM clustering method of microbiota, with the use of the average Silhouette score, revealed four microbiota profiles (supplementary figure E3) similar to those in MARC-35, i.e. three profiles were dominated by one of *Haemophilus*, *Moraxella* or *Streptococcus* genera, while the fourth profile (i.e. mixed profile) had the highest bacterial richness and alpha-diversity levels (supplementary table E7). In addition, the sub-clustering of the mixed profile identified a sub-cluster with a relatively high abundance of *Streptococcus*, *Prevotella* and *Alloprevotella* (supplementary table E8). Likewise, the significant associations

TABLE 1 Characteristics and clinical presentation of infants hospitalised for bronchiolitis by nasopharyngeal microbiota profile in the MARC-35 cohort

Variables	<i>Haemophilus</i> -dominant profile	<i>Moraxella</i> -dominant profile	<i>Streptococcus</i> -dominant profile	Mixed profile	p-value
Subjects	193 (19.2)	220 (21.9)	283 (28.2)	309 (30.7)	
Characteristics					
Median age months (IQR)	4 [3–8]	3 [2–6]	3 [1–5]	3 [2–6]	<0.001
<2	39 (20.2)	69 (31.4)	108 (38.2)	93 (30.1)	<0.001
2–5.9	82 (42.5)	92 (41.8)	127 (44.9)	149 (48.2)	
6–12	72 (37.3)	59 (26.8)	48 (17.0)	67 (21.7)	
Male sex	112 (58.0)	127 (57.7)	172 (60.8)	192 (62.1)	0.70
Race/ethnicity					0.02
Non-Hispanic white	71 (36.8)	89 (40.5)	145 (51.2)	123 (39.8)	
Non-Hispanic black	39 (20.2)	58 (26.4)	57 (20.1)	79 (25.6)	
Hispanic	76 (39.4)	65 (29.5)	72 (25.4)	93 (30.1)	
Other	7 (3.6)	8 (3.6)	9 (3.2)	14 (4.5)	
Parental history of asthma	58 (30.1)	68 (30.9)	109 (38.8)	106 (34.4)	0.16
Maternal smoking during pregnancy	21 (11.3)	25 (11.7)	40 (14.3)	58 (19.0)	0.051
Mode of birth					0.95
Vaginal birth	122 (63.2)	143 (65)	179 (63.3)	199 (64.4)	
C-section	64 (34.4)	72 (33.5)	101 (36.1)	106 (34.8)	
Prematurity [32–37 weeks]	33 (17.1)	35 (15.9)	51 (18.0)	64 (20.7)	0.52
Previous breathing problems before the index hospitalisation [#]	49 (25.4)	38 (17.3)	55 (19.4)	61 (19.8)	0.42
History of eczema	27 (14.0)	30 (13.6)	37 (13.1)	52 (16.8)	0.58
Ever attended nursery	52 (26.9)	60 (27.3)	50 (17.7)	68 (22.0)	0.03
Aeroallergen sensitisation	1 (0.7)	3 (1.8)	4 (1.7)	5 (2.1)	0.75
Food sensitisation	37 (19.2)	42 (19.1)	45 (15.9)	55 (17.8)	0.75
Sibling at home	144 (74.6)	182 (82.7)	223 (78.8)	250 (80.9)	0.20
Mostly breastfed for the first 3 months of life	87 (49.7)	94 (49.2)	123 (50.6)	115 (42.9)	0.29
Smoke exposure at home	30 (15.5)	33 (15.0)	39 (13.8)	51 (16.5)	0.83
Antibiotic use before index hospitalisation	92 (47.7)	46 (20.9)	84 (29.7)	90 (29.1)	<0.001
Corticosteroid use before index hospitalisation	32 (16.6)	34 (15.5)	35 (12.4)	45 (14.6)	0.60
Clinical presentation					
Month of hospitalisation					0.005
November	13 (6.7)	29 (13.2)	18 (6.4)	22 (7.1)	
December	26 (13.5)	56 (25.5)	46 (16.3)	58 (18.8)	
January	59 (30.6)	56 (25.5)	91 (32.2)	98 (31.7)	
February	42 (21.8)	45 (20.5)	76 (26.9)	78 (25.2)	
March	37 (19.2)	23 (10.5)	34 (12.0)	36 (11.7)	
April	16 (8.3)	11 (5.0)	18 (6.4)	17 (5.5)	
Median duration in days of breathing problem before the index hospitalisation (IQR)	4 [2–5]	3 [2–5]	3 [2–5]	3 [2–4]	0.14
Median weight at presentation kg (IQR)	7.0 [5.2–8.2]	6.1 [4.80–7.93]	5.5 [4.4–7.2]	5.9 [4.8–7.4]	<0.001
Median respiratory rate at presentation per minute (IQR)	48 [40–60]	50 [40–60]	48 [40–60]	48 [40–60]	0.48
Oxygen saturation at presentation					0.20
<90%	26 (13.8)	12 (5.6)	24 (8.7)	27 (8.9)	
90–93%	33 (17.6)	28 (13.1)	47 (17.0)	45 (14.8)	
≥94%	129 (68.6)	174 (81.3)	205 (74.3)	233 (76.4)	
Retractions on examination					0.003
None	28 (14.5)	49 (22.5)	66 (23.4)	47 (15.3)	
Mild	74 (38.3)	105 (48.2)	116 (41.1)	131 (42.5)	
Moderate/severe	83 (43.0)	55 (25.2)	95 (33.7)	121 (39.3)	
Wheezing on examination	106 [54.9]	144 [65.5]	154 [54.4]	192 [62.1]	0.10
Received antibiotics during pre-hospitalisation visit	45 [23.3]	13 [6.0]	58 [20.7]	59 [19.4]	<0.001
Received corticosteroids during pre-hospitalisation visit	21 [10.9]	16 [7.3]	26 [9.3]	26 [8.6]	0.64

