Haemophilus-Dominant Nasopharyngeal Microbiota Is Associated With Delayed Clearance of Respiratory Syncytial Virus in Infants Hospitalized for Bronchiolitis

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The relation of nasopharyngeal microbiota to the clearance of respiratory syncytial virus (RSV) in infants hospitalized for bronchiolitis is not known. In a multicenter cohort, we found that 106 of 557 infants (19%) hospitalized with RSV bronchiolitis had the same RSV subtype 3 weeks later (ie, delayed clearance of RSV). Using 16S ribosomal RNA gene sequencing and a clustering approach, infants with a *Haemophilus*-dominant microbiota profile at hospitalization were more likely than those with a mixed profile to have delayed clearance, after adjustment for 11 factors, including viral load. Nasopharyngeal microbiota composition is associated with delayed RSV clearance.

Keywords. Airway microbiota; bronchiolitis; children; infant; microbiome; respiratory infection, respiratory syncytial virus.

Bronchiolitis is the leading cause of infant hospitalization in the United States and respiratory syncytial virus (RSV) is the most common etiology [1]. Although critical for understanding the pathogenesis of RSV infection and its transmissibility, studies examining RSV clearance in naturally infected infants are sparse. In a 1976 study of 40 hospitalized children with RSV, Hall et al detected RSV up to 3 weeks after hospitalization, using viral culture [2]. More recently, Brint et al, in a single-center study of 51 hospitalized infants, used quantitative reverse transcription–polymerase chain reaction (qRT-PCR) to demonstrate RSV detection up to 1 month after symptom onset [3]. The underlying pathobiology of RSV clearance in infants has

almost exclusively focused on the relation of the host response to RSV clearance [4].

However, emerging evidence suggests that the composition of the nasopharyngeal microbiota is associated with susceptibility to acute respiratory infections [5], the severity of bronchiolitis [6, 7], and, importantly, the host immune response [6, 8]. Indeed, it has become clear that airway bacteria are not merely bystanders during acute respiratory infections, but rather have a complex interplay with infecting viruses and the host immune response [5–8]. However, no study has examined the association between the nasopharyngeal microbiota and RSV clearance in infants with bronchiolitis. To address this knowledge gap, we conducted a planned secondary data analysis of our multicenter study of severe bronchiolitis (ie, requiring hospitalization) to examine the association between the nasopharyngeal microbiota upon hospitalization and delayed clearance of RSV.

METHODS

Study Design, Setting, and Participants

As previously described [7], the 35th Multicenter Airway Research Collaboration (MARC-35) is an ongoing 17-center prospective cohort study of hospitalized infants (age <1 year) with an attending physician's diagnosis of bronchiolitis, as defined by the American Academy of Pediatrics [9]. Researchers at the 17 sites enrolled hospitalized infants into MARC-35 over the 3 consecutive winter seasons (ie, November–April) from 2011 to 2014 (Supplementary Table). The institutional review board at each site approved this study.

Data Collection

Site teams not only extracted clinical data from the emergency department and inpatient charts, but also conducted structured interviews with parents during the hospitalization for demographic, historical, and environmental information.

Nasopharyngeal Aspirate Collection

All site researchers collected nasopharyngeal aspirates (NPAs) within 24 hours of hospitalization, using the same equipment type (eg, sample traps and suction catheters from Medline Industries [Mundelein, IL]) and a standardized protocol [10]. These samples were immediately placed on ice, refrigerated at 4°C within an hour, and frozen at –80°C within 24 hours. The NPAs were shipped on dry ice to Baylor College of Medicine (Houston, TX), where they underwent microbiota and viral testing.

Clearance Nasal Swab Collection

During the hospitalization, site teams taught parents how to collect the nasal swab sample. Three weeks after the date of hospitalization, parents collected the “clearance swab” from

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both anterior nares, using a single nylon, pediatric FLOQSwab (Copan, Brescia, Italy) [10]. Parents mailed the clearance swabs to Massachusetts General Hospital (Boston, MA) at ambient temperature. Upon receipt, the swabs were stored at -80°C until they were shipped on dry ice to Baylor College of Medicine, where they underwent viral testing.

16S Ribosomal RNA (rRNA) Gene Sequencing and Compositional Analysis

As previously described [10], we sequenced the 16S rRNA gene V4 region of the NPA bacteria on the Illumina MiSeq platform and only used microbiota data with sufficient sequence depth (ie, rarefaction cutoff 2128 reads per sample). Using partitioning around medoids with weighted UniFrac distances, we identified 4 distinct and reproducible NPA microbiota profiles: *Haemophilus*-dominant, *Moraxella*-dominant, *Streptococcus*-dominant, and mixed [7]. These 4 microbiota profiles served as the primary exposure.

