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MAJOR ARTICLE

Nasopharyngeal Codetection *of Haemophilus influenzae* and *Streptococcus pneumoniae* Shapes Respiratory Syncytial Virus Disease Outcomes in Children

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Background. The role of nasopharyngeal bacteria in respiratory syncytial virus (RSV) disease has been underestimated. We measured the frequency and burden of respiratory bacteria in the upper respiratory tract of infants with RSV infection over 7 respiratory seasons, and their impact on clinical outcomes.

Methods. Children <2 years old with mild (outpatients, n = 115) or severe (inpatients, n = 566) RSV infection, and matched healthy controls (n = 161) were enrolled. Nasopharyngeal samples were obtained for RSV, *Streptococcus pneumoniae, Staphylococcus aureus, Moraxella catarrhalis,* and *Haemophilus influenzae* detection and quantitation by PCR. Multivariable models were constructed to identify variables predictive of severe disease.

Results. *S. pneumoniae*, *H. influenzae,* and *M. catarrhalis,* but not *S. aureus,* were detected more frequently in RSV-infected children (84%) than healthy controls (46%; $P < .001$). Detection of *S. pneumoniae* and/or *H. influenzae* was associated with fever, more frequent antibiotic treatment, worse radiologic findings, and higher neutrophil counts ($P < .01$). In adjusted analyses, *S. pneumoniae/H. influenzae* codetection was independently associated with greater odds of hospitalization, higher disease severity scores, need for supplemental oxygen, and longer hospitalization.

Conclusions. Nasopharyngeal codetection of *S. pneumoniae* and *H. influenzae* in infants with RSV infection is associated with increased disease severity.

Keywords. RSV; nasopharyngeal bacterial colonization; bacterial PCR; disease severity; infants.

Respiratory syncytial virus (RSV) is the leading cause of bronchiolitis in infants, of pneumonia in children <5 years of age, and the second cause of death in the first year of life in resource-limited countries [1, 2]. There are groups of children at high risk for severe RSV disease; however, the majority of infants hospitalized with RSV infection are previously healthy [3, 4]. Studies have shown that viral factors, a dysregulated host immune response, and genetic predisposition play a role in RSV disease severity [5, 6]. Nevertheless, none of these factors can entirely explain the wide differences in clinical presentations and outcomes observed in infants with RSV infection.

Our previous studies in small cohorts of children with RSV infection, also confirmed by others, suggested that detection of *Streptococcus pneumoniae* or *Haemophilus influenzae* in the upper respiratory tract was associated with differences in host immune responses and clinical outcomes [7–10]. Nevertheless, there are still significant knowledge gaps regarding the frequency, potential additive effect, and clinical implications of bacterial detection in the respiratory tract of children with RSV infection. In particular, the role of these bacteria alone or in combination, and whether bacterial loads influence RSV disease pathogenesis and severity, have not been fully defined.

The aims of this study were (1) to define the frequency of nasopharyngeal bacterial detection in young children with mild (outpatients) and severe (inpatients) RSV infection, compared to healthy age- and season-matched controls across several respiratory seasons; and (2) to define whether specific bacteria, bacterial loads, and/or combination of specific bacteria were associated with enhanced disease severity.

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METHODS

Study Design

From December 2010 to December 2018, we conducted a prospective observational study and enrolled a convenience sample of previously healthy children <2 years of age hospitalized or evaluated in the outpatient setting with RSV infection. RSV was diagnosed per standard of care by rapid antigen or polymerase chain reaction (PCR) test. We excluded premature children (≤36 weeks' gestation), those with previous history of wheezing or chronic medical conditions (ie, congenital heart disease, chronic lung disease, immunodeficiency). Inpatients were enrolled within medians 25%–75% interquartile range (IQR) 24 (18–34) hours of hospitalization to Nationwide Children's Hospital (NCH) inpatient units, and outpatients at the time of presentation at the emergency department or primary care clinics.

Each respiratory season, we also enrolled healthy control children of similar age, sex, and race/ethnicity compared to RSV cases, with no respiratory symptoms or treated with antibiotics within 2 weeks of enrollment. Controls were enrolled in the operating room while undergoing minor scheduled surgical procedures, or at primary care offices during well-child visits. The study was approved by the institutional review board at NCH, and informed consent obtained from legal guardians before study participation.

Data and Sample Collection

In all study subjects, we collected demographic and clinical information using a standardized questionnaire designed for the study. We also collected 2 upper respiratory swabs for bacterial detection and RSV quantitation respectively, and a blood sample for white blood cell (WBC) count with differential analyses. Disease severity was assessed by the need for hospitalization and a standardized clinical disease severity score (CDSS) that included 5 parameters (transcutaneous oxygen saturation, respiratory rate, chest wall retractions, auscultation, and level of activity) [11]. The CDSS ranged from 0 (normal) to 15 (most severe disease) and has been validated internally and externally in other studies [12–15]. In hospitalized infants, we analyzed additional criteria of severity including duration of hospitalization, oxygen administration, and need for pediatric intensive care unit (PICU) care.

Nasopharyngeal bacterial swabs were obtained in RSV patients and healthy controls as described [9] and aliquots stored at −80°C until further quantitation by real time PCR (qRT-PCR) for *S. pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and *H. influenzae* using published primers (Supplementary Table 1) [8]. Bacterial loads were measured in copies/mL and $\log_{10}$ transformed for analyses purposes. These bacteria were selected based on previous studies that showed their relevant role in acute and long-term respiratory morbidity in children [9, 16–18]. Midturbinate swabs were also obtained in RSV patients and controls for RSV detection, quantitation, and typing using qRT-PCR targeting the N gene as described [5, 9, 13, 14].

Statistical Analysis

Baseline characteristics are reported using means with standard deviation, medians with 25%–75% interquartile ranges, or frequencies with percentages. For continuous variables, group comparisons were assessed using 2-tailed *t* test with a Satterthwaite correction for unequal group variance, Mann-Whitney test, 1-way analysis of variance (ANOVA), or Kruskal-Wallis tests with Dunn correction for multiplicity when appropriate. Categorical data were analyzed using χ^2 or Fisher exact tests.

