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Research Paper

Development of Upper Respiratory Tract Microbiota in Infancy is Affected by Mode of Delivery

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ABSTRACT

Birth by Caesarian section is associated with short- and long-term respiratory morbidity. We hypothesized that mode of delivery affects the development of the respiratory microbiota, thereby altering its capacity to provide colonization resistance and consecutive pathobiont overgrowth and infections. Therefore, we longitudinally studied the impact of mode of delivery on the nasopharyngeal microbiota development from birth until six months of age in a healthy, unselected birth cohort of 102 children (n = 761 samples). Here, we show that the respiratory microbiota develops within one day from a variable mixed bacterial community towards a *Streptococcus viridans*-predominated profile, regardless of mode of delivery. Within the first week, rapid niche differentiation had occurred; initially with in most infants *Staphylococcus aureus* predominance, followed by differentiation towards *Corynebacterium pseudodiphteriticum/propinquum*, *Dolosigranulum pigrum*, *Moraxella catarrhalis/nonliquefaciens*, *Streptococcus pneumoniae*, and/or *Haemophilus influenzae* dominated communities. Infants born by Caesarian section showed a delay in overall development of respiratory microbiota profiles with specifically reduced colonization with health-associated commensals like *Corynebacterium* and *Dolosigranulum*, thereby possibly influencing respiratory health later in life.

1. Introduction

The total number of Caesarian sections (C-sections) has dramatically increased during the last decades; from 15% in 1990 to 27% in 2011 of all live births in industrialized countries (Mueller et al., 2015; OECD Publishing, 2013). This is worrisome as delivery by C-section has been associated with early life morbidity, including respiratory distress directly after birth (Karlström et al., 2013), hospitalization for respiratory syncytial virus infection (Kristensen et al., 2015), and long-term health problems, including development of asthma later in life (Guibas et al., 2013; Thavagnanam et al., 2008). One hypothesis that explains the association between the increase of infant disease and the mode of delivery is a disrupted mother-to-child bacterial transmission and thereby altered microbial colonization patterns in children born by C-section (Kristensen et al., 2015). Depending on mode of delivery, children are exposed to either the maternal vaginal and intestinal microbiota (vaginal delivery) or skin and environmental microbiota (C-section), leading to distinct microbial acquisition shortly after birth (Dominguez-Bello et al., 2010; Penders et al., 2013). This suggests that mode of delivery is likely to have profound impact on both the structure of early and late microbiota, as well as on processes depending on microbiota development, i.e. immune maturation, epithelial integrity, microbial tolerance, and pathogen resistance (Hooper et al., 2012).

The upper respiratory tract is the natural niche for respiratory bacterial and viral pathogens and the origin for consecutive respiratory tract infections (RTI). Potential pathogenic bacteria are embedded in a community of commensals, jointly forming the nasopharyngeal microbiota. Microbial colonization succession is highly influenced by environmental factors, host factors, and bacterial acquisition during the first years of life (Koppen et al., 2015). Recently, we published a first crude picture of microbiota development in children over the first two years of life (Biesbroek et al., 2014b). Despite large sampling intervals, the composition of the nasopharyngeal microbiota appeared highly dynamic, especially in the first six months of life. As we hypothesized that a

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balanced microbiota is more resilient to bacterial acquisition and overgrowth, we focused on the stability of the microbial profiles. Intriguingly, early microbiota composition (six weeks of life) predicted microbiota stability over the first two years of life: stable profiles were associated with (exclusive) breastfeeding and fewer respiratory tract infections in the consecutive period (Biesbroek et al., 2014a, 2014b). These findings indicate that there is a window of opportunity during early life where a stable microbial signature is formed, that is associated with protection against respiratory symptoms. These data are supported by both epidemiological (Vissing et al., 2013) and murine (Gollwitzer et al., 2014) data. Although timing and order of exposure to specific groups of microbes, like mediated by mode of delivery, may have crucial consequences on development of the microbial profile, studies investigating the respiratory microbiota of healthy young children in detail in a longitudinal fashion are lacking.

We therefore decided to study the dynamics of the nasopharyngeal microbiota in relation to mode of delivery in detail, from birth until the age of six months in 62 vaginally delivered children and 40 children born by C-section.

2. Materials and Methods

Details can be found in the Supplemental data.

2.1. Study Population

Upper respiratory (nasopharyngeal) samples were obtained from 102 healthy children who participated in an ongoing prospective birth cohort study. The primary aim of this study is to characterize the development of the respiratory microbiota in term children born vaginally (n = 62) compared to children born by C-section (n = 40). Inclusion criteria at baseline were term birth (gestational age > 37 weeks). Exclusion criteria were major congenital anomalies, severe maternal or neonatal complications during birth, language barrier, intention to move outside the research area, or parents under 18 years of age. All participants were born between December 2012 and June 2014. Written informed consent was obtained from both parents before birth of the child. Participants did not receive any financial compensation. An acknowledged national ethics committee in the Netherlands (METC Noord-Holland, committee on research involving human subjects) approved the study (M012-015, NTR3986) and the study was conducted in accordance with the European Statements for Good Clinical Practice.