Continued

TABLE 1 Continued

Variables	<i>Haemophilus</i> -dominant profile	<i>Moraxella</i> -dominant profile	<i>Streptococcus</i> -dominant profile	Mixed profile	p-value
Virology					<0.001
Sole RSV infection	83 (43.0)	119 (54.1)	202 (71.4)	176 (57.0)	
Sole RV infection	24 (12.4)	15 (6.8)	7 (2.5)	14 (4.5)	
RSV + RV co-infection	20 (10.4)	30 (13.6)	24 (8.5)	46 (14.9)	
RSV + non-RV pathogens	32 (16.6)	21 (9.5)	27 (9.5)	33 (10.7)	
RV + non-RSV pathogens	9 (4.7)	13 (5.9)	4 (1.4)	5 (1.6)	
Neither RSV nor RV [†]	19 (9.8)	18 (8.2)	12 (4.2)	25 (8.1)	
No viral pathogens	6 (3.1)	4 (1.8)	7 (2.5)	10 (3.2)	
Median viral genomic load CT-value (IQR)					
RSV	23 (21–26)	22 (20–24)	23 (21–25)	23 (21–26)	<0.001
RV	29 (26–34)	28 (26–35)	28 (27–36)	30 (27–35)	0.89 ^f
Outcomes					
Intensive care use [§]	39 (20.2)	27 (12.3)	48 (17.0)	47 (15.2)	
Hospital length of stay ≥3 days	99 (51.3)	73 (33.2)	111 (39.2)	112 (36.2)	
Median hospital length of stay days (IQR)	3 (2–4)	2 (1–3)	2 (1–3)	2 (1–3)	

Data are presented as n (%) of infants unless otherwise indicated. Percentages may not equal 100, because of missingness. Patient characteristics and hospital course were compared using the Chi-squared test or Kruskal–Wallis test across the identified nasopharyngeal microbiota profiles. IQR: interquartile range; RSV: respiratory syncytial virus; RV: rhinovirus; CT: cycle threshold. [‡]: Defined as an infant having cough that wakes him/her at night and/or causes emesis, or when the child has wheezing or shortness of breath without cough. [¶]: Defined by having one or more positive values for allergen-specific IgE. ^{*}: Consisted of adenovirus (n=6), coronavirus (n=15), human bocavirus (n=9), human metapneumovirus (n=38), influenza virus (n=11), and parainfluenza virus (n=13). Note that 18 patients had co-infection. [§]: Defined as admission to the intensive care unit and/or use of mechanical ventilation (continuous positive airway pressure and/or intubation during inpatient stay, regardless of location) at any time during the index hospitalisation. ^f: Unadjusted p-values are presented in table 2.