To assess the role of nasopharyngeal bacteria on clinical outcomes we conducted multivariate logistic regression for binary outcomes (need for hospitalization and oxygen administration) and ordinal logistic regression for continuous outcomes (duration of hospitalization and the CDSS). For analyses purposes, children were categorized based on the CDSS as mild (≤3), which represents the mildest disease phenotype and did not include administration of supplemental oxygen, moderate (4–7), or severe (≥8–15). Covariates were included in the models if they were clinically meaningful or had univariate *P* values of <.15, and were retained if they had an adjusted *P* value of <.1 or if their inclusion had a substantial impact on the models' goodness of fit, based on Akaike information criterion (AIC) [19]. These covariates included age, sex, race, antibiotic use at enrollment, blood neutrophil counts, RSV loads, and bacterial detection. Statistical analyses were conducted using GraphPad Prism version 8.0 and SAS version 9.4, with a 2-sided *P* value < .05 considered statistically significant.

RESULTS

Nasopharyngeal Detection of Potentially Pathogenic Bacteria in Infants with RSV Infection

From December 2010 to December 2018, we enrolled 878 children <2 years of age, and excluded 36 children for the reasons outlined in Figure 1, leaving a total of 681 children with RSV infection and 161 healthy controls. Within the RSV cohort, 566 children (83%) were hospitalized (ward n = 375, 55%; PICU n = 191, 28%) and 115 (17%) were managed as outpatients (Table 1). The majority of RSV patients (n = 620, 91%) were <12 months of age. Median age for healthy controls and RSV outpatients was comparable (6–7 months), but higher than for inpatients (2.5 months). Inpatients also showed significantly lower RSV loads than outpatients (*P* < .001).

First, we compared the frequency of *S. aureus*, *S. pneumoniae*, *H. influenzae,* and *M. catarrhalis* detection by qRT-PCR in children with RSV infection versus healthy controls. Detection of at least 1 of the 4 potentially pathogenic bacteria was more common in RSV patients, irrespective of the need for hospitalization, than in healthy controls (89% vs

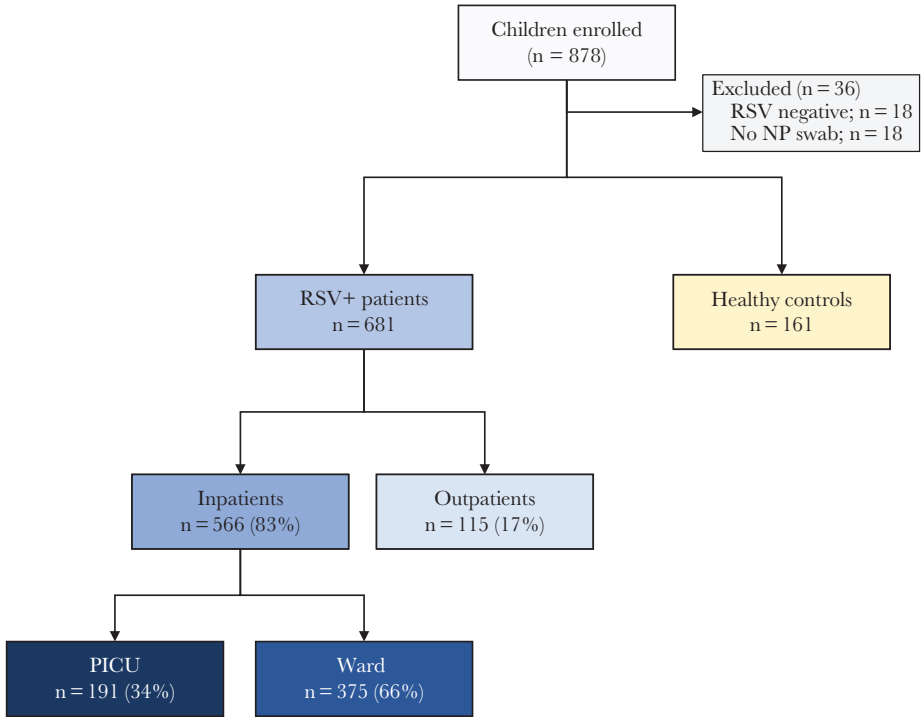

Figure 1. Flowchart of study participants. The upper boxes in gray indicate the number of patients and controls enrolled and the reasons for exclusion. Children with RSV infection included in the study (blue boxes) were classified according to disease severity in RSV outpatients and RSV inpatients (ward and PICU). Yellow box indicates the number of healthy controls enrolled. Abbreviations: NP, nasopharyngeal; PICU, pediatric intensive care unit; RSV, respiratory syncytial virus.

65%; $P < .0001$; Figure 2A). Detection of >1 of these 4 potentially pathogenic bacteria was also more common in children with RSV infection (58% inpatients, 59% outpatients) than in controls (27%; $P < .0001$; Figure 2A and 2B).

Analyses of the contribution of these bacteria as a single pathogen or combined with other bacteria (any detection) is shown in Figure 2B and 2C. *S. aureus* detection alone was more common in healthy controls than RSV patients (19%

Table 1. Demographic Characteristics of RSV Patients and Healthy Controls

	Healthy Controls (n = 161)	All RSV Patients (n = 681)	RSV Inpatients (n = 566)	RSV Outpatients (n = 115)	*P* Value[a]	*P* Value[b]
Age, mo, median (25%–75% IQR)	7.0 (4–10.2)	2.9 (1.5–6.5)	2.5 (1.4–5.4)	6.0 (3.4–10)	<.0001	<.0001
Sex, male	117 (73)	372 (55)	315 (56)	57 (50)	<.0001	.258
Race						
White	103 (64)	454 (67)	401 (71)	53 (46)	.251	<.0001
Black	38 (24)	124 (18)	81 (14)	43 (37)		
Other	20 (12)	103 (15)	84 (15)	19 (16)		
Delivery, vaginal	118 (73)	509 (75)	419 (74)	90 (78)	.689	.409
Daycare attendance	31 (19)	202 (30)	159 (28)	43 (37)	.008	.056
Smoke exposure	31 (19)	233 (34)	199 (35)	34 (30)	.0002	.281
Breastfeeding	60 (37)	237 (35)	188 (33)	49 (43)	.582	.067
Immunizations	146 (91)	578 (85)	481 (85)	97 (84)	.058	.886
Parental asthma	82 (51)	347 (51)	287 (51)	60 (52)	1.000	.838
RSV A	…	393 (59)	343 (63)	50 (43)		.0002
RSV B	…	270 (41)	205 (37)	65 (57)		
RSV loads, $\log_{10}$ copies/mL, median (25%–75% IQR)[c]	Negative	7.5 (5–7.6)	7.0 (6.2–7.8)	7.9 (6.9–8.4)		<.0001

Data are No. (%) except where indicated. We used the χ^2 or Fisher exact tests for categorical variables, and Mann-Whitney *U* or Kruskal-Wallis tests for continuous variables.

