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The Nasopharyngeal Microbiota of Children with Respiratory Infections in Botswana

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Abstract

Background—Nearly half of child pneumonia deaths occur in sub-Saharan Africa. Microbial communities in the nasopharynx are a reservoir for pneumonia pathogens and remain poorly described in African children.

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Methods—Nasopharyngeal swabs were collected from children with pneumonia (N=204), children with upper respiratory infection symptoms (N=55), and healthy children (N=60) in Botswana between April 2012 and April 2014. We sequenced the V3 region of the bacterial 16S ribosomal RNA gene and used partitioning around medoids to cluster samples into microbiota biotypes. We then used multivariable logistic regression to examine whether microbiota biotypes were associated with pneumonia and upper respiratory infection symptoms.

Results—Mean ages of children with pneumonia, children with upper respiratory infection symptoms, and healthy children were 8.2, 11.4, and 8.0 months, respectively. Clustering of nasopharyngeal microbiota identified five distinct biotypes: *Corynebacterium/Dolosigranulum*-dominant (23%), *Haemophilus*-dominant (11%), *Moraxella*-dominant (24%), *Staphylococcus*-dominant (13%), and *Streptococcus*-dominant (28%). The *Haemophilus*-dominant [odds ratio (OR): 13.55, 95% confidence interval (CI): 2.10–87.26], the *Staphylococcus*-dominant (OR: 8.27, 95% CI: 2.13–32.14), and the *Streptococcus*-dominant (OR: 39.97, 95% CI: 6.63–241.00) biotypes were associated with pneumonia. The *Moraxella*-dominant (OR: 3.71, 95% CI: 1.09–12.64) and *Streptococcus*-dominant (OR: 12.26, 95% CI: 1.81–83.06) biotypes were associated with upper respiratory infection symptoms. In children with pneumonia, HIV infection was associated with a lower relative abundance of *Dolosigranulum* ($P=0.03$).

Conclusions—Pneumonia and upper respiratory infection symptoms are associated with distinct nasopharyngeal microbiota biotypes in African children. A lower abundance of the commensal genus *Dolosigranulum* may contribute to the higher pneumonia risk of HIV-infected children.

Keywords

microbiota; microbial communities; respiratory infections; pneumonia; children

Introduction

Pneumonia is the leading infectious killer of children, accounting for roughly 920,000 child deaths each year.¹ More than 99% of these deaths occur in low- and middle-income countries, and nearly half are in sub-Saharan Africa.² Historically, the lower respiratory tract was believed to be sterile, with pneumonia resulting from the invasion of the lung by a single pathogen. However, over the past decade, studies employing high-throughput sequencing methods have identified diverse microbial communities in both the upper and lower respiratory tracts.^{3,4} More recent conceptual models of pneumonia pathogenesis postulate that overgrowth of a single bacterial species within the respiratory tract is a function of complex interactions between the microbiota, potential pneumonia pathogens, and the host immune system.⁴ In particular, the nasopharyngeal microbiota provides a barrier to colonization and invasion by bacterial respiratory pathogens through competition for nutrients, production of inhibitory substances, and modulation of the local immune response.^{5,6} Thus, the composition of the nasopharyngeal microbiota may influence pneumonia risk and also provide insight into pneumonia pathogenesis and etiology. To date, no studies investigated associations between the nasopharyngeal microbiota and respiratory infections in African children.

In this study, we used nasopharyngeal samples from children with pneumonia, children with upper respiratory infection (URI) symptoms, and healthy children in Botswana to assess whether pneumonia and URI symptoms are associated with specific alterations of the nasopharyngeal microbiota. As secondary objectives, we sought to determine the effect of HIV infection or exposure on the nasopharyngeal microbiota and to describe age-related differences in the nasopharyngeal microbiota of African children.