PCR Assays and Delayed Clearance

qRT-PCR assays were conducted at Baylor College of Medicine for RSV types A and B, using both the NPA and the clearance swab, as previously described [1]. Delayed RSV clearance, the primary outcome, was defined as an infant having the same RSV subtype at hospitalization and 3 weeks after the date of hospitalization. To determine the presence of viral coinfections at hospitalization, we used qRT-PCR to test the NPA for an additional 15 respiratory viruses.

RSV Sequencing

Using the Sanger method (Supplementary Materials), we sequenced the second hypervariable region of the RSV G gene in NPAs and clearance swabs with sufficiently high genomic load. We confirmed the initial delayed clearance designation based on RSV subtype, when infants had NPA and clearance swab sequences that were identical or differed by 1 amino acid.

Statistical Analyses

To examine the association of the 4 microbiota profiles with delayed clearance, we constructed 2 generalized linear mixed-effects models with a binary response, accounting for potential patient clustering at the hospital level. First, we fitted an unadjusted model that included only microbiota profiles as the independent variable with the mixed profile as the reference [5]. Second, we constructed a multivariable model adjusting for 11 patient-level variables (ie, age, sex, gestational age, delivery mode, daycare attendance, lifetime history of antibiotic use, history of corticosteroid use, intensive care use, NPA RSV-cycle threshold [Ct] value, NPA CCL-5 levels, and serum LL-37 levels). Covariates were selected on the basis of a priori knowledge and biological plausibility. To examine the robustness of our findings, we performed a sensitivity analysis examining infants with solo RSV infections [1]. Data were analyzed using R, version 3.4.4. All *P* values were 2 tailed, with a *P* value of $<.05$ considered statistically significant.

RESULTS

Of the 921 children in the MARC-35 cohort, 673 (73%) had both a NPA at hospitalization and a clearance swab collected a median of 3.1 weeks (interquartile range [IQR], 3.0–3.6 weeks) after the first hospital day. Of these 673 infants, 557 (83%) had both RSV infection and microbiota data; 3 infants had RSV data but no microbiota data. These 557 infants composed the analytic cohort. There were 411 (74%) with RSV-A infection and 146 (26%) with RSV-B infection. The median age at hospitalization was 3 months (IQR, 2–6 months), 58% were male, and 54% were non-Hispanic white.

Delayed Clearance of RSV

Overall, 106 children (19%) had delayed clearance of RSV (ie, the same RSV subtype was detected at hospitalization and 3 weeks after date of hospitalization). We confirmed the delayed clearance categorization by comparing the G gene sequences in the 106 NPA–clearance swab pairs. We successfully sequenced 105 of 106 NPAs (99%). The median clearance swab cycle threshold (Ct) was 33.6 (IQR, 29.8–36.2). There were 54 clearance swabs with a $\text{Ct} \leq 33.6$ (a lower Ct indicates higher genomic load), and we successfully sequenced 44 (81%). The remaining 52 clearance swabs had a $\text{Ct} \geq 34$; we successfully sequenced 2 (4%). Among the 46 pairs that were successfully sequenced, 40 (93%) had identical RSV sequences at both time points; 3 had identical but incomplete sequences ($\geq 79\%$ base pairs were sequenced); and 3 pairs had a single amino acid change.

Table 1 shows patient characteristics, clinical variables, laboratory data, and nasopharyngeal microbiota clusters at hospitalization among infants with and those without delayed clearance. In general, compared with infants without delayed clearance, those with delayed clearance of RSV were younger, were less likely to have a previous breathing problem, weighed less, and had higher RSV genomic load (all *P* $< .05$).

Nasopharyngeal Microbiota Profiles and Delayed Clearance of RSV

In unadjusted analysis, there was no statistically significant association between the microbiota profiles and delayed clearance (Figure 1). However, in the multivariable model adjusting for 11 patient characteristics (eg, age, viral load, antibiotic use, and intensive care use) and clustering at the hospital level, the risk of delayed clearance was significantly higher in infants with a *Haemophilus*-dominant microbiota profile (odds ratio [OR] for comparison with mixed profile, 2.77; 95% confidence interval [CI], 1.35–5.71; *P* = .006; Figure 1). In the sensitivity analysis excluding viral coinfections, infants with solo RSV infections (*n* = 405) had consistent results—that is, the risk of delayed clearance remained significantly higher in infants with a *Haemophilus*-dominant profile (OR for comparison with mixed profile, 4.27; 95% CI, 1.77–10.29; *P* = .001).