Abbreviations: IQR, interquartile range; RSV, respiratory syncytial virus.

[a] *P* value for comparisons between healthy controls and RSV patients.

[b] *P* value for comparisons between RSV inpatients and RSV outpatients.

[c] Data not available for 18 RSV inpatients.

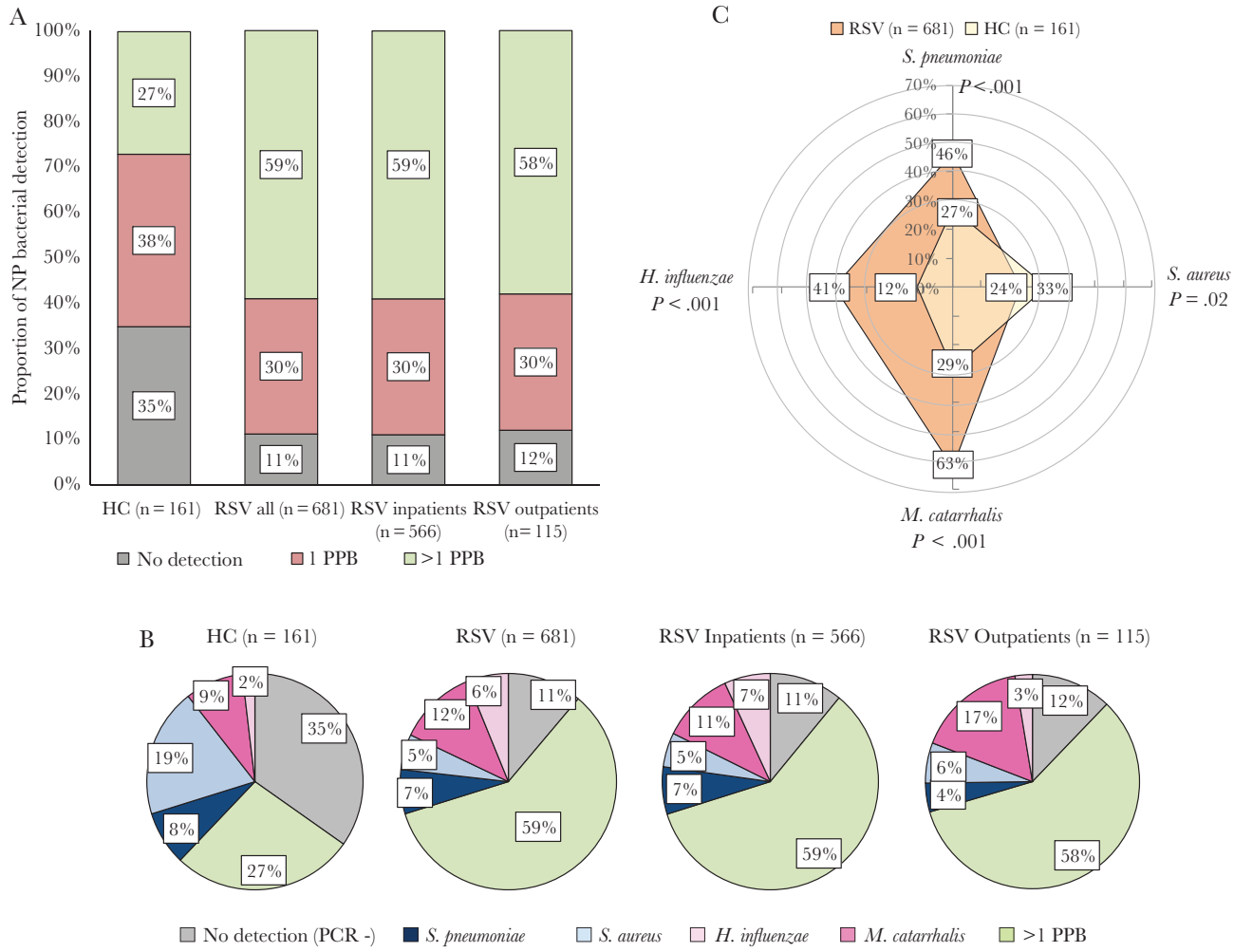

Figure 2. Detection of potentially pathogenic bacteria (PPB) in the upper respiratory tract of young children with respiratory syncytial virus (RSV) infection and healthy age controls. *A*, Frequency of nasopharyngeal (NP) bacterial detection in study patients. The horizontal axis represents the study groups and the vertical axis represents the percentage of NP bacterial detection (*Streptococcus pneumoniae, Staphylococcus aureus, Moraxella catarrhalis, Haemophilus influenzae*): negative detection for any of the 4 bacteria (gray), detection of 1 of these 4 PPB (red), and detection of >1 PPB (green). *B*, Frequency of NP detection of specific bacteria. Pie charts for healthy controls and children with RSV infection (all, inpatients, and outpatients) showing the percentage detection of none of the 4 bacteria by PCR (gray), *S. pneumoniae* only (dark blue), *S. aureus* only (light blue), *M. catarrhalis* only (dark pink), *H. influenzae* only (light pink), and >1 PPB (green). *C*, Radar plot depicting the frequency of any detection of *S. pneumoniae, S. aureus, M. catarrhalis,* and *H. influenzae* in children with RSV infection (orange) compared to healthy controls (HC; light yellow). *P* values indicate the comparisons between RSV and HC for any detection (alone and in combination with other bacteria) of each of the 4 bacteria.

vs 5%, respectively; $P < .001$), while *H. influenzae* alone was more frequently detected in RSV patients than controls (6% vs 2%, respectively; $P = .03$; Figure 2B). Any detection of *S. aureus* was also more common in healthy controls than RSV patients (33% vs 24%, respectively; $P = .02$; Figure 2C). On the other hand, any detection of *S. pneumoniae* (46% vs 27%; $P < .001$) and *M. catarrhalis* (63% vs 29%; $P < .001$) was more common in RSV patients that in healthy controls. We also assessed the types of bacterial associations. The likelihood of *S. pneumoniae*, *M. catarrhalis*, and *H. influenzae* codetections was high, while *S. aureus* detection was inversely associated with *H. influenzae* and not associated with *M. catarrhalis* or *S. pneumoniae* detection (Supplementary Figure 1).