Materials and Methods

Setting

Botswana is located in southern Africa and has a semi-arid climate, with a short rainy season that typically occurs from November to March. The country's HIV prevalence among adults 15 to 49 years of age was 22.2% in 2015.⁷ *Haemophilus influenzae* type B (Hib) and 13-valent pneumococcal conjugate (PCV-13) vaccinations were introduced in November 2010 and July 2012, respectively. Gaborone, the capital and largest city of Botswana, is located in the country's South-East District and had a population of 231,626 in 2011.⁸

Study population

We conducted prospective cohort and case-control studies of pneumonia between April 2012 and June 2016, as described elsewhere in detail.^{9,10} Briefly, we recruited children one to 23 months of age with pneumonia, defined by the World Health Organization as "a cough or difficult breathing with lower chest wall indrawing," at a tertiary hospital in Gaborone.¹¹ We enrolled these children within six hours of the triage time in the Emergency Department and collected a nasopharyngeal swab at enrollment. For controls, we recruited children one to 23 months of age without pneumonia at 18 public clinics in Gaborone. We further classified controls based on the presence or absence of URI symptoms, defined as "a cough or nasal congestion or discharge." For children who were enrolled in the study more than once as either a case or control, only the first enrollment and nasopharyngeal swab were included. Although we matched controls to pneumonia cases by date (within 2 weeks) and primary care clinic, the analyses presented herein were not limited to matched case-control pairs but rather included all pneumonia cases and controls enrolled between April 2012 and April 2014 for which sufficient nasopharyngeal swab specimen was available. To minimize the potential for sampling variability, nasopharyngeal samples from pneumonia cases and controls were collected by the same research team using a standard operating procedure.

Data collection

Sociodemographic and clinical data were collected at enrollment from physical examination, infant and maternal medical records, and a face-to-face interview with the child's caregivers. HIV exposure status was classified through review of HIV test results in maternal and child medical records, as previously described.¹² We recorded antibiotics received within the seven days preceding enrollment (including cotrimoxazole prophylaxis) from child medical records. However, we excluded doses within the 12 hours before nasopharyngeal swab collection because we perceived that these would be unlikely to affect the composition of the nasopharyngeal microbiota measured using molecular methods. We evaluated the validity of this assumption by comparing the nasopharyngeal microbiota of children with pneumonia

who did not receive antibiotics prior to nasopharyngeal swab collection to the nasopharyngeal microbiota of children with pneumonia who were started on antibiotics less than 12 hours prior to nasopharyngeal swab collection. The nasopharyngeal microbiota of these two groups was similar, with no significant differences in alpha diversity (measured using the Chao1 and Shannon diversity indices), the relative abundances of highly abundant genera, or community similarity based on PERMANOVA testing.¹³

Laboratory methods

Nasopharyngeal specimens were collected using flocked swabs and universal transport medium (Copan Italia, Brescia, Italy). These samples were transported to the National Health Laboratory in Gaborone, transferred to cryovials, and stored at -80°C . Samples were shipped in batches on dry ice at six-month intervals to St. Joseph's Healthcare (Hamilton, ON, Canada). Total genomic DNA was extracted from nasopharyngeal specimens using a protocol involving mechanical and enzymatic lysis.¹⁴ Polymerase chain reaction (PCR) was used to amplify the variable V3 region of bacterial 16S ribosomal RNA (rRNA) gene using broad-range primers and an Illumina MiSeq instrument.^{14,15} These same nasopharyngeal specimens were tested by real-time PCR for respiratory syncytial virus, influenza viruses A and B, parainfluenza virus types 1–4, human metapneumovirus, adenovirus, coronaviruses (NL63, 229E, OC43, HKU1), bocavirus, and rhinovirus/enterovirus using nucleic acid extracted on the NucliSens easyMAG platform (bioMérieux, Marcy l'Etoile, France). This extract was also tested for *S. pneumoniae* using a quantitative PCR assay targeting the autolysin (lytA) gene.¹⁴

Bioinformatics

Raw sequences were analyzed through a pipeline that included Cutadapt and PANDAseq tools.¹⁴ Sequences were clustered at 97% identity using AbundantOTU+, and given taxonomic assignments using the Ribosomal Database Project classifier against the Greengenes 2011 database.¹⁴ Each read was grouped into an operational taxonomic unit (OTU), a cluster of similar 16S rRNA sequences corresponding roughly to a species or a group of closely related species. OTUs with fewer than ten counts across all samples or that did not appear in at least 10% of samples were removed. Sample reads were then normalized to the cumulative 75th percentile using the metagenomeSeq package in R.¹⁶ We calculated the relative abundance of each OTU in each nasopharyngeal sample. Because in most cases, each genus was dominated by a single OTU, we aggregated OTUs at the genus level for compositional analyses (unless otherwise specified). Genera referred to as “commensal” do not contain species known as common etiologies of upper or lower respiratory infections in children.