between the *Haemophilus*-dominant profile and the higher rate of severity outcomes were also observed, with larger effect sizes, in this nested case–control sample with a greater severity contrast, for example, the adjusted odds ratio for intensive care use of *Haemophilus*-dominant profile in comparison with *Moraxella*-dominant profile was 5.34 (95% CI 1.96–14.5, p=0.001; supplementary table E9).

Discussion

In this prospective multicentre cohort of 1005 infants with severe bronchiolitis, we identified four distinct microbiota profiles in their nasopharynx. Infants with a *Haemophilus*-dominant profile had significantly higher rates of intensive care use and prolonged hospital length of stay than those with a *Moraxella*-dominant profile. In contrast, the rate of outcomes in those with a *Streptococcus*-dominant or mixed profile was not significantly different. These findings were externally validated in a separate multicentre study of severe bronchiolitis. To our knowledge, this is the first study to have examined the association of airway microbiota with disease severity in infants with severe bronchiolitis. Our data corroborate and build on previous reports linking bacteria composition in the airway to ARI outcomes [15, 16, 36–39], findings of both research and clinical importance.

Previous reports on ARI in young children have reported inconsistent relationships between airway bacteria and ARI incidence and severity. For example, KLOEPFER *et al.* [36] applied quantitative PCR to nasal samples (n=380) and found no associations between *H. influenzae* detection alongside rhinovirus and ARI severity in school-age children. Similarly, CARLSSON *et al.* [37], using a culture-dependent technique in the COPSAC₂₀₀₀ cohort (n=283), found no association between *H. influenzae* detection in the hypopharynx and duration of wheezing episodes in children aged <3 years. By contrast, TEO *et al.*, using 16S rRNA gene sequencing in the Childhood Asthma Study cohort (n=234) in Western Australia, reported six nasopharyngeal microbiota profiles (*Haemophilus*, *Moraxella*, *Streptococcus*, *Corynebacterium*, *Alloiococcus* and *Staphylococcus*) in infants with a high risk of atopy, and that a *Haemophilus*-dominant nasopharyngeal microbiota was associated with higher incidence of ARI and higher severity [16]. In addition to the apparent inconsistency in the *Haemophilus*–ARI link, we also observed a lower abundance of *Corynebacterium*, *Dolosigranulum* and *Staphylococcus* genera in our population (*i.e.* young infants hospitalised with bronchiolitis) compared to that in previous studies of healthy children [15] and children with mild upper respiratory infection [40, 41]. Potential reasons for these discrepancies across studies include differences in patient populations, clinical settings (*e.g.* community *versus* inpatient setting), sampling (*e.g.* anterior nasal swab *versus* nasopharyngeal aspirate) and laboratory techniques (*e.g.* culture, PCR) for bacteria identification. By contrast, the validity of our findings is buttressed by the use of 16S

TABLE 2 Unadjusted and multivariable associations of nasopharyngeal microbiota profiles with bronchiolitis outcomes, MARC-35 cohort

Outcome by microbiota profile	Unadjusted model		Adjusted model [#]	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Intensive care use[†]				
<i>Haemophilus</i> -dominant profile	1.81 (1.07–3.11)	0.03	1.98 (1.08–3.62)	0.03
<i>Moraxella</i> -dominant profile	Reference		Reference	
<i>Streptococcus</i> -dominant profile	1.46 (0.88–2.45)	0.14	1.32 (0.74–2.34)	0.34
Mixed profile	1.28 (0.78–2.16)	0.34	1.19 (0.68–2.09)	0.54
Hospital length of stay ≥ 3 days				
<i>Haemophilus</i> -dominant profile	2.12 (1.43–3.17)	<0.001	2.47 (1.60–3.83)	<0.001
<i>Moraxella</i> -dominant profile	Reference		Reference	
<i>Streptococcus</i> -dominant profile	1.30 (0.90–1.88)	0.16	1.06 (0.71–1.57)	0.78
Mixed profile	1.14 (0.80–1.65)	0.47	1.01 (0.68–1.48)	0.97