DISCUSSION

In this multicenter study of infants hospitalized with bronchiolitis, we found that having a *Haemophilus*-dominant

Table 1. Patient Characteristics of 557 Infants Hospitalized for Respiratory Syncytial Virus (RSV) Bronchiolitis, According to Delayed Clearance of RSV

Variable	No Delayed Clearance (n = 451)	Delayed Clearance (n = 106)	P
Participants, % of sample	81	19	
Primary exposure			
Nasopharyngeal microbiota profile			.18
<i>Haemophilus</i> dominant	72 (16.0)	20 (18.9)	
Mixed	146 (32.4)	23 (21.7)	
<i>Moraxella</i> dominant	89 (19.7)	26 (24.5)	
<i>Streptococcus</i> dominant	144 (31.9)	37 (34.9)	
Characteristic			
Age, mo	3.0 (1.6–5.9)	2.3 (1.3–4.4)	.006
Female sex	190 (42.1)	42 (39.6)	.72
Race/ethnicity			.32
Non-Hispanic white	241 (53.4)	58 (54.7)	
Non-Hispanic black	77 (17.1)	19 (17.9)	
Hispanic	115 (25.5)	21 (19.8)	
Other	18 (4.0)	8 (7.5)	
Parental history of asthma	138 (30.6)	37 (34.9)	.08
Parental history of eczema	76 (16.9)	25 (23.6)	.03
Maternal smoking during pregnancy	54 (12.0)	10 (9.4)	.052
Mode of birth (cesarean section)	164 (36.4)	34 (32.1)	.37
Premature birth (32–37 wk of gestation)	83 (18.4)	23 (21.7)	.52
Previous breathing problems	78 (17.3)	7 (6.6)	.009
History of eczema	59 (13.1)	11 (10.4)	.55
Ever attended daycare	110 (24.4)	14 (13.2)	.02
Household sibling	354 (78.5)	79 (74.5)	.45
Breastfed	226 (50.1)	57 (53.8)	.71
Smoke exposure at home	59 (13.1)	14 (13.2)	.99
Corticosteroid use in lifetime	64 (14.2)	9 (8.5)	.16
Presentation and course at hospitalization for bronchiolitis			
Weight at presentation, kg	6.00 (4.80–7.70)	5.38 (4.50–6.60)	.004
Received antibiotics ^a	86 (19.1)	17 (16.0)	.72
Received corticosteroids ^a	44 (9.8)	3 (2.8)	.064
Laboratory testing			
Virology			
RSV solo infection	325 (72.1)	80 (75.5)	.56
Rhinovirus coinfection	64 (14.2)	12 (11.3)	.54
RSV Ct	22.8 (20.8–25.6)	21.6 (20.0–23.3)	.001
Blood eosinophilia (≥4% eosinophils)	32 (7.1)	10 (9.4)	.36
Serum total 25OHD level, ng/mL	26.6 (17.5–32.9)	26.0 (17.0–32.7)	.64
Serum LL37 level, ng/mL	46.0 (34.0–58.0)	44.0 (32.5–58.0)	.32
sIgE sensitization ^b	85 (18.8)	19 (17.9)	.94
Food sensitization	76 (16.9)	18 (17.0)	.99
Aeroallergen sensitization	11 (2.4)	1 (0.9)	.56
Nasopharyngeal CCL5 level, pg/mL	41.5 (18.4–93.0)	36.7 (19.2–99.9)	.90
Nasopharyngeal microbiota			
Observed OTUs	16.0 (9.0–24.0)	16.0 (8.3–24.8)	.88
Shannon index	0.9 (0.6–1.4)	0.9 (0.5–1.4)	.60
Clinical course			
Intensive care use ^c	73 (16.2)	14 (13.2)	.54
Admission to ICU	70 (15.5)	14 (13.2)	.65
Use of mechanical ventilation	25 (5.5)	5 (4.7)	.92
Hospitalization duration, d	2 (1–3)	2 (1–4)	.60

Data are no. (%) of infants or median value (interquartile range), unless otherwise indicated. Percentages may not equal 100, because of rounding and missing values.

Abbreviations: Ct, cycle threshold; ICU, intensive care unit; OTU, operational taxonomic unit; sIgE, specific immunoglobulin E; 25OHD, 25-hydroxyvitamin D.

^aDefined as any use of antibiotics or corticosteroids up until the time the child was hospitalized, including use in the emergency department.

^bDefined by having ≥1 positive value for serum allergen-specific IgE at the index hospitalization.

^cDefined as admission to the ICU 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 hospitalization.