Statistical analysis

Characteristics of children with pneumonia, children with URI symptoms, and healthy children were compared using Chi square or Fisher's exact tests (for categorical variables) and one-way analysis of variance (for continuous variables). We used zero-inflated Gaussian distribution mixture models to examine whether the proportion of genera differed between groups.¹⁶ We considered p-values <0.05 after adjustment for the false discovery rate to be significant. We used partitioning around medoids using Jensen-Shannon divergence to

identify co-associated organisms as community profiles, herein referred to as “biotypes.” We used logistic regression to estimate odds ratios for associations between nasopharyngeal microbiota biotypes and disease status (pneumonia, URI symptoms, healthy). Adjusted analyses included season, enrollment year, age, sex, HIV infection or exposure, current breastfeeding, household use of wood as a cooking fuel, PCV-13 doses, receipt of antibiotics within the prior seven days, and detection of one or more respiratory viruses, which were potential confounding variables identified based on subject matter knowledge and a literature review.^{6,17–20} Statistical analyses were conducted using R version 3.3.1. This study was approved by the Health Research and Development Committee (Botswana Ministry of Health), the Princess Marina Hospital ethics committee, and institutional review boards at the University of Pennsylvania, Children’s Hospital of Philadelphia, McMaster University, and Duke University.

Results

Three hundred nineteen children were included in these analyses, including 204 (64%) children with pneumonia, 55 (17%) children with URI symptoms, and 60 (19%) healthy children. Characteristics of the study population are shown in Table 1. Age, HIV exposure status, season, enrollment year, household use of wood as a cooking fuel, PCV-13 doses, receipt of antibiotics within the prior seven days, and respiratory virus detection differed significantly by disease status. Of the 14 HIV-infected children with pneumonia, four (29%) were on antiretroviral therapy and two (14%) had received cotrimoxazole prophylaxis or other antibiotics in the seven days preceding enrollment.

Sequence diversity

A total of 15,016,857 high-quality 16S rRNA sequences (mean of 47,075 sequences per sample) were obtained from the 319 nasopharyngeal samples included in this study. All samples had sufficient sequencing depth to obtain a high degree of sequence coverage and were included in analyses. Rarefaction curves were constructed to ensure coverage of the bacterial diversity present (see Figure, Supplemental Digital Content 1). The mean (standard deviation) Chao1 and Shannon diversity indices in the overall population were 206.0 (89.3) and 1.26 (0.62). Species richness, as measured by the Chao1 index, was lower in children with pneumonia than healthy children ($P<0.0001$), but the Shannon diversity index did not differ in these groups (Figure 1). The Shannon diversity index of samples from children with URI symptoms was higher than that of samples from healthy children ($P=0.02$). However, when we excluded children who had received antibiotics in the prior seven days, no significant differences in richness or diversity were observed between healthy children and children with either pneumonia or URI symptoms.

Sequences were classified into 285 bacterial OTUs (after filtering), representing 145 genera from 12 phyla. Domination of the nasopharyngeal microbiota – defined as the occupation of >50% by a single genus – was frequent in children with pneumonia (69%), children with URI symptoms (56%), and healthy children (77%). We compared relative abundances of the most frequently occurring genera in children by disease status (Figure 2A). Compared with healthy children, children with pneumonia had higher relative abundances of *Haemophilus*

($P<0.0001$), *Streptococcus* ($P<0.0001$), *Escherichia* ($P=0.03$), and *Klebsiella* ($P=0.02$), and lower relative abundances of *Corynebacterium* ($P=0.0001$) and *Dolosigranulum* ($P=0.0005$). Among children with pneumonia, we observed differences in nasopharyngeal microbial composition by HIV exposure status (Figure 2B). Compared with unexposed children, HIV-exposed, uninfected (HIV-EU) children had a higher relative abundance of *Klebsiella* ($P=0.003$), while HIV-infected children had a lower relative abundance of *Dolosigranulum* ($P=0.03$). Children with URI symptoms had higher relative abundances of *Haemophilus* ($P<0.0001$), *Streptococcus* ($P=0.01$), and *Moraxella* ($P=0.01$), and a lower relative abundance of *Staphylococcus* ($P=0.01$) than healthy children (Figure 2C). In healthy children, the composition of the nasopharyngeal microbiota differed by age (Figure 2D). Compared to children one to 11 months of age, children 12 months of age or older had a higher relative abundance of *Moraxella* ($P=0.0001$) and a lower relative abundance of *Staphylococcus* ($P=0.02$).

