Dynamics of the nasal microbiota in infancy: A prospective cohort study

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Background: Understanding the composition and dynamics of the upper respiratory tract microbiota in healthy infants is a prerequisite to investigate the role of the microbiota in patients with respiratory diseases. This is especially true in early life, when the immune system is in development.
Objective: We sought to describe the dynamics of the upper respiratory tract microbiota in healthy infants within the first year of life.
Methods: After exclusion of low-quality samples, microbiota characterization was performed by using 16S rDNA pyrosequencing of 872 nasal swabs collected biweekly from 47 unselected infants.
Results: Bacterial density increased and diversity decreased within the first year of life ($R^2 = 0.95$ and 0.73, respectively). A distinct profile for the first 3 months of life was found with increased relative abundances of Staphlyococcaceae and Corynebacteriaceae (exponential decay: $R^2 = 0.94$ and 0.96, respectively). In addition, relative bacterial abundance and composition differed significantly from summer to winter months. The individual composition of the microbiota changed with increasing time intervals between samples and was best modeled by an exponential function ($R^2 = 0.97$). Within-subject dissimilarity in a 2-week time interval was consistently lower than that between subjects, indicating a personalized microbiota.
Conclusion: This study reveals age and seasonality as major factors driving the composition of the nasal microbiota within the first year of life. A subject's microbiota is personalized but dynamic throughout the first year. These data are indispensable to interpretation of cross-sectional studies and investigation of the role of the microbiota in both healthy subjects and patients with respiratory diseases. They might also serve as a baseline for future intervention studies. (J Allergy Clin Immunol 2015;135:905-12.)

Key words: *Nasal microbiota, bacterial families, toddlers, cohort study, season, age*

The prevalence and incidence of asthma in children continue to increase, leading to efforts to curb the so-called epidemic, even while its causes remain ambiguous.[1] An association between bacterial colonization of the airways in children and later development of asthma has been suggested, particularly for the potentially pathogenic *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Haemophilus influenzae*.[2] However, exposure to a wider range of microbes seems to have a protective effect on the development of asthma in children by activating the innate immune system.[3] This finding would support the hygiene hypothesis, according to which asthma is partly caused by a lack of microbial exposure early in life and its subsequent influence on the developing immune system.[4] Indeed, the presence of commensal bacteria was shown to be critical in the control of allergic airway inflammation in a mouse model.[5,6] Therefore it is assumed that the respiratory tract microbiota plays a crucial role in immune development early in life. In particular, the ages between early infancy and 3 years are suggested to be important for the long-term development of immune responses and asthmatic airway disease. Therefore the best possibility for intervention to change the natural trajectory of the disease exists within this window of time.[7]

In recent studies the respiratory tracts of healthy adult subjects were found to harbor a homogeneous microbiota that decreases in biomass from the upper to the lower respiratory tract. The bacteria present in the lungs were suggested to originate from the upper respiratory tract microbiota through microaspiration.[8,9] Therefore the upper respiratory tract is considered the entry point of commensals and potential pathogens into the airways. The nasopharynx was described as an ecologic reservoir consisting of a broad variety of commensal bacteria and potential pathogens.[10] Recent studies revealed a complex and highly variable nasopharyngeal microbiota in children,[11,12] which

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Abbreviations used
nMDS: Nonmetric multidimensional scaling
rRNA: Ribosomal RNA
SDI: Shannon Diversity Index

undergoes significant changes during disease, exposure to antimicrobials, and vaccination.[13,14] Despite these significant findings, a detailed description of the nasopharyngeal microbiota in healthy children was not previously available.

Previous studies assumed an association between the early composition of the upper airway microbiota as a marker for later immune development and subsequent development of allergy and asthma.[2] However, before any conclusions regarding the role of the microbiota on later asthma and other respiratory diseases can be drawn, this hypothesis must be tested in longitudinal measurements.

The aim of this study was to analyze biweekly serial dynamics of the nasal microbiota in healthy infants within the first year of life. In particular, we revealed the influence of age and seasonality on the personalized composition of the microbiota and investigated its dissimilarity throughout the first year of life.

METHODS

Study design

A total of 48 healthy infants from the prospective Basel Bern Infant Lung Development cohort (www.birthcohorts.net) study[15] were enrolled and followed weekly within their first year of life. Pregnant mothers were recruited from the 4 major maternity hospitals and practices of obstetricians in the agglomeration of Bern, Switzerland, through advertisements and interviews. Exclusion criteria for the study were as follows: ethnicity other than white, preterm delivery (<37 weeks' gestation), major birth defects, disease or later diagnosis of airway malformation, or specific chronic respiratory disease. The study was approved by the Ethics Committee of the Canton of Bern.