Figure 1. Unadjusted and adjusted associations between nasopharyngeal microbiota profiles and delayed clearance of respiratory syncytial virus (RSV; n = 557). *A*, The unadjusted association between the 4 microbiota profiles and the risk of delayed clearance of RSV. *B*, The multivariable model adjusting for 11 patient-level variables (ie, age, sex, gestational age, delivery mode, daycare attendance, lifetime history of antibiotic use, history of corticosteroid use, intensive care use, RSV-cycle threshold [CT] value, nasopharyngeal CCL-5 levels, and serum LL-37 levels).

nasopharyngeal microbiota at hospitalization was associated with a higher risk of delayed clearance of RSV, even after adjustment for viral load, acute severity, and 9 other patient-level factors. These results not only confirm that delayed clearance occurs in approximately 20% of infants hospitalized with RSV bronchiolitis, but also extend this finding by demonstrating, for the first time, that the composition of the nasopharyngeal microbiota is related to delayed RSV clearance.

In 1976, Hall et al used viral culture to describe delayed RSV clearance for up to 21 days among 23 children hospitalized for either RSV pneumonia or bronchiolitis [2]. More recently, in 2017, Brint et al reported that 6 of 41 infants (15%) hospitalized with RSV continued to be RSV qRT-PCR positive 1 month after hospitalization [3]. Our larger, diverse sample from multiple hospitals across the United States found that almost 20% of infants hospitalized with RSV bronchiolitis continue to shed RSV for at least 3 weeks after hospitalization. Although the ability of RSV to replicate for weeks after hospitalization has implications for RSV transmission and pathobiology of long-term sequelae, the underlying pathobiology of this observation remains largely unclear.

One possible factor affecting viral clearance is the airway microbiome. Indeed, emerging evidence suggests that a *Haemophilus*-dominant nasopharyngeal microbiota is associated with neutrophil recruitment and activation [6], which is intriguing given the beneficial (eg, limiting viral replication) and harmful (eg, airway damage) effects of neutrophils in RSV bronchiolitis [11]. Despite this complexity, *Haemophilus* dominance was associated with acute disease severity (ie, a need for hospitalization) in children aged <2 years with RSV infection [6]. Moreover, in 2 separate large, multicenter infant cohorts,

our group showed that *Haemophilus* dominance was related to higher risks of intensive care [7]. Although the mechanism of this microbiota-acute severity association remains unclear, Følsgaard et al demonstrated that nasal *Haemophilus* colonization in asymptomatic infants was associated with a distinct immune profile that may inhibit the T-helper type 1 response needed to clear RSV [8]. In the present analysis, we extend this burgeoning line of inquiry by demonstrating that *Haemophilus* dominance in the nasopharynx is associated with delayed clearance of RSV. Taken together, these results suggest a complex interplay between RSV infection, the host innate and adaptive immune response, and the nasopharyngeal microbiota.

Adding to this complexity is the uncertainty about the directionality of the relationship between viral infection and airway microbiota composition. Does viral infection enhance or select for a microbial relative abundance, or does the extant microbiota increase susceptibility to specific viral infections? More likely is that the relationship between viral infections and the nasopharyngeal microbiome is bidirectional. Indeed, RSV infection may create the local environmental conditions for an increase in the abundance of *Haemophilus* [12, 13]. And the reverse may also be true; the nasopharyngeal microbiome may influence the susceptibility to viral infections [5], including bronchiolitis [14]. Adding to the viral-microbial literature are the present results, which suggest that the microbiota is associated with viral clearance. It is becoming increasingly clear that understanding the underlying pathobiology of “viral” bronchiolitis will require some understanding of the microbial ecosystem within which the virus infects the child.

This study has potential limitations. First, although some have questioned whether a positive qRT-PCR result reflects

active RSV replication, one trial demonstrated that once replication ceases, RSV RNA is no longer present in samples [15]. Second, the study population consists of infants hospitalized with bronchiolitis, and the results may not apply to older children with RSV infections. Nonetheless, bronchiolitis is the leading cause of infant hospitalization, and RSV is the most common cause [1]. Third, we collected samples from only 2 time points. Finally, the present analysis did not examine the relationship between RSV gene sequences and delayed RSV clearance. We plan to pursue this important research question.

In summary, in a large, prospective, multicenter cohort of infants hospitalized with RSV bronchiolitis, we found that 19% of infants have delayed RSV clearance and that a *Haemophilus*-dominant nasopharyngeal microbiota is associated with this delay. Although causal inference remains premature, these findings support further investigation into the complex interplay between viral infection, host response, and airway microbiota, as well as further clinical correlates with delayed clearance of RSV after bronchiolitis hospitalization.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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