Association of microbiota biotypes with disease status

Hierarchical clustering identified five distinct nasopharyngeal microbiota biotypes: *Corynebacterium/Dolosigranulum*-dominant ($n=73$, 23%), *Haemophilus*-dominant ($n=35$, 11%), *Moraxella*-dominant ($n=78$, 24%), *Staphylococcus*-dominant ($n=43$, 13%), and *Streptococcus*-dominant ($n=90$, 28%). We compared characteristics of children with these microbiota biotypes (see Table, Supplemental Digital Content 2). Four microbiota biotypes were dominated by one genus and a single biotype was co-dominated by *Corynebacterium* and *Dolosigranulum* (Figure 3A). The distribution of microbiota biotypes by disease status is shown in Figure 3B. The most frequent biotype in children with pneumonia was the *Streptococcus*-dominant biotype (36%). Among children with URI symptoms, most samples were classified as either the *Moraxella*-dominant (44%) or the *Streptococcus*-dominant (27%) biotypes. The majority of samples from healthy children had either the *Corynebacterium/Dolosigranulum*-dominant (53%) or the *Moraxella*-dominant (27%) biotypes. Multivariable analyses for associations between nasopharyngeal microbiota biotypes and disease status are shown in Table 2. Compared with healthy children, children with pneumonia were more likely to have the *Haemophilus*-dominant [odds ratio (OR): 13.55, 95% confidence interval (CI): 2.10–87.26], the *Staphylococcus*-dominant (OR: 8.27, 95% CI: 2.13–32.14), or the *Streptococcus*-dominant biotype (OR: 39.97, 95% CI: 6.63–241.00). The *Moraxella*-dominant (OR: 3.71, 95% CI: 1.09–12.64) and *Streptococcus*-dominant (OR: 12.26, 95% CI: 1.81–83.06) biotypes were associated with URI symptoms.

Detection of *Streptococcus pneumoniae* by PCR

We next sought to more precisely define the role of *S. pneumoniae* in the differences in relative abundances of streptococci observed by disease status. To identify the OTU corresponding to *S. pneumoniae*, we compared the relative abundances of OTUs within the genus *Streptococcus* according to *S. pneumoniae* detection by species-specific PCR (see Figure, Supplemental Digital Content 3). The most abundant streptococcal OTU (#2) was also strongly associated with detection of *S. pneumoniae* by PCR ($P=7.11 \times 10^{-27}$), indicating a high likelihood that this OTU contained *S. pneumoniae*. This OTU was also represented in nasopharyngeal samples that tested negative for *S. pneumoniae* by PCR, consistent with prior studies indicating that *S. pneumoniae* is not reliably distinguished from

Streptococcus pseudopneumoniae and other closely related species by 16S rRNA sequencing.²¹ We observed a higher relative abundance of this *S. pneumoniae* complex OTU in children with URI symptoms ($P=0.002$) or pneumonia ($P<0.0001$) compared with healthy children. Similarly, the association between disease status and detection of *S. pneumoniae* by species-specific PCR was strong (healthy children, 38%; children with URI symptoms, 67%; children with pneumonia, 63%; $P=0.001$).

Discussion

To our knowledge, this is the first study to examine the nasopharyngeal microbiota of African children with and without respiratory infections. Our results indicate that pneumonia and URI symptoms are associated with distinct nasopharyngeal microbiota biotypes in these children.