Bold results are statistically significant. Full adjusted models are included in supplementary table E3. [#]: Mixed-effects logistic regression model adjusting for 11 patient-level variables (age, sex, race/ethnicity, gestational age, history of breathing problems, nursery attendance, siblings at home, lifetime history of antibiotic use, history of corticosteroid use, use of antibiotics during the pre-hospitalisation visit and respiratory viruses detected by PCR) and sites as random effect. [†]: Defined as admission to the intensive care unit and/or use of mechanical ventilation (continuous positive airway pressure and/or intubation during inpatient stay, regardless of location) at any time during the index hospitalisation.

rRNA gene sequencing of the nasopharyngeal microbiota, a sample size that is many times larger than any other prior study on this topic, and validation in an additional multicentre cohort of severe bronchiolitis.

The observed microbiota–severity association challenges the conventional virus-centric view of bronchiolitis. However, the nature of this microbial association warrants clarification. It is possible that there is a causal relationship, *i.e.* the *Haemophilus*-dominant microbiota in the infant’s airway alters immune responses to increase the severity of bronchiolitis. Indeed, FØLSGAARD *et al.* reported that *H. influenzae* colonisation of the airways in asymptomatic neonates is associated with an upregulated T-helper (Th)1/Th2/Th17-type inflammatory response of the upper airway mucosa [11]. The presence of

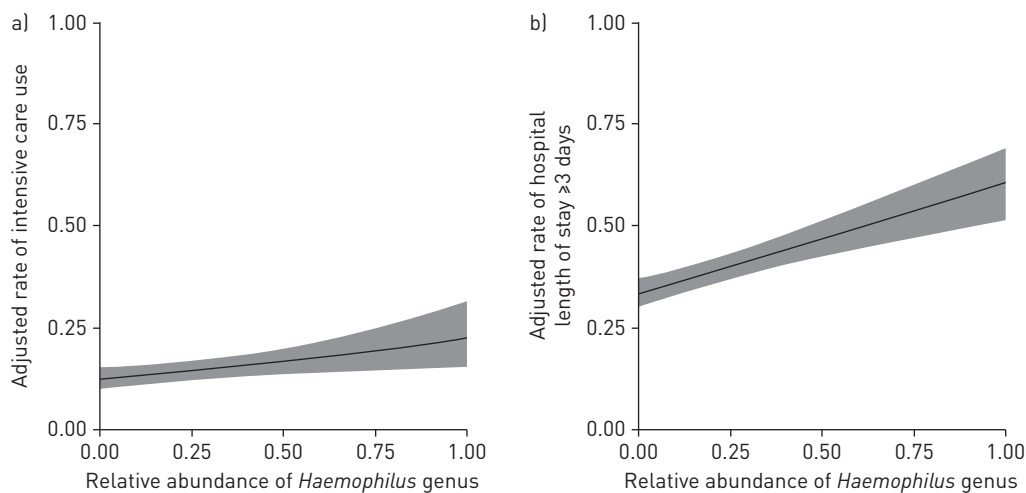


FIGURE 2 Multivariable association of relative abundance of *Haemophilus* genus with the rate of severity outcomes in infants hospitalised for bronchiolitis, MARC-35. Two-level mixed-effects models were constructed to account for patient clustering at the hospital level. The models adjusted for 11 patient-level variables (*i.e.* age, sex, race/ethnicity, gestational age, history of breathing problems, nursery attendance, siblings at home, lifetime history of antibiotic use, history of corticosteroid use, use of antibiotics during the pre-hospitalisation visit and respiratory viruses detected by PCR). a) There was a positive linear association between relative abundance of *Haemophilus* genus and the rate of intensive care use [adjusted OR 1.07 [per 0.1 increase in the relative abundance of *Haemophilus*], 95% CI 1.01–1.13, $p=0.03$]. b) There was a positive linear association between relative abundance of *Haemophilus* genus and the rate of a hospital length of stay of ≥ 3 days [adjusted OR 1.11 [per 0.1 increase in the relative abundance of *Haemophilus*], 95% CI 1.06–1.17, $p<0.001$]. The grey shaded areas represent the 95% confidence intervals.

