# Nasopharyngeal Microbiota in Healthy Children and Pneumonia Patients

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Our study is the first to compare the nasopharyngeal microbiota of pediatric pneumonia patients and control children by 454 pyrosequencing. A distinct microbiota was associated with different pneumonia etiologies. Viral pneumonia was associated with a high abundance of the operational taxonomic unit (OTU) corresponding to *Moraxella lacunata*. Patients with nonviral pneumonia showed high abundances of OTUs of three typical bacterial pathogens, *Streptococcus pneumoniae* complex, *Haemophilus influenzae* complex, and *Moraxella catarrhalis*. Patients classified as having no definitive etiology harbored microbiota particularly enriched in the *H. influenzae* complex. We did not observe a commensal taxon specifically associated with health. The microbiota of the healthy nasopharynx was more diverse and contained a wider range of less abundant taxa.

Pneumonia is the leading cause of childhood mortality worldwide, claiming 2 million lives yearly among young children (1). The etiology of pediatric pneumonia is complex and not routinely determined in clinical practice (2). Its definitive determination remains challenging (3). Clinical research shows that *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* are the leading pathogens of bacterial pneumonia (1, 4). Respiratory syncytial virus (RSV), parainfluenza, and influenza viruses are the main causes of pediatric viral pneumonia (e.g., see references 5 and 6). *S. pneumoniae* and *H. influenzae* frequently colonize the nasopharynx of young children, and the nasopharyngeal (NP) carriage of *S. pneumoniae* is considered key to pneumonia and other pneumococcal diseases (7). However, the mere nasopharyngeal presence of bacterial pathogens does not necessarily lead to invasive lung infection. Various mechanisms, such as competition with resident nonpathogenic microbiota, viral coinfection, or host immune factors are likely to affect the transition from nasopharyngeal colonization to pneumonia (8). The increasing accessibility of culture-independent sequencing methods has permitted microbiota description at various body sites (9), and the potential of commensal microbiota to modify the disease process is receiving increasing attention (10).

Previous culture-independent studies of the nasopharyngeal microbiota of young children looked at healthy children (11) and compared the microbiota composition of healthy children and children with acute otitis media (12). In the present study, we analyzed the nasopharyngeal microbiota composition, including the presence of respiratory viruses, in pediatric pneumonia patients and matched healthy controls and looked for microbiota associations with the disease status.

## MATERIALS AND METHODS

**Patients and clinical samples.** A prospective case-control study conducted in 2008 to 2009 in 3 major hospitals in Switzerland (Geneva, Lausanne, and Sion) to investigate pediatric community-acquired pneumonia etiology was described in detail elsewhere (13). Briefly, pneumonia cases were diagnosed according to the WHO criteria (14) in children 2 months to 16 years old presenting with fever (temperature > 38°C), increased respiratory rate for age or respiratory distress, and infiltrates on chest X-ray. Controls were age matched and recruited from children undergoing minor elective surgery under anesthesia. Patients with respiratory tract infection (RTI) within the past 3 weeks and any history of chronic disease were excluded. From this case-control study population we selected 50 children with pneumonia and 50 control children who were age and sex matched. The median age of the included children was 2.7 years; 41% were girls.

Signed informed parental consent was obtained before enrollment. Ethics approval was obtained from the research ethics committees at the 3 hospitals (authorization number CER 07-269).

The nasopharynx samples were collected from all patients using swabs with liquid Amies preservation medium (Copan) and immediately frozen at −80 °C. Blood cultures were performed, and C-reactive protein (CRP) concentrations were determined for all pneumonia patients.

**Detection of respiratory viruses and *S. pneumoniae*.** Twenty-five common respiratory viruses were detected in nasopharyngeal samples using a PCR- and microarray-based assay (Infinity RVP Plus; Auto-Genomics, Inc., Vista, CA) (15) following the instructions of the manufacturer. Detection of *S. pneumoniae* was done by PCR targeting *autolysin* (*lytA*) and *pneumolysin* (*ply*) genes (13).