Nasopharyngeal Bacterial Detection According to Age

To evaluate differences in bacterial detection according to age, RSV patients and healthy controls were stratified in 4 age groups: <3 months, 3 to <6 months, 6 to <12 months, and 12–24 months (Figure 3). Single detection of *S. pneumoniae, M. catarrhalis,* and *H. influenzae* remained stable in RSV patients and healthy controls across age groups. *S. aureus* detection decreased significantly with increasing age in RSV patients (9% in <3 months vs 0% in >12 months; $P = .005$) and controls (31% in <3 months vs 3% in >12 months; $P < .0001$). Detection of more than 1 of the 4 potentially pathogenic bacteria remained higher in RSV patients than in controls across all age groups, and increased with age with 47% detection of >1 bacterium in RSV infants <3 months and 72% in the >12–24 months age group ($P < .0001$).

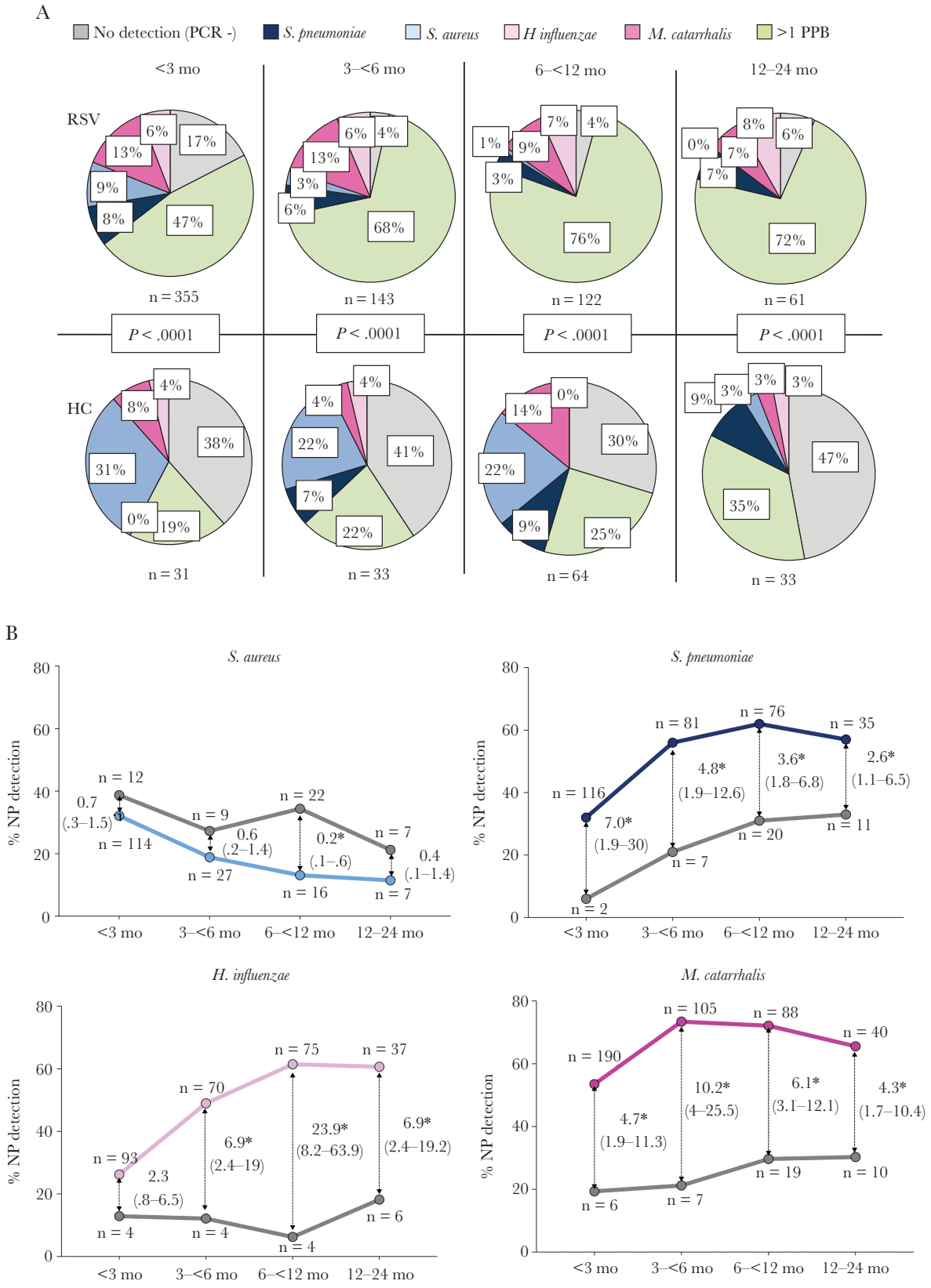

Figure 3. Nasopharyngeal (NP) bacterial detection stratified by age in children with respiratory syncytial virus (RSV) infection in relation to healthy controls. *A*, Pie charts depicting the proportion of nasopharyngeal bacterial detected in RSV patients (upper panels) and healthy controls (HC; lower panels) stratified by age: no detection of the 4 bacteria (PCR–; gray), single detection of *Streptococcus pneumoniae* (dark blue), *Staphylococcus aureus* (light blue), *Moraxella catarrhalis* (dark pink), *Haemophilus influenzae* (light pink), and >1 potentially pathogenic bacteria (PPB; green). Comparisons by χ^2 for each age group. *B*, Odds of *S. aureus*, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* detection in RSV patients (colored lines) in relation to healthy controls (gray lines) according to age. Thin arrows indicate the odds ratio (95% confidence interval) for each age group comparison. Asterisks indicate the time points that are significantly different. The numbers of RSV patients and controls per bacteria and age group are depicted in each plot. Analyses by χ^2 test for trends.

Any detection of *S. pneumoniae, H. influenzae,* or *M. catarrhalis* was consistently higher in RSV patients versus controls ($P < .0001$). While *S. pneumoniae* and *H. influenzae* detection increased with age, detection of *M. catarrhalis* remained stable (Figure 3B). Rates of bacterial detection across the 7 respiratory seasons was not significantly different (Supplementary Figure 2).