The nasopharynx is the site of dynamic interactions between commensal bacteria and potential respiratory pathogens. Current models of pneumonia pathogenesis maintain that disturbances of the microbiota facilitate nasopharyngeal colonization, overgrowth, and invasion of the lower respiratory tract by potential pathogens.⁴ Consistent with these models, we found that *S. pneumoniae* was detected frequently by species-specific PCR in children with pneumonia, children with URI symptoms, and healthy children. However, a *Streptococcus*-dominant biotype, indicative of nasopharyngeal overgrowth, was strongly associated with pneumonia or URI symptoms and was only rarely observed in healthy children. Such a biotype was also characterized by low relative abundances of *Corynebacterium* and *Dolosigranulum*, suggesting that losses of these commensal bacteria may have facilitated overgrowth by *S. pneumoniae*. Similarly, we found that the *Haemophilus*-dominant and *Staphylococcus*-dominant biotypes were also more frequent in children with pneumonia, consistent with culture-based studies that previously established bacteria from these genera (*H. influenzae* and *Staphylococcus aureus*) as common causes of pneumonia in African children.^{22,23} In contrast, the *Moraxella*-dominant biotype was associated with URI symptoms but not pneumonia. This conflicts with findings from a recent study conducted in Australia in which *Moraxella* was more strongly associated with pneumonia than URI when identified using molecular methods.⁶ However, our results are consistent with culture-based studies that suggest that *Moraxella* species are infrequent causes of pneumonia in African children and children from other developed countries.^{22–25} Interestingly, nasopharyngeal microbial diversity did not differ between healthy children and children with either pneumonia or URI symptoms after excluding children who had received antibiotics in the preceding seven days. These findings challenge the traditional dogma that disease states are associated with a loss of biodiversity and differ from several prior studies of childhood respiratory infections in developed countries, and perhaps indicate different mechanisms of susceptibility to pneumonia in African children.^{21,26,27} Finally, we observed strong associations between nasopharyngeal microbiota biotype and disease status despite inclusion of detection of one or more respiratory viruses in multivariable models. This indicates that there are changes in the composition of the nasopharyngeal microbiota in children with pneumonia or URI symptoms that reflect the disease state and occur independent of the effect of respiratory virus infection.

We did not identify an association between nasopharyngeal microbiota biotype and HIV infection or exposure in children with or without pneumonia. However, in analyses conducted at the genus level, the nasopharyngeal microbiota of HIV-infected children with pneumonia was characterized by the near-complete absence of *Dolosigranulum*. Species within this genus are common commensals in the nasopharynx and are believed to protect from respiratory infections.¹⁹ Biesbroek *et al.* reported that lower relative abundances of *Dolosigranulum* were associated with a higher frequency of mild respiratory infections in Dutch infants.¹⁷ Similarly, Laufer *et al.* found that a principal component-based factor including *Dolosigranulum* was associated with a lower risk of otitis media and *S. pneumoniae* colonization among children in the United States.²⁶ Our findings suggest that the lower relative abundance of *Dolosigranulum* in HIV-infected children may contribute to the higher risk of pneumonia and invasive pneumococcal disease in this group, although a mechanistic relationship between *Dolosigranulum* and *S. pneumoniae* has not been described.²⁸ We also observed higher relative abundances of *Escherichia* and *Klebsiella* in children with pneumonia, particularly among HIV-infected or HIV-EU children. Pathogens from these genera (*Escherichia coli* and *Klebsiella pneumoniae*) were common causes of pneumonia in a study of South African children and were only identified in children with HIV infection or exposure.²² In Botswana, oropharyngeal colonization with Gram-negative bacteria was also frequent in healthy HIV-exposed infants, particularly those who were formula feeding.²⁹ Consistent with this observation, the vast majority of HIV-infected (79%) and HIV-EU (97%) children in our study were formula-fed. However, we did not observe differing relative abundances of *Escherichia* and *Klebsiella* by HIV exposure status in children without pneumonia (data not shown). These data associate these genera with pneumonia in HIV-infected and HIV-EU children while excluding them as major components of the healthy nasopharyngeal microbiota of African children.

The composition of the nasopharyngeal microbiota of healthy infants and children in Botswana was similar to a prior study of African children and previous reports from developed countries. Most notably, we found that the nasopharyngeal microbiota was comprised primarily of bacteria from six genera: *Corynebacterium*, *Dolosigranulum*, *Haemophilus*, *Moraxella*, *Staphylococcus*, and *Streptococcus*. This is largely consistent with the findings of a study of 60 healthy children in Kenya, as well as prior studies conducted in North America, Europe, and Australia.^{6,19,20,26,30} Moreover, as in several of these studies, we observed a redistribution of the nasopharyngeal microbiota during infancy, with a lower relative abundance of *Staphylococcus* and a higher relative abundance of *Moraxella* observed with increasing age.^{6,31} This indicates that the nasopharynx is a relatively preserved ecological niche during early childhood despite substantial differences in host and environmental factors.