2.2. Data Collection

At baseline, data were collected on prenatal and perinatal characteristics. Follow-up of participants in the current study included visits directly post-partum, 24–36 h after delivery, and at 7 days, 14 days, and one, two, three, four, and six months of age. Deep nasopharyngeal swabs and a questionnaire on the health status of the child, including respiratory symptoms, were obtained during each visit by a trained research team of doctors and research nurses.

2.3. Construction of the Phylogenetic Library

Bacterial DNA of the nasopharyngeal samples was isolated using a mechanic disruption method as described previously (Biesbroek et al., 2012; Wyllie et al., 2014). Only samples with a bacterial density of at least 0.3 pg/μl above the background (DNA quantity of the negative controls) as measured with Real-Time PCR were analyzed to avoid interference of background DNA. After amplification of the hypervariable V4 region of the 16S rRNA gene, samples were sequenced by Illumina MiSeq (Illumina Inc., San Diego, CA, USA). Since the samples collected postpartum and at day one generally had very low DNA density, we further analysed 12 samples obtained at those time points that did not meet the inclusion criteria, however had shown to generate an amplicon in the16S qPCR and had generated >200 reads in the sequence run. We used complete linkage hierarchical clustering based on the Bray-Curtis dissimilarity matrix including a broad set of negative controls (blanks) which had also generated >200 reads (n = 16). We were able to make a distinction between samples that had significant different microbiota profiles (n = 9) and samples that did not (n = 3). Based on these results, we excluded the latter samples from further analyses. Sequences were processed using modules implemented in Btrim (Kong, 2011) and Mothur V1.31.2 ((Schloss et al., 2009), for modules see supplemental methods). The unsupervised method Minimum Entropy Decomposition (MED) was used to assemble the unique sequences into high resolution oligotypes ((Eren et al., 2014) and oligotyping.org; default settings were used except for the minimal substantive abundance which was set at 100). Taxonomic classification of oligotype node representatives was performed using a naive Bayesian RDP classifier. We calculated the relative abundance of oligotypes per sample and determined the Shannon diversity index to describe the microbial diversity.

2.4. Spectral Clustering

To analyze the trajectory of the microbiota composition of individuals over time, an unsupervised co-regularized spectral clustering algorithm was applied to all data of the 102 children according to previously described methods (Biesbroek et al., 2014b; Imangaliyev et al., 2015; Tsivtsivadze et al., 2013). In short, this multiview clustering algorithm allows for 1) the identification of clusters comprised of individuals with similar microbial profiles in an unbiased and robust manner and 2) the detection of bio-marker species by using an unsupervised feature selection approach (Tsivtsivadze et al., 2014). For further detail, see supplemental methods.

2.5. Statistical Analyses

For all analyses, we used normalized microbial abundance (Biesbroek et al., 2014b). Differences in baseline characteristics and metadata were statistically tested using the 2-sided Chi-square or Fisher's exact test, and Students *T*-test where appropriate (SPSS version 21). p-Values < 0.05 were considered significant. Calculations on Shannon diversity and observed oligotypes were performed using a Kruskal-Wallis test with Dunn's correction for multiple testing in GraphPad Prism (version 6).

Nonmetric multidimensional scaling plots based on Bray-Curtis dissimilarity of log-transformed relative abundances were used to visualize the differences between mode of delivery and time-dependent microbiota development. Statistical significance of the difference in the overall microbiota composition driven by mode of delivery was assessed using PERMANOVA.

To describe intra-individual changes in relative abundance and presence of microbial species over time, we calculated a relative change matrix (Harville, 1997) per oligotype for two-month timeframes. From this relative change matrix per timeframe, we calculated the magnitude of microbiota change (norm value) using L2 norm (Biesbroek et al., 2014b; Harville, 1997). The higher this norm value, the higher the magnitude of change.

To investigate whether there is a statistically significant association between colonization trajectory (e.g. the change of the clustering profile in time) and the mode of delivery, we conducted a randomization test (Biesbroek et al., 2014b).

To identify biomarker species that are associated with modus partus, we evaluated oligotypes that varied in relative abundance over time between vaginally and C-section born children, independent of feeding type (EDGE package R((Storey et al., 2005) and tutorial "Extraction of Differential Gene Expression Version 2.0.0" March 2015, R version 3.2.0)). We conducted the analysis described above for the total dataset (n = 102 children) and for a subset of children who were exclusively