Nasopharyngeal Bacterial Loads and Clinical Outcomes in Children with RSV Infection

Next, we evaluated whether the pattern and loads of nasopharyngeal bacteria were associated with clinical parameters (Table 2). Children with RSV infection with any of the bacteria identified had fever at presentation (> 38°C) more frequently (67% vs 30% for negative PCR; $P < .0001$), higher CDSS ($P = .03$), higher WBC

Table 2. Demographic and Clinical Characteristics of RSV Patients Stratified by Potentially Pathogenic Bacteria

Characteristic	PCR– (n = 76)	Any Bacteria (n = 605)	*S. pneumoniae* (n = 45)	*S. aureus* (n = 36)	*M. catarrhalis* (n = 81)	*H. influenzae* (n = 42)	>1 Bacteria (n = 401)	*P* Value[a]	*P* Value[b]
Demographic characteristics									
Age, mo, median (25%–75% IQR)	1.4 (0.9–2.3)	3.2 (1.6–6.8)	2.2 (1–4)	1.6 (0.9–2.5)	2.4 (1.6–4.8)	3.2 (1.6–7.1)	3.8 (2–7.8)	<.0001	<.0001
Sex (male)	44 (58)	328 (54)	23 (51)	14 (39)	45 (56)	27 (64)	219 (55)	.625	.330
Race, white	57 (75)	397 (66)	36 (80)	24 (67)	49 (61)	35 (83)	253 (63)	.047	.018
Vaginal delivery	53 (70)	456 (75)	30 (67)	26 (72)	59 (74)	34 (81)	307 (76)	.326	.528
Daycare	11 (14)	191 (32)	9 (20)	2 (6)	24 (30)	11 (26)	145 (36)	<.0001	.002
Smoke exposure	26 (34)	207 (34)	12 (27)	11 (31)	27 (33)	20 (48)	137 (34)	1.000	.451
Breastfeeding	37 (49)	200 (33)	21 (47)	17 (47)	27 (33)	14 (33)	121 (30)	.010	.008
Parental asthma	28 (37)	319 (53)	23 (51)	18 (50)	38 (47)	23 (55)	217 (54)	.010	.133
Immunizations	66 (87)	513 (85)	38 (84)	32 (89)	71 (88)	39 (93)	333 (83)	.734	.502
Clinical parameters									
Duration of symptoms, d, median (25%–75% IQR)[c]	5 (4–6)	5 (3–6)	5 (3.5–5.5)	4 (3–5.7)	5 (4–6)	5.5 (4–6)	4 (3–6)	.095	.088
Presence of fever[c]	23 (30)	403 (67)	34 (76)	10 (28)	39 (48)	33 (79)	287 (72)	<.0001	<.0001
Hospitalized	62 (81)	504 (83)	40 (89)	29 (81)	62 (76)	39 (93)	334 (83)	.745	.243
Supp. O_2 need	41 (54)	369 (61)	33 (73)	19 (53)	43 (53)	31 (74)	243(60)	.263	.060
Ventilatory support[d]	17 (42)	134 (36)	10 (30)	4 (21)	19 (45)	9 (29)	92 (38)	.609	.423
Supp. O_2 duration, h, median (25%–75% IQR)	48 (22–76)	48 (24–95)	36 (23–114)	24 (12–72)	48 (35–73)	45 (24–72)	48 (24–96)	.622	.584
CDSS, median (25%–75% IQR)	6 (4–9)	7 (5–10)	8 (5–10.5)	6 (3–8)	7 (4–9)	7 (5–10)	7 (5–10)	.037	.027
Antibiotic treatment[c]	30 (39)	271 (45)	28 (62)	9 (25)	30 (37)	28 (67)	176 (44)	.393	.001
PICU need	23 (30)	168 (28)	14 (31)	6 (17)	20 (25)	14 (33)	114 (28)	.684	.583
PICU stay, h, median (25%–75% IQR)	54 (32–101)	88 (54–146)	98 (53–154)	63 (46–156)	89 (39–110)	76 (41–175)	92 (57–159)	.002	.049
Hospitalization, d, median (25%–75% IQR)	2.7 (1.7–4)	2.8 (1.8–4.5)	2.6 (1.8–4.7)	2 (1.5–3.5)	2.7 (1.6–4.1)	2.7 (1.–5.8)	2.9 (1.8–4.5)	.625	.560
Laboratory and radiologic studies									
WBC 10^3/mL, median (25%–75% IQR)	8.5 (7–10)	10.6 (8–13)	11.5 (7–13)	8.2 (7–11)	10 (8–12)	11 (9–15)	11.2 (9–14)	<.0001	<.0001
Neutrophils %, median (25%–75% IQR)	19 (14–28)	35 (25–47)	36.5 (29–49)	21 (13–31)	29 (21–45)	42 (29–56)	36 (26–48)	<.0001	<.0001
Lymphocytes %, median (25%–75% IQR)	65 (56–73)	51 (39–61)	49 (39–55)	65 (55–75)	54 (38–64)	51 (33–58)	50 (39–60)	<.0001	<.0001
Radiology									
Normal	7 (14)	26 (6	1 (3)	1 (6)	5 (11)	0 (0)	19 (7)	.026	.043
BWT/hyperinflation	29 (57)	187 (45	21 (55)	12 (71)	22 (47)	13 (41)	119(42)		
Atelectasis	7 (13)	118 (28)	6 (16)	4 (23)	11 (24)	11 (34)	86 (30)		
Lobar consolidation	8 (16)	86 (21)	10 (26)	0 (0)	8 (18)	8 (25)	60 (21)		
RSV load, log10 copies/mL, median (25%–75% IQR)	7.5 (6.5–8.2)	7.1 (6.3–7.9)	6.8 (5.8–7.9)	7.5 (6.4–8.4)	7.1 (6.4–7.9)	6.8 (6.1–7.8)	7.1 (6.3–7.9)	.185	.178

Data are No. (%) except where indicated. We used the χ^2 test for categorical variables, and the Kruskal-Wallis test for continuous variables to calculate *P* values.

Abbreviations: BWT, bronchial wall thickening; CDSS, clinical disease severity score; IQR, interquartile range; PCR, polymerase chain reaction; PICU, pediatric intensive care unit; PPB, potentially pathogenic bacteria; RSV, respiratory syncytial virus; WBC, white blood cell.

[a] *P* value for comparisons between PCR– and any PPB.

[b] *P* value for comparisons between PCR–, individual bacteria, and >1 PPB (6 groups).

[c] Presence of fever, duration of symptoms, and antibiotic treatment at enrollment.

[d] Indicates invasive and noninvasive ventilatory support.

