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Intestinal Microbial Ecology in Premature Infants Assessed Using Non-Culture Based Techniques

Maka Mshvildadze[1,2], **Josef Neu**[2], **Jonathan Shuster**[3,4], **Douglas Theriaque**[4], **Nan Li**[2], and **Volker Mai**[5]

[1]Chachava Scientific-Research Institute of Perinatal Medicine Obstetrics and Gynecology, Tbilisi, Georgia

[2]Department of Pediatrics, University of Florida, Gainesville, Florida, USA

[3]Departments of Epidemiology and Health Policy Research, University of Florida, Gainesville, Florida, USA

[4]General Clinical Research Center, University of Florida, Gainesville, Florida, USA

[5]Microbiology and Cell Sciences and Emerging Pathogens Institute, University of Florida, Gainesville, Florida, USA

Abstract

Objectives—To utilize high throughput techniques to analyze intestinal microbial ecology in premature neonates, who are highly susceptible to perturbations of the luminal environment associated with necrotizing enterocolitis (NEC) and late onset sepsis..

Study design—Using non-culture based techniques we evaluated intestinal microbiota shortly after birth and during hospitalization in 23 neonates born at 23-32 weeks gestational age. Microbiota compositions were compared in six preterm infants who developed NEC and/or signs of systemic inflammation versus matched controls using 16S rRNA pyrosequencing.

Results—Microbial DNA was detected in meconium suggesting an intrauterine origin. Differences in diversity were detected in infants whose mothers intended to breast feed (p=0·03), babies born to mothers with chorioamnionitis (p=0·06) and in babies born at less than 30 weeks gestation (p=0·03). A 16S rRNA sequence analysis detected Citrobacter-like sequences only in cases with NEC (three out of four) as well as an increased frequency of Enterococcus-like sequences in cases and Klebsiella in controls (p=0·06). The overall microbiota profiles in cases with NEC were not distinguishable from that in controls.

Conclusions—Microbial DNA in meconium of premature infants suggests prenatal influences. Microbial ecology alterations prior to NEC and sepsis suggest intestinal microbial origins.

Keywords

preterm infants; necrotizing enterocolitis; systemic inflammation; intestinal microbiota; bacteria

The authors declare no conflicts of interest.

The human adult harbors an estimated 1×10^{14} cells, ~10% belong to the host and the remaining 90% represent the microbiota in or on the host, most of which are found in the intestinal tract.[1-4] Although little data exist from human studies, animal studies suggest that the intestinal microbiota during the neonatal period has a profound effect on nutritional status, gastrointestinal (GI) tract development, and maintenance of mucosal surface integrity.[2,5-7] Our understanding of the establishment and evolution of the intestinal microbiota during early infancy in humans has been limited because classical cultivation techniques allow the identification of a limited number of bacteria. In the adult human gut, 60-80% of the total microbiota can not be cultivated.[8,9]

In premature infants, the GI environment and susceptibility to disease are different compared with that in term infants.[10] There is little information pertaining to the development of the intestinal microbiota using non-culture based techniques in infants. A few studies have monitored the bacterial communities in preterm infants.[11-15] Application of high throughput 16S rRNA based techniques is needed to accurately assess the developing GI microbiota. 16S rRNA is part of the small ribosomal subunit that is preserved among all living organisms. It is the most universally used molecule for phylogenetic analysis as the gene encoding it contains conserved as well as variable regions. Similarity in the 16S rRNA genes is a measure of the relatedness of a bacterium of interest to other bacteria. Advantages of phylogenetic analysis using a 16S rRNA approach include its high sensitivity and the ability to analyze organisms without a need to first culture them. 454 pyrosequencing allows for an in depth analysis of 16S rRNA gene sequences derived from community DNA, such as that present in human feces. The large number of sequences obtained using the 454 pyrosequencing approach, which exceeds conventional Sanger based sequencing by magnitudes, permits the detection of abundant but also rare bacteria.

This research is critical because these infants have several immaturities of the GI tract,[16-18] including hyperactive inflammatory responses[19] to microbial antigens that may predispose the premature infant to intestinal injury including necrotizing enterocolitis (NEC) and intestine-derived systemic inflammation.[7] This study was designed to use non-culture based technology to evaluate intra- and interindividual variation in early intestinal microbial ecology in premature infants to help guide investigations in larger, more extensive studies.