Our study has several limitations. First, given the cross-sectional design, we were unable to assess whether the alterations of the nasopharyngeal microbiota associated with pneumonia and URI symptoms preceded disease onset. Moreover, although we observed nasopharyngeal overrepresentation of genera containing common pneumonia pathogens in children with pneumonia, we were unable to definitively determine pneumonia etiology given the lack of lower respiratory tract samples. Also, the use of 16S rRNA sequencing precluded determination of bacterial composition at the species level, although we were able

to identify the taxon that contained *S. pneumoniae* with a high degree of confidence using a species-specific PCR assay. We adjusted for a large number of potential confounders in the multivariable models examining associations between nasopharyngeal microbiota composition and disease status, but unmeasured confounding factors remain possible. In addition, it is possible that nasopharyngeal sampling may have varied by disease state or in the presence of respiratory virus infection. While we cannot exclude this possibility, we minimized the potential for sampling variability by having the same research team collect nasopharyngeal swab samples from pneumonia cases and controls and by using a standard operating procedure. Finally, all of the HIV-infected children in our study had pneumonia, and we are thus unable to determine whether the loss of *Dolosigranulum* is a characteristic of the nasopharyngeal microbiota of HIV-infected children or a consequence of pneumonia in this group.

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Figure 1. Comparison of bacterial alpha diversity measures by disease status
Nasopharyngeal microbiota richness (a), as measured by the Chao1 index, and diversity (b), as measured by the Shannon Diversity Index, among children with URI symptoms, healthy children, and children with pneumonia. URI, upper respiratory infection

Figure 2. Relative abundances of frequently occurring genera by disease status

Relative abundances of genera in healthy children and children with pneumonia (a), by HIV exposure status in children with pneumonia (b), in healthy children and children with URI symptoms (c), and by age categories in healthy children (d). URI, upper respiratory infection, HIV-EU, HIV-exposed uninfected

Figure 3. Nasopharyngeal microbiota biotypes by disease status

Relative abundances of frequently occurring genera in microbiota biotypes (a), and the distribution of nasopharyngeal samples from children with pneumonia, children with URI symptoms, and healthy children with these microbiota biotypes (b). In panel (b), the medoids are shown as large points with white centers; all other points correspond to individual nasopharyngeal swab samples. URI, upper respiratory infection

Table 1
 Characteristics of healthy children, children with URI symptoms, and children with pneumonia.

Demographics	Healthy Children (N=60)		Children with URI Symptoms (N=55)		Children with Pneumonia (N=204)		p ^a
Mean (SD) age, months	8.0	(5.2)	11.4	(5.7)	8.2	(6.1)	0.001
Sex							0.14
Female	36	60%	23	42%	101	50%	
Male	24	40%	32	58%	103	50%	
Low birthweight (<2500 grams) ^b	14	23%	10	18%	42	21%	0.79
HIV exposure status							0.01
Unexposed	44	73%	40	73%	134	66%	
HIV-exposed, uninfected	10	17%	5	9%	54	26%	
HIV-infected	0	0%	0	0%	14	7%	
Unable to determine	6	10%	10	18%	2	1%	
Current breastfeeding	29	48%	26	47%	86	42%	0.62
Season							0.003
Dry (April – October)	29	48%	43	78%	136	67%	
Rainy (November – March)	31	52%	12	22%	68	33%	
Enrollment year							0.01
2012	12	20%	8	15%	73	36%	
2013	41	68%	44	80%	115	56%	
2014	7	12%	3	5%	16	8%	
Socioeconomic Factors							
Maternal education level							0.09
None or primary	0	0%	2	4%	18	9%	
Secondary	46	77%	38	69%	137	67%	
Tertiary	14	23%	15	27%	49	24%	
Mean (SD) household members	10.6	(2.3)	9.9	(2.8)	10.1	(3.4)	0.49
Electricity in home	38	63%	37	67%	138	68%	0.82