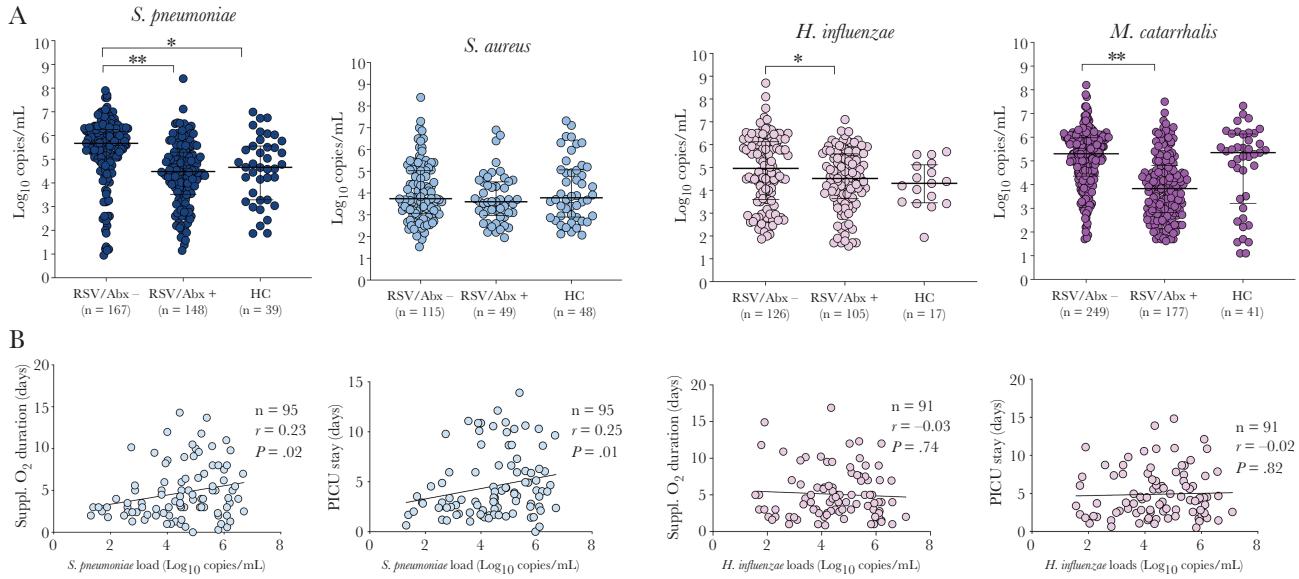

Figure 4. Bacterial loads in children with respiratory syncytial virus (RSV) infection and healthy controls. *A*, Bacterial loads in healthy controls and children with RSV infection according to antibiotic use at study enrollment. The horizontal axis represents RSV-infected children not treated with antibiotics (RSV/Abx–), those treated (RSV/Abx+), and healthy controls (HC). The Y-axis represent bacterial loads in $\log_{10}$ copies/mL for each bacterium. Comparisons by Mann-Whitney *U* test between RSV/Abx– and RSV/Abx+, and between RSV/Abx– and HC. **P* < .05; ***P* < .01. *B*, Spearman correlations between *Streptococcus pneumoniae* (blue) and *Haemophilus influenzae* (pink) loads and duration of supplemental oxygen and pediatric intensive care unit (PICU) stay among patients admitted to the PICU. The number of patients (n), correlation coefficient (*r*), and *P* value are included in each plot.

counts and neutrophil percentages ($P < .0001$), and atelectasis and/or consolidation by chest X-ray ($P = .02$). Similar results were identified in children with RSV infection with *S. pneumoniae* or *H. influenzae* detection, but not in those with *S. aureus* detection or negative PCR results for the 4 bacteria. In fact, children with *S. aureus* detection or negative bacterial PCR had the lowest CDSS compared to children with other bacteria ($P = .02$; Supplementary Figure 3).

We then analyzed the role of bacterial loads on clinical outcomes. Although bacterial detection was conducted by PCR and patients were enrolled early in the disease course, we assessed whether prior antibiotic treatment (any dose) had an impact on bacterial detection and quantitation. Forty-four percent of RSV patients, the majority in the PICU, had received antibiotics for a median of 25%–75% interquartile range of 24 (24–48) hours at the time of study enrollment (Supplementary Table 2). Rates of bacterial detection in children with RSV infection were similar, irrespective of antibiotic treatment (antibiotic treated, 87% and nontreated, 91%; $P = .14$). None of the healthy controls had received antibiotics as this was an exclusion criterion. *S. pneumoniae, H. influenzae,* and *M. catarrhalis* bacterial loads, measured in $\log_{10}$ copies/mL, were lower in children that received antibiotics with activity against these bacteria (Figure 4A and Supplementary Table 3). Excluding children who had received antibiotics, we found that *S. pneumoniae* loads were significantly higher ($P = .0003$) and *H. influenzae* loads modestly higher ($P = .05$) in children with RSV infection than in healthy controls (Figure 4A), with no differences in *M. catarrhalis* and *S. aureus* loads. In addition, *S. pneumoniae*, but not *H. influenzae,* loads in children with severe RSV infection that required PICU care were significantly correlated with duration of oxygen and PICU length of stay (Figure 4B).

Influence of *S. pneumoniae* and *H. influenzae* Detection on RSV Clinical Outcomes

As we found that *S. pneumoniae* and/or *H. influenzae* were associated with differences in clinical parameters, we analyzed the possible synergistic effect of these bacteria on disease severity. To this end, RSV patients were stratified according to the presence/absence of these bacteria: no detection (*S. pneumoniae* negative/*H. influenzae* negative; n = 232), detection of one but not the other (*S. pneumoniae* positive/*H. influenzae* negative; n = 172) or (*S. pneumoniae* negative/*H. influenzae* positive; n = 134), and codetection of both (*S. pneumoniae* positive/*H. influenzae positive*; n = 143; Supplementary Table 4).