**454 pyrosequencing.** Two hundred microliters of each sample was lysed in 1× Tris-EDTA (TE) buffer with 0.5% Tween and 200 μg/ml of proteinase K (Fermentas). Proteinase K was inactivated by a 10-min incubation at 95°C (16). PCR and pyrosequencing of the variable region of V1-V2 of the 16S rRNA gene was performed as previously described using Roche 454 FLX chemistry (17), except that the requirement for a minimum of 2 ng of DNA template per reaction was not followed. PCR products suitable for sequencing were obtained from samples of 27 of 50 pneumonia and 22 of 50 control patients. A total of 26,955 good-quality sequencing reads were obtained for controls and 80,022 were obtained for pneumonia patients.

**FIG 1** The composition of nasopharyngeal microbiota of control children (left) and children with nonviral (center) or viral (right) pneumonia. Nonviral-pneumonia patients were further subdivided according to *S. pneumoniae* diagnosis (patients with both PCR positivity and CRP levels of >40 mg/dl were grouped apart from patients with only one or none of these features). The 12 most abundant operational taxonomic units (OTUs) are shown. Sequences belonging to OTUs which contain <0.5% of total number of sequences were pooled and are labeled "Remaining OTUs." The samples where respiratory viruses were detected are labeled accordingly, i.e., AdV (adenovirus), RV-EV (rhinovirus or enterovirus), HRSV (human respiratory syncytial virus), and HMPV (human metapneumovirus). "NEG" along the *x* axis indicates negativity for virus detection. ND, not determined; NA, not available.

**Taxonomic classification.** The initial sequence reads underwent quality checks to remove sequencing artifacts and low-quality sequences, as described in reference 17. High-quality reads were classified into Bergey's taxonomy using the RDP-Classifier version 2.3 (60% confidence cutoff). Classification at the genus level was used for the analysis. Reads were also grouped into operational taxonomic units (OTUs) based on their best BLAST hit to reference type strain 16S DNAs using a 98% sequence identity cutoff. Some of the reference sequences representing type strains of distinct species were over 98% identical and hence were pooled in a single OTU. All OTUs for which the reference sequences were similar at a >98% level over the sequenced region (V1-V2) were summed up and labeled with the name of the dominant taxon. For example, the *S. pneumoniae* complex OTU was a cluster grouping *Streptococcus pneumoniae*, *S. mitis*, and *S. pseudopneumoniae* sequences. Table S1 in the supplemental material contains the list of OTUs and corresponding reference taxa.

**Statistical analysis.** The Wilcoxon rank sum test was used to assess whether individual taxa/OTUs were statistically different between the groups. The 12 most abundant OTUs were used for principal component analysis (PCA). PCA biplots were used to visualize the differences between the multivariate means and the multivariate variability (dispersion of points) and which taxa contributed to this difference (arrows with corresponding label). Nonparametric multivariate analysis of variance (NP-MANOVA) was used to assess the difference between groups. All analyses were performed using R 2.12.0.

## RESULTS

The nasopharyngeal microbiota of children with community-acquired pneumonia cases was compared with that of matched children undergoing minor surgery (controls). Three dominant bacterial genera, namely, *Moraxella*, *Haemophilus*, and *Streptococcus*, represented 92% of reads in the pneumonia patients and 76% of reads in the controls (see Fig. S1 in the supplemental material). Despite the dominance of just 3 genera, high variability among the patients was observed. Twenty samples were dominated (>50% of all reads) by *Moraxella*, 17 by *Haemophilus*, and 4 by *Streptococcus*. Only 2.1% of reads were unclassified at the genus level.

**Richness and diversity was higher in control than in pneumonia patients.** For each sample, the number of genera present among 300 randomly chosen reads was counted, omitting all genera that were represented by ≤5 reads per sample. Control patients, with 14.7 genera, showed significantly greater genus richness than pneumonia cases, with 7.7 genera ($P < 0.01$, Mann-Whitney U test). Diversity, measured by the Shannon index, was also higher in controls than in patients (2.31 versus 1.71, $P < 0.002$, Mann-Whitney U test).

**Viral diagnostics and presumed pneumonia etiology.** Eight of 27 pneumonia patients compared to only 1 of 22 control patients showed a respiratory viral pathogen in the nasopharynx ($P = 0.03$, Fisher exact test). The viruses detected in nasopharyngeal samples of the pneumonia patients included respiratory syncytial virus, adenovirus, human metapneumovirus, and rhinovirus (Fig. 1). The only virus-positive sample from a control patient contained a rhinovirus. We tentatively assumed that patients with respiratory virus suffered from viral pneumonia (6).