	Healthy Children (N=60)		Children with URI Symptoms (N=55)		Children with Pneumonia (N=204)		<i>P</i> ^a
	55	92%	51	93%	179	88%	
Municipal or private water source	36	60%	37	67%	128	63%	0.72
Refrigerator in home	8	13%	9	16%	58	28%	0.02
Household use of wood as a cooking fuel							
Clinical Factors							
Pneumococcal conjugate vaccine (PCV-13) ^c							0.03
0 doses	30	50%	21	38%	120	59%	
1 dose	7	12%	12	22%	19	9%	
2 doses	23	38%	22	40%	64	31%	
Receipt of antibiotics within the prior 7 days ^d	0	0%	4	7%	44	22%	<0.0001
Detection of one or more respiratory viruses	14	23%	24	44%	167	82%	<0.0001
Adenovirus	0	0%	0	0%	4	2%	
Bocavirus	0	0%	0	0%	2	1%	
Coronaviruses (NL63, 229E, OC43, HKU1)	1	2%	2	4%	0	0%	
Human metapneumovirus	0	0%	0	0%	11	5%	
Influenza viruses A and B	0	0%	2	4%	9	4%	
Parainfluenza virus types 1-4	0	0%	1	2%	17	8%	
Respiratory syncytial virus	0	0%	4	7%	76	37%	
Rhinovirus/enterovirus	13	22%	17	31%	70	34%	

URI, upper respiratory infection; SD, standard deviation; PCV-13, 13-valent pneumococcal conjugate vaccine

^a *P* values were calculated using Chi square or Fisher's exact tests for categorical variables and one-way analysis of variance for continuous variables

^b Birthweight data were missing from two children with pneumonia

^c Immunization records were unavailable for one child with pneumonia

^d Excludes antibiotic doses received within the 12 hours preceding nasopharyngeal swab sample

Table 2

Association of nasopharyngeal microbiota biotypes with disease status in multivariable models.

	Crude OR	(95% CI)	P	Adjusted OR	(95% CI)	P
Children with pneumonia vs. healthy children						
<i>Corynebacterium/Dolosigranulum</i> -dominant	1	Ref	-	1	Ref	-
<i>Haemophilus</i> -dominant	16.00	(3.52, 72.83)	0.0003	13.55	(2.10, 87.26)	0.01
<i>Moraxella</i> -dominant	2.53	(1.18, 5.46)	0.02	2.58	(0.86, 7.71)	0.09
<i>Staphylococcus</i> -dominant	4.40	(1.76, 11.03)	0.002	8.27	(2.13, 32.14)	0.002
<i>Streptococcus</i> -dominant	38.93	(8.77, 172.83)	<0.0001	39.97	(6.63, 241.00)	<0.0001
Children with URI symptoms vs. healthy children						
<i>Corynebacterium/Dolosigranulum</i> -dominant	1	Ref	-	1	Ref	-
<i>Haemophilus</i> -dominant	4.36	(0.64, 29.64)	0.13	2.23	(0.21, 23.15)	0.50
<i>Moraxella</i> -dominant	4.36	(1.72, 11.09)	0.002	3.71	(1.09, 12.64)	0.04
<i>Staphylococcus</i> -dominant	0.73	(0.13, 3.96)	0.71	1.35	(0.17, 10.46)	0.77
<i>Streptococcus</i> -dominant	21.82	(4.29, 110.99)	0.0002	12.26	(1.81, 83.06)	0.01
Children with pneumonia vs. children with URI symptoms						
<i>Corynebacterium/Dolosigranulum</i> -dominant	1	Ref	-	1	Ref	-
<i>Haemophilus</i> -dominant	3.67	(0.93, 14.48)	0.06	3.38	(0.64, 17.94)	0.15
<i>Moraxella</i> -dominant	0.58	(0.25, 1.37)	0.21	0.70	(0.24, 2.09)	0.53
<i>Staphylococcus</i> -dominant	6.05	(1.24, 29.54)	0.03	6.18	(0.88, 43.20)	0.07
<i>Streptococcus</i> -dominant	1.78	(0.74, 4.33)	0.20	1.02	(0.34, 3.07)	0.97

OR, odds ratio; CI, confidence interval; URI, upper respiratory infection

Odds ratios and 95% confidence intervals estimated from logistic regression models adjusting for season, enrollment year, age, sex, HIV infection or exposure, current breastfeeding, household use of wood as a cooking fuel, pneumococcal conjugate vaccine (PCV-13) doses, antibiotic use in the prior 7 days, and detection of one or more respiratory viruses