RSV patients with *S. pneumoniae*/*H. influenzae* codetection, followed by the *H. influenzae* or *S. pneumoniae* groups had fever more frequently (88% vs 75% vs 73% respectively) compared with the *S. pneumoniae* negative/*H. influenzae* negative group (35%; $P < .0001$). Oxygen administration was also more frequent in the codetection (66%) and the *S. pneumoniae* negative/*H. influenzae* positive (66%) groups versus the *S. pneumoniae* negative/*H. influenzae* negative group (53%; $P = .03$). In addition, children with RSV infection with *S. pneumoniae*/*H. influenzae* codetection versus no detection had higher CDSS (8 vs 6; $P = .001$), increased rates of PICU admission (37% vs 25%;

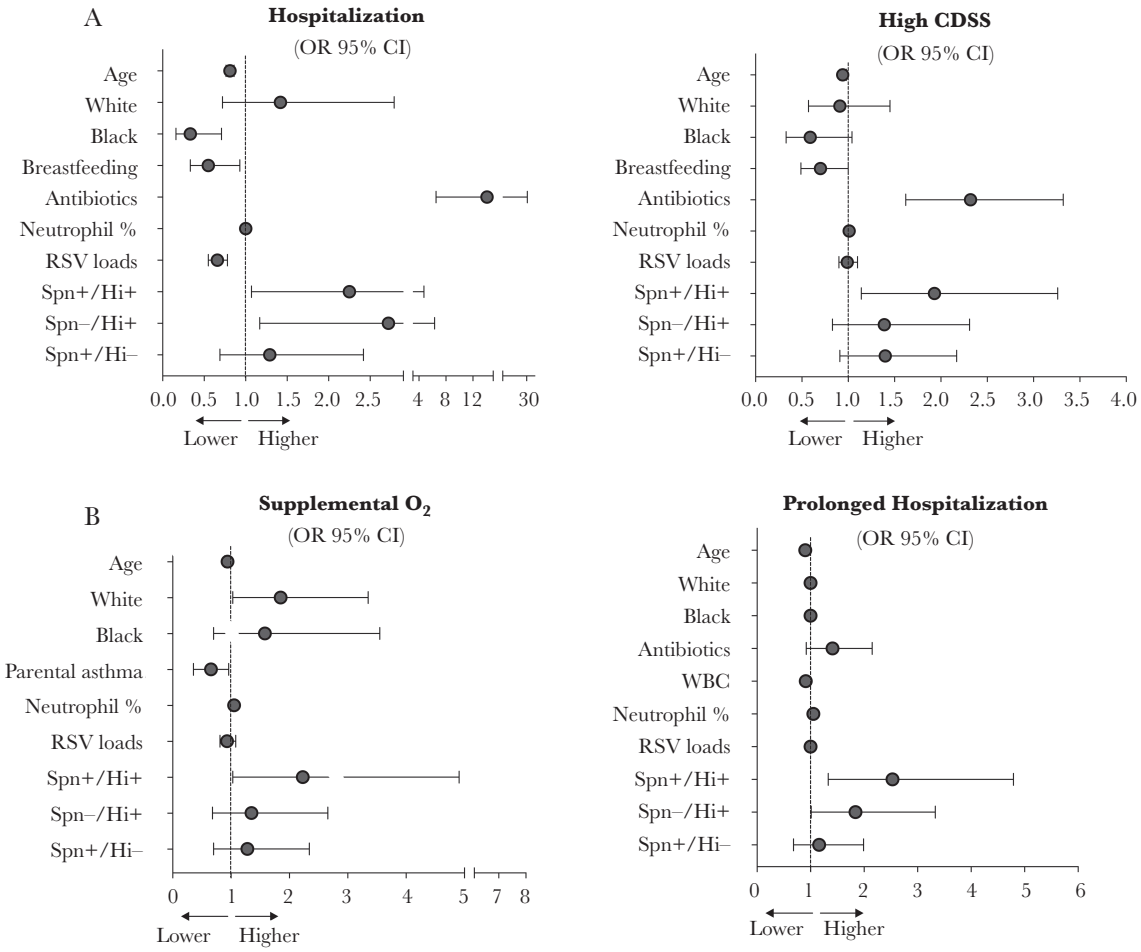

Figure 5. Predictors of disease severity in children with RSV infection. *A,* Adjusted need for hospitalization and high CDSS evaluated in the whole cohort of RSV patients. High CDSS was defined as a CDSS >3. *B,* Adjusted odds of oxygen use and prolonged hospital stay defined by a duration of hospitalization of ≥3 days. Reference group for race is other race. Reference group for bacterial classification is no detection of *Streptococcus pneumoniae* and *Haemophilus influenzae* (Spn–/Hi–). Data are adjusted ORs with point estimates and 95% CIs. Abbreviations: CDSS, clinical disease severity score; CI, confidence interval; Hi, *H. influenzae*; OR, odds ratio; RSV, respiratory syncytial virus; Spn, *S. pneumoniae*.

P = .03), higher WBC and neutrophil counts (P < .0001), and atelectasis/consolidation documented by chest X-ray more frequently (56% vs 31%; P < .001).

To confirm these findings, we conducted multivariable analyses and calculated the odds ratio (OR) and 95% confidence interval (CI) of hospitalization and a high CDSS in all RSV patients (Supplementary Table 5), and the need for oxygen and duration of hospitalization in RSV inpatients (Figure 5 and Supplementary Table 6). We included in all models demographic data, clinical variables, RSV loads, and detection of *S. pneumoniae* and *H. influenzae* according to the scheme described above.

After controlling for other factors, children with RSV infection in the *S. pneumoniae* negative/*H. influenzae* positive group (OR, 2.72; 95% CI, 1.17–6.33), and in the codetection *S. pneumoniae* positive/*H. influenzae* positive group (OR, 2.25; 95% CI, 1.07–4.74) had greater odds of hospitalization. Codetection of *S. pneumoniae*/*H. influenzae* was also associated with high CDSS (OR, 1.93; 95% CI, 1.14–3.26). In hospitalized children, *S. pneumoniae*/*H. influenzae* codetection was associated with higher odds of oxygen administration (OR, 2.23; 95% CI, 1.01–4.91). In addition, *S. pneumoniae* negative/*H. influenzae* positive (OR, 1.84; 95% CI, 1.01–3.33) and *S. pneumoniae* positive/*H. influenzae* positive (OR, 2.53; 95% CI, 1.33–4.79) detection were associated with increase odds of prolonged hospitalization. Together, these data indicate that detection of *H. influenzae,* and especially the simultaneous detection of *S. pneumoniae* and *H. influenzae,* in children with RSV infection is associated with increased disease severity, even after adjusting for other covariables such as age and viral load.

DISCUSSION

In this study we analyzed a large cohort of young children with mild or severe RSV infection over several respiratory seasons to explore whether the detection and loads of specific bacteria in the upper respiratory tract were associated with clinical

outcomes. We found that compared with healthy controls of similar demographic characteristics, *M. catarrhalis, S. pneumoniae,* and *H. influenzae,* but not *S. aureus*, were frequently identified in RSV-infected children, both outpatients and inpatients. We also found that the detection of *S. pneumoniae* or *H. influenzae* was associated with worse laboratory, radiologic, and clinical parameters. However, it was the codetection of *S. pneumoniae* and *H. influenzae* that consistently increased the risk for severe disease defined by greater odds of hospitalization, clinical scores, oxygen administration, and prolonged hospital stay.