both Th2 and Th17 mediators suggests that colonisation with *H. influenzae* could counteract the Th1 response needed to eradicate viral pathogens of bronchiolitis. Similarly, studies of primary bronchial epithelial cells demonstrated that exposure to *H. influenzae* upregulates intercellular adhesion molecule-1 expression and enhances chemokine release induced by subsequent infection by RSV [42] and rhinovirus [42, 43]. Alternatively, a *Haemophilus*-dominant microbiota could simply be a marker of an infant who is prone to develop more-severe bronchiolitis. Additionally, reverse causation, *i.e.* more-severe illness that results in a rapid overgrowth of *Haemophilus* in the airway, is also possible [44]. These possibilities are not mutually exclusive. Notwithstanding the complexity, the identification and validation of a *Haemophilus*-dominant profile as the primary culprit in the association between airway microbiota and bronchiolitis severity is an important finding. Our data should facilitate further investigations (*e.g.* animal models, metagenomics, proteomics, metabolomics) to disentangle the complex web of the airway microbiome, viral pathogens, host immune responses, and bronchiolitis pathogenesis [14].

Potential limitations

The study has several potential limitations. First, bronchiolitis is a disease of the lower airways, and our study was based on samples obtained from the infant's nasopharynx. However, lower airway sampling in infants is technically and ethically challenging. Nevertheless, prior studies have shown strong correlations between upper and lower airway microbiology [45] and virology [46] in children. Therefore, we believe that the nasopharyngeal microbiota in the infant is likely indicative of that in the lower respiratory tract. Second, with the use of 16S rRNA gene sequencing, we did not analyse the microbiota at the species level; therefore, we were limited in the confirmation of their phenotypic features. Third, the study design precluded us from evaluating the relationship between succession of the airway microbial ecosystem and respiratory health in children. To address this important question, the study population is currently being followed longitudinally up to age 6 years with airway specimen sampling at multiple time points. Fourth, as with any observational studies, the associations of microbiota profiles with bronchiolitis severity do not necessarily prove causality and could be confounded by unmeasured factors such as virus genotypes and institutional variation in resource use. One could surmise that our inferences are biased by co-circulating viruses within hospitals or regions. However, MARC-35 enrolled patients at 17 hospitals across 14 US states across three consecutive bronchiolitis seasons (2011–2014). Their findings were further validated in a separate 16-centre cohort of severe bronchiolitis during 2007–2010. Furthermore, the observed association between the microbiota and bronchiolitis severity remained significant after accounting for patient-level clustering within hospitals using a mixed-effects model. Fifth, we did not have information from a 'control' group (*e.g.* infants hospitalised for non-respiratory events). However, the study objective was not to assess the role of microbiota on the development of bronchiolitis (yes/no) but to investigate its relationship with disease severity. Finally, although the study cohorts consisted of a racially, ethnically and geographically diverse US sample of severe bronchiolitis, our inferences might not be generalisable to those in the ambulatory setting. Nevertheless, our data remain highly relevant for 130 000 children hospitalised yearly in the USA; a vulnerable population with high morbidity [3].

Conclusions

In this prospective multicentre cohort of 1005 infants hospitalised for bronchiolitis, we identified four distinct microbiota profiles in their nasopharynx. Infants with a *Haemophilus*-dominant profile had greater bronchiolitis severity, *i.e.* significantly higher rates of intensive care use and prolonged length of stay, than those with a *Moraxella*-dominant profile. These findings were validated in a separate multicentre study of severe bronchiolitis. The findings should serve as an important starting point for further mechanistic and interventional investigations of the interplay between the airway microbiome, viral pathogens, host immune responses and bronchiolitis pathogenesis.

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