Nasal swab procedure

Nasal swabs (Verridial E. Mueller, Blonay, Switzerland) were collected biweekly by the parents of the study infants, starting in the fifth week of life. Parents were personally instructed by the study nurses on how to obtain the nasal samples in a standardized way. Immediately after acquisition, nasal swabs were sent in transport tubes and medium (UTM tubes, Verridial E. Muller) to the study center and frozen at −80°C within 10 days or less. In addition, study nurses made weekly telephone calls, during which study parents were interviewed by using a standardized questionnaire. The study infant's health status, as well as respiratory symptoms and antibiotic therapy, were assessed.[16] Nasal swabs taken during upper or lower respiratory tract infection or while the infant received antibiotic therapy were excluded from the analysis because the goal of the present study was to analyze swabs of clinically healthy infants at the time of sampling.

PCR amplification of 16S ribosomal RNA genes and 454 amplicon sequencing

Amplification by using PCR and amplicon sequencing was described elsewhere.[13] In brief, DNA was extracted with 200 μL of transport medium, followed by amplification of the bacterial 16S ribosomal RNA (rRNA) variable regions V3 to V5 by using the multiplex identifier tagged primer pair 341F/907R. PCR reactions were eluted by 40 μL of double-distilled water, and the concentration was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). PCR products with a concentration of less than 1.0 ng/μL were excluded from the study. This corresponds to less than 1 pg/μL bacterial DNA, as evaluated by using quantitative PCR of pneumococcal DNA at different dilutions (data not shown). A minimum of 1 pg/μL bacterial DNA was recently recommended as the threshold when working with low-density materials.[17] Of each purified PCR product, 40 ng/μL was pooled, whereas every multiplex identifier was used once, resulting in 8 amplicon pools.[13] The amplicon pools were sequenced with the 454 sequencing platform. The reads were submitted to the National Center for Biotechnology Information Sequence Read Archive (accession no. SRP041616). Analysis of sequencing products was performed by using PyroTagger, which comprises the definition of operational taxonomic units based on 97% sequencing identity, estimation of chimeras, and taxonomic assignments.[18]

Quality control

In addition to the exclusion of PCR products with a concentration of less than 1 ng/μL, we performed the following quality control steps. First, a cutoff value of 70 reads per sample was defined. Second, 2 negative control samples were sequenced, and those samples displaying greater than 5% sequence reads identical to these negative control samples were excluded.

Bacterial density and α diversity calculations

Bacterial density was estimated based on the concentration of the PCR product. α Diversity, which was referred to as within-community diversity,[19] was assessed by using the Shannon Diversity Index (SDI). The SDI was calculated in R, version 3.02 (http://www.R-project.org.), by using the function "diversity" of the "vegan" package. For analyses of bacterial density and SDI, samples were binned according to the date of acquisition, either referring to the month of the year or to the age of the study infant, resulting in 1 to 3 samples per infant for every month.

β Diversity analyses

β Diversity, which is referred to as community comparison,[19] was calculated by using the function "vegdist" of the "vegan" package in R. We used the Manhattan-type dissimilarity Jaccard to calculate the weighted beta diversity indices (abundance-based). For each study infant, Jaccard dissimilarity was calculated by means of pairwise comparison of 2 samples. Time intervals (Δt) between the samples were subsequently increased, ranging from 2 to 44 weeks. Values from each infant were binned according to the time interval between the samples, as previously described.[20] Short-term within-subject dissimilarity includes all pairwise comparisons of samples from a subject in 2-week intervals. Resulting values were grouped according to the month of age or season. Between-subject dissimilarity includes pairwise comparisons of all samples from 1 subject with all samples from all the other subjects within the corresponding month. "metaMDS" of the "vegan" package was used for nonmetric multidimensional scaling (nMDS), as previously described.[13] As an input matrix, we used the abundance-based Jaccard dissimilarities, as described above. Statistical analysis on nMDS clustering was done with the function "adonis" of the "vegan" package. Vectors were fitted by using "envfit": the resulting arrows point to the direction of the most rapid change in the variable, whereas the length indicates the strength of this gradient.[21] Age in months and month of sampling (seasonality) was investigated for clustering.

Calculation of relative bacterial abundances

Prevalence and relative abundances of bacterial families were obtained based on the taxonomic assignment of PyroTagger, as described previously.[13,18] For every bacterial family, the mean and 95% CI per sample were calculated. The 5 most abundant families were analyzed separately, whereas all the remaining families were grouped as "others." Again, samples were binned according to the date of acquisition, either referring to the month of the year or to the age of the study infant.

Additional statistical analyses

Regression models were then fitted for the relative bacterial abundance, bacterial density, SDI value, and β diversity by using either a linear or an exponential function for age and a second-order polynomial function for the