Methods

The study protocol was approved by the ethics committee at the University of Florida. Informed consent was obtained from the parents. The study protocol involved all the babies less than 32 weeks gestational age at birth. Parents of three infants refused to participate in the study. The first stool sample (meconium) and weekly samples thereafter were collected from 27 preterm infants, born at 23 to 32 weeks of gestational age, who had been admitted to the Neonatal Intensive Care Unit. Four of these babies' samples did not have denaturing gradient gel electrophoresis (DGGE) profiling but were used only for pyrosequencing. For monitoring the GI microbiota profile, DGGE was performed on multiple fecal samples from 23 infants that were born at gestational ages that ranged from 23-32 weeks with birth weight ranging from 520g to 1997g (Table I).

In order to evaluate in greater depth the intestinal microbial composition in infants with i) NEC and ii) presumptive sepsis with signs of systemic inflammatory response, we performed 454-based 16S rRNA pyrosequencing in a subset of 12 infants. Prospective stool samples were chosen approximately one week (range three to nine days) before onset of the disease from four subjects who developed NEC and two additional subjects who showed signs of sepsis and systemic inflammation (elevated C-reactive protein (CRP>10), elevated

white blood cell counts and I/T ratio > 20, or culture positive sepsis). Six controls were matched by birth weight, week of delivery and the day of life of stool collection to each of the cases (Table II). All fecal samples were frozen at –80°C immediately after collection until analyses were performed.

Microbiota analysis

DNA extraction and DGGE—DNA was extracted from 200-300mg fecal samples using a modified Qiagen stool DNA extraction protocol.[20] Primers were used to amplify the V6-V8 region as described by Zoetendal et al[21] DGGE was performed in an 8% (wt/vol) polyacrylamide gel with a denaturing gradient ranging from 40% to 50% at the top and bottom of the gel, respectively (100% denaturing conditions were defined as 7M urea and 40% formamide). After electrophoresis (16h, 65V, 60°C), the gels were stained with SYBER Gold (Novex, San Diego, CA), scanned with Quantity One, and analyzed with Diversity Database software (Bio-Rad, Hercules, CA).

Estimation of microbial richness and diversity—The richness and diversity of the preterm infants' fecal microbiota was determined from the number and intensity of PCR-DGGE bands present in each profile. Profiles for various time points were compared for each infant and between different infants. We calculated Simpson diversity index (SDI)[22] and compared microbial diversity using 1/SDI for individual time points as well as the mean for each infant.

454 based 16S rRNA pyrosequencing—DNA from the fecal samples was amplified using a barcoded pyrosequencing primer based on universal primers 27F and 338R as described by Hamady et al[23] Sequences of low quality or with a length of less than 150 nucleotides were removed from the analysis. Sequences were analyzed using the RDP pyrosequencing pipeline[24] including features to calculate diversity indices and rarefaction curves. We used weighted UNIFRAC to generate a principal component analysis to compare overall similarity in microbiota composition.[25]

Statistics

The associations of prognostic factors (such as breast or formula feeding) with the Simpson diversity Index (SDI) were evaluated by the two-sample two-sided t-test. Where repeated measures were used, we calculated a personal mean of the SDI and compared the mean of the means between the two groups. Descriptive data are expressed as mean± S.D. In addition to the mean of mean analyses, which weigh all subjects equally, we conducted secondary nested repeated (mixed random effects) measures ANOVAs. The two give basically the same qualitative conclusions, and therefore only the mean of mean analyses are reported in detail. Trends over time were assessed by first obtaining a personal slope, and comparing these with a median of zero by a two-sided Wilcoxon sign-rank test. Nonparametric methods were used here as personal slopes tend to be outlier prone.

Student t-test was used to test the difference between two independent groups or samples and to determine the significance of differences in the number of specific OTU's (operational taxonomic unit) that were detected in the sequence analysis. P< 0·05 was considered statistically significant. Similarity matrices for comparing overall microbiota composition were generated using weighted as well as unweighted UNIFRAC analysis, and they were further analyzed by principal component analysis.[25]

Results

Over a period of approximately one year, 27 preterm infants were enrolled (Table I). The time of first stool collection varied from second day of life to day 22 of life, one infant born at 23 weeks gestational age had a delayed passage of meconium on day of life 22. Depending on the length of hospital stay the number of stool samples collected per infant varied from one to 15.