There is an increasing interest in understanding the interplay between the host, RSV, and the respiratory microbiota, which has been recognized as an important contributor to respiratory morbidity in children [20–22]. Studies conducted in selected and unselected populations of children showed the presence of a stable respiratory microbiome during health that was altered with incursions of *Streptococcus* spp., *Moraxella* spp., or *Haemophilus* spp. during acute respiratory infections. These changes in microbiome composition were also influenced by age and seasonality [21, 23–25]. Our study design differs from the aforementioned studies, as we studied exclusively children with acute RSV infection in a cross-sectional mode and compared them with contemporary heathy controls of similar ages. Despite these differences, we also found that detection of *M. catarrhalis,* and especially of *S. pneumoniae* and *H. influenzae,* was more frequent in RSV-infected children and increased with age. On the other hand, *S. aureus* detection was not common in children with RSV infection and it was not associated with worse clinical outcomes. This is different from other respiratory viral infections, like influenza, where *S. aureus* is a risk factor for severe pneumonia [26].

The interactions between RSV and *S. pneumoniae* or *H. influenzae* have been studied in in vitro systems, animal models, and in children [27–31]. In human bronchial epithelial cells, *S. pneumoniae* colonization enhanced RSV replication [32, 33]. In animal models, a preceding RSV infection predisposed to invasive pneumococcal disease [33]. In children, epidemiologic studies showed an association between the peak activity of RSV and the incidence of invasive pneumococcal disease [34, 35]. In addition, a reduction in pneumococcal carriage through vaccination led to decreased rates of hospitalization for RSV pneumonia [36, 37]. Also, *S. pneumoniae*- or *H. influenzae*-dominated microbiome profiles in children have been associated with enhanced mucosal proinflammatory responses [22, 29–31, 36, 38].

Initial studies conducted in small numbers of low- and high-risk children hospitalized with RSV infection in the PICU showed high rates of bacterial detection in the lower respiratory tract that was associated with worse clinical outcomes [10, 39–41]. Subsequent studies in children hospitalized with bronchiolitis showed that nasopharyngeal detection of *H. influenzae* was associated with longer duration of hospitalization and increased rates of PICU admission [42–44]. Nevertheless, invasive bacterial infections, such as bacteremia or meningitis, are rare in children with RSV infection, thus antibiotics are not routinely recommended [3, 45]. A recent large retrospective study conducted in children <2 years of age with RSV lower respiratory tract infection in the PICU showed that early antibiotic treatment was associated with shorter duration of mechanical ventilation and hospital stay [46]. It is possible that PICU patients represent a unique subset, in whom respiratory bacteria play a more significant role in disease severity, and that the benefit associated with antibiotic therapy could be related to a reduction in bacterial burden. Nevertheless, this observation deserves further prospective randomized studies.

In our previous studies using bacterial 16S-rRNA sequencing in a small cohort of children with RSV infection, we found that compared to *S. aureus* or *M. catarrhalis,* nasopharyngeal detection of *Haemophilus* spp. or *Streptococcus* spp. enriched profiles were associated with distinct systemic immune transcriptional profiles and more frequent hospitalization [8]. The present study confirms and expands those findings as we targeted using qRT-PCR the four most common respiratory bacteria that have been identified in children with RSV infection. We found that detection of *S. pneumoniae*, *H. influenzae, M. catarrhalis*, but not *S. aureus,* was frequent and consistent in children with RSV infection, and that the vast majority had more than one bacterium identified. The number of RSV patients and healthy controls enrolled allowed us to define the differences in rates of bacterial detection (alone or in combination) based on age. In addition, univariate followed by multivariable analysis showed that although *S. pneumoniae* or *H. influenzae*, were associated with significant clinical and laboratory changes, it was the concomitant detection of both bacteria that was associated with worse clinical outcomes. Last, we also found that antibiotic treatment was associated with greater odds of hospitalization and a higher initial CDSS. At the same time, antibiotic treatment was more frequently administered to children with RSV infection and *S. pneumoniae* and/or *H. influenzae* detection, by physicians who were unaware of the results of bacterial detection. Further controlled studies are needed to understand the impact of antibiotics on clinical outcomes in young children with RSV infection.

Our study has limitations. The use of antibiotics before sample collection may have altered the bacterial composition or nasopharyngeal bacteria loads. However, we used targeted qRT-PCR that increased the yield of bacterial detection and quantitation, and the multivariable models were adjusted for antibiotic therapy. In addition, we conducted a sensitivity analysis that showed that antibiotic administration decreased bacterial loads but did not influence the rates of bacterial detection. We did not collect sequential samples during the acute disease to assess whether delay of bacterial clearance could have influenced our results. Nevertheless, we prospectively enrolled

a large cohort of young children with RSV infection over multiple respiratory seasons, including a representative number of outpatients, that allowed us to compute fair comparisons. While the current study did not allow us to assess whether RSV infection favored the acquisition of the bacteria analyzed, or whether the preexisting microbiota composition predisposed to a more severe RSV infection, we were able to identify a significant association between *S. pneumoniae* and *H. influenzae* nasopharyngeal detection and RSV disease pathogenesis and severity, especially when detected together. Last, although biomarkers indicative of a proinflammatory state in the blood or respiratory mucosa were not studied, the proportion of blood neutrophils was consistently increased in infants with RSV infection and codetection of *S. pneumoniae* and/or *H. influenzae*, suggesting that nasopharyngeal detection of specific bacteria, in the context of RSV infection, is more than a passive phenomenon.

In summary, our findings contribute to the growing evidence of the complex interactions between RSV and respiratory bacterial communities in children with acute RSV infection. Further studies are needed to help elucidate the mechanisms involved in these interactions and which ones contribute to greater disease severity, which may be amenable to targeted interventions.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. A. M. has received research grants from Janssen; and fees for participation in advisory boards from Janssen, Sanofi-Pasteur, Merck, and Roche. O. R. has received research grants from the Bill and Melinda Gates Foundation, Janssen, and Merk; fees for participation in advisory boards from Merck, Sanofi-Pasteur, Lilly, Pfizer, and Adagio; and fees for lectures from Pfizer, unrelated to the current work. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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