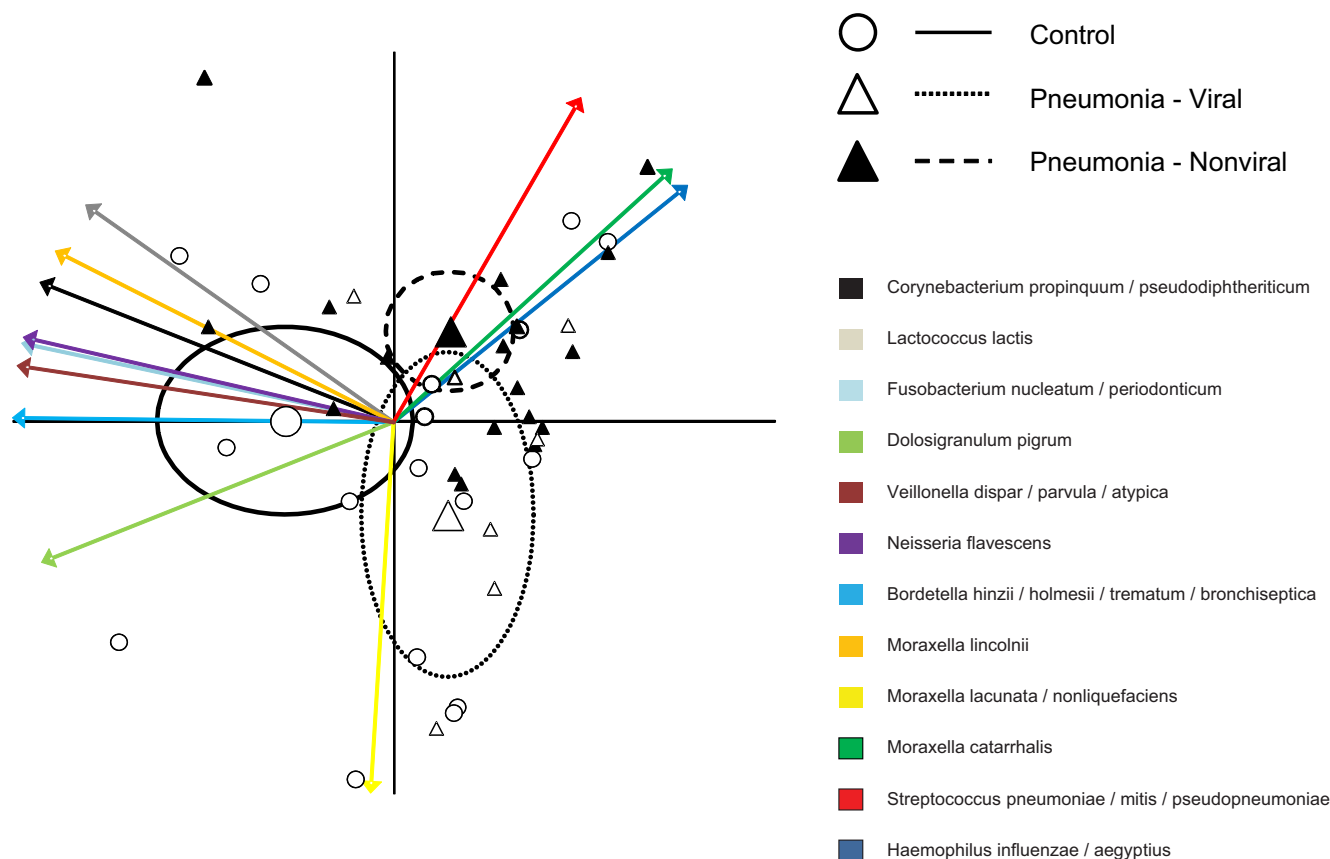Blood culture was negative for all patients. Eighteen out of 26

**FIG 2** Multivariate analysis including 12 most common OTUs. PCA-biplot representation of microbiota of control children and children with bacterial (nonviral) and viral pneumonia. Arrows represent projections of the taxa that are responsible for the differences between groups. Large points represent the group means, and ellipses represent 95% confidence limits.

patients had nasopharyngeal samples which were positive for *S. pneumoniae* by PCR. We considered patients who showed both a CRP concentration of >40 mg/dl and a positive test for *S. pneumoniae* in the nasopharynx by PCR to suffer from pneumococcal pneumonia (18). For a mixed infection, the two above criteria would have to be fulfilled, in addition to the detection of respiratory virus in the nasopharynx. According to these criteria, none of the patients suffered from mixed infection. The remaining patients were considered to show no definitive etiology (Fig. 1).

**Presence of respiratory virus was associated with distinct microbiota.** A great majority (92%) of reads were assigned to an OTU with 98% identity cutoff, i.e., produced a BLAST hit to a reference sequence of a type strain. In total, 250 OTUs were identified (see Table S1 in the supplemental material). Out of 250 identified OTUs, only 12 showed >0.5% average relative abundance. Many samples (35 out of 49) were dominated (>50% of all reads) by a single OTU (Fig. 1).

Multivariate analysis performed on the 12 most common OTUs, (Fig. 2) suggested that nonviral pneumonia patients had a high abundance of the three typical pneumonia pathogens (*S. pneumoniae* complex, *H. influenzae* complex, and *M. catarrhalis*), while viral pneumonia was distinguished by increased presence of the *M. lacunata* complex. NP-MANOVA indicated no difference between the control group and the viral pneumonia group ($P = 0.86$) and trends for a difference between the control group and the nonviral pneumonia group ($P = 0.12$) and between the bac-

terial and viral pneumonia groups ($P = 0.11$). Subsequent univariate analysis confirmed these observations. The *M. lacunata* complex was nearly 7-fold more abundant in viral pneumonia patients than in nonviral pneumonia patients (Fig. 3) ($P = 0.005$). When OTUs corresponding to the three main bacterial pathogens (*S. pneumoniae* complex, *H. influenzae* complex, and *M. catarrhalis*) were combined, nonviral pneumonia patients had a higher proportion of the three bacterial pathogens than did both the controls ($P = 0.0005$) and the viral pneumonia patients (Fig. 3) ($P = 0.025$). Four OTUs showed a trend toward a higher abundance in control patients than in nonviral pneumonia patients (*M. lacunata* complex, $P = 0.075$; *Dolosigranulum pigrum*, $P = 0.058$; *Fusobacterium nucleatum* complex, $P = 0.065$; *Lactococcus lactis*, $P = 0.074$). There were no statistically significant differences or trends ($P < 0.15$) between controls and viral pneumonia patients. We also observed that patients for whom no definitive etiology was detected showed particularly high abundances of *H. influenzae* complex (Fig. 1).

When all pneumonia patients were compared with controls, the only significant difference between the groups was a higher relative abundance of OTUs corresponding to the combined three pathogens (*S. pneumoniae* complex, *H. influenzae* complex, and *M. catarrhalis*) (66% versus 36%, $P = 0.005$) (Fig. 3). *F. nucleatum* complex ($P = 0.055$) and *D. pigrum* ($P = 0.084$) showed trends toward higher abundances in control patients.
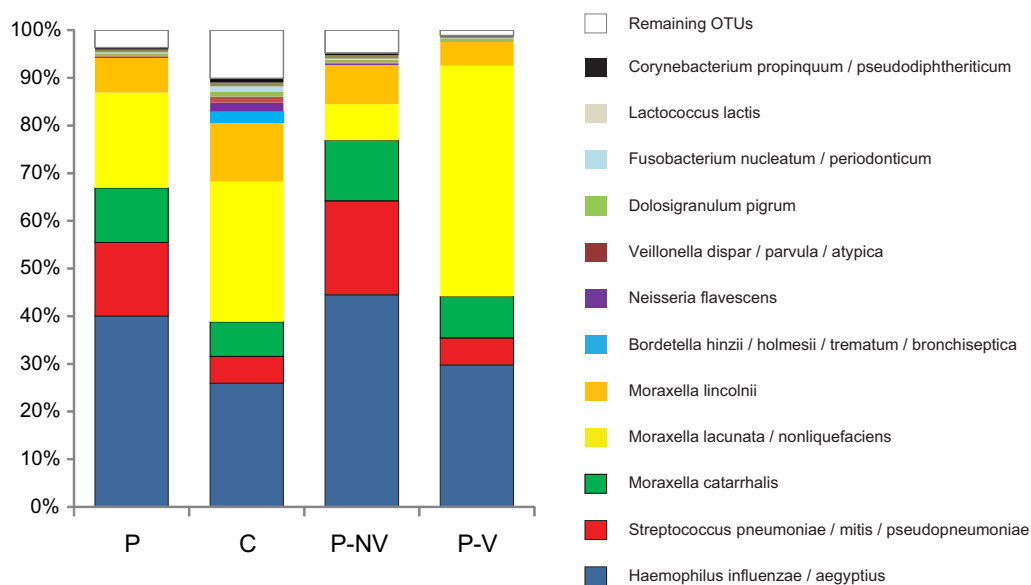
**FIG 3** Mean relative abundance of 12 most common OTUs in samples from pneumonia patients (P) and controls (C). Pneumonia patients are further differentiated as having nonviral pneumonia (P-NV) or viral pneumonia (P-V). OTUs containing <0.5% of the total number of sequences were pooled and are labeled "Remaining OTUs."

## DISCUSSION

In this study, we used the culture-independent technique of pyrosequencing to analyze the nasopharyngeal microbiota of pediatric pneumonia patients and compare it to the microbiota of control children. Three highly dominant genera, namely, *Moraxella*, *Streptococcus*, and *Haemophilus*, were observed, which was in agreement with two previous studies surveying the nasopharynxes of children (11, 12). Likewise, the abundances of *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* in the nasopharynx in children are well known from previous culture-based studies. Here we identified other prevalent taxa, namely, *M. lacunata* and *Moraxella lincolnii*.

High dissimilarity of microbiota among patients was striking. Dominance of just one taxon in a sample was common, and more so in pneumonia patients. However, on average, the nasopharyngeal microbiota of control and pneumonia patients did not display prominent differences. In terms of abundance, the only clear difference was the increased presence of the bacterial pathogens *S. pneumoniae* complex, *H. influenzae*, and *M. catarrhalis* in pneumonia patients. Comparison between viral and nonviral pneumonia revealed that nonviral (and thus presumably bacterial) pneumonia patients harbored a microbiota which was particularly enriched in these three bacterial pathogens, as expected. In viral pneumonia patients the three pathogenic taxa were not more abundant than in control patients; instead, the increased presence of the little-known *M. lacunata* was observed. Although we think it is unlikely that *M. lacunata* is a pathogen on its own, its association with true respiratory pathogens, in this case respiratory viruses, should be further investigated. Different etiologies of pneumonia appear to be associated with specific patterns of microbiota dysbiosis. Patients without definitive etiology appeared to harbor a particularly high abundance of *H. influenzae* complex. Nontypeable (NT) *H. influenzae* has increasingly been considered an important pathogen of pediatric pneumonia (19). Because it is a very frequent colonizer of the nasopharynx and it does not appear to be associated with elevated CRP concentrations, definitive attribution of etiology to this species may prove difficult. Recent studies, where samples taken from the lung were investigated in cases of recurrent or severe pneumonia, showed high prevalence of NT *H. influenzae*. Our results suggest that this pathogen should be considered in uncomplicated pneumonia. The nasopharyngeal microbiota of children in our study showed low taxon complexity and low bacterial abundance. Although we did not perform a direct assessment of bacterial abundance on our samples, low (50%) success in amplification implies low bacterial colonization density, as previously observed (11). Moreover, higher amplification success of pneumonia samples implied denser colonization in disease than in health, as already suggested for acute otitis media (12).

The microbiota of control children was more diverse than that of pneumonia patients. The association of decreased richness and diversity of microbiota with disease appears to be a common theme, reported for a number of conditions, and also specifically for nasopharyngeal (12) and nasal (20) microbiota in children. One might speculate that the decrease in diversity is caused by the outgrowth of pathogens, which predisposes to invasive infection, but this hypothesis can be tested only in a longitudinal study. Our initial working hypothesis was that commensal bacteria keep the facultative pathogens colonizing the nasopharynx in check (21). This could prevent their descent into the lungs and invasive infection. However, we did not find evidence for a protective commensal taxon associated with health. Instead, the healthy nasopharynx was colonized with a wider range of less numerous taxa.

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