Early Fecal Microbiota

In order to determine whether microbes were present in the meconium and to evaluate diversity depending on pre- and perinatal factors, we compared microbiota diversity in all initial fecal samples using DGGE profiling. For two of the 23 very premature infants we did not detect DNA in the first stool sample, they were therefore not included in this analysis. Microbiota diversity in the samples was compared using the SDI. Table III (left column) shows differences in diversity index from stool microbiota from the first stool samples from 21 babies. Lower gestational age (<30 compared with 30 weeks gestation) was associated with lower diversity index (p=0·03). Trends toward lower diversity index were evident in babies whose mothers had prolonged rupture of membranes (PROM) and who received intrapartum antibiotics (p=0·09 and p=0·06, respectively). Mothers' intent to breast feed was associated with a lower diversity index (p=0·03). No significant differences in microbiota diversity were seen in babies of mothers who delivered by Cesarean section versus vaginal delivery (p=0·5).

Diversity over time under different conditions

Of the 23 infants initially included, one was transferred to another hospital after the first stool collection and was not available for longitudinal microbiota profiling. Microbiota profiles determined in the remaining 22 infants by DGGE revealed unique compositions for each infant. Although composition changed over time, characteristic bands were observed for each infant at multiple time points. There was a trend towards increased diversity over time (data not shown). After removing samples collected during the first week of life from the analysis, the association between microbiota diversity and type of feeding breast milk (the infants who were in the intent to breast feed group remained in breast fed group) vs formula disappeared (Table III, right column). No significant associations were detected between microbiota diversity and sex, birth weight, gestational age (p=0·42), PROM (p=0·79), maternal intrapartum antibiotics (p=0·75), or mode of delivery (p=0·14)

454 pyrosequencing: NEC and/or suspected sepsis with signs of inflammation

In addition to microbiota profiling by DGGE, we performed an in depth 16S rRNA analysis for a 6 infants with NEC and/or suspected sepsis with signs of inflammation (cases) and six matched samples without signs of NEC or sepsis (controls) (Table II). Fecal samples were collected prospectively and 16S rRNA sequence analysis was performed on samples collected approximately one week (range three to nine days) before onset of symptoms. For the 12 samples we obtained a total of 13377 16S rRNA sequences with an average length of 244 nucleotides (Min = 46, Max = 319). We used the RDP pyrosequencing pipeline to remove low quality and short reads, retaining a total of 10656 reads (average of 888 sequences/sample). The number of OTU's in each of the samples at the level of 100%, 99% and 97% identity ranged from 107-260; 48-116 and 18-46 respectively.

Differences were obvious at the level of bacterial family and were more pronounced at the level of bacterial genus (Figure; for the subject characteristics refer to Table II). When OTU's were grouped by bacterial family ten families represented at least 98 % of all sequences observed (Figure). Each sample varied in the proportion of OTU's belonging to a

particular bacterial family. When OTU's were grouped by genus level some similarities could be observed in cases and controls respectively (Figure). Higher numbers of Enterococcus were more frequently detected in cases with NEC and SIRS (systemic inflammatory response syndrome) compared with controls. In contrast, Klebsiella was more frequently detected in controls compared with both cases of NEC and SIRS (p=0·06). In addition we detected two OTUs with closest matches to Enterobacteriaceae that were detected in higher proportion in controls (p<0·05). Sequences matching Citrobacter species were detected, although present in low numbers, in 3 out of 4 cases of NEC but in none of the controls.

When overall microbiota composition was evaluated using a weighted Unifrac analysis,[25] the resulting principal component analyses did not indicate that microbiota composition in cases is more similar than in controls. Cases and controls did not cluster into distinct groups as would be expected if overall microbiota composition was more similar within cases or controls.

Discussion

The goal of this study was to gain insight into the establishment of intestinal microbiota composition in premature infants born under 32 weeks of gestational age. We investigated changes in microbiota over time to evaluate potential associations with environmental factors such as gestational age, mode of delivery, premature rupture of membranes, use of maternal antibiotics, and breast versus formula feeding. In addition, we generated initial data on potential contributions of distortions in microbial ecology to the onset of the NEC or late onset sepsis. Our findings, based on non-culture based techniques, show that microbiota in preterm infants shows a high inter-individual variability that develops surprisingly rapidly after birth. In the earliest stool samples, diversity differed depending on gestational age at delivery, use of maternal antibiotics, prolonged rupture of membranes, and intended feeding type (breast versus formula). No significant differences were detected in these early samples between C-section versus vaginally delivered infants. Comparison of infants stool microbiota prior to the development of inflammatory processes such as NEC or sepsis using 454 pyrosequencing suggested differences in colonization patterns. One of the findings using both DGGE and 454 pyrosequencing is the marked variability of microbial composition between infants. However, within individual babies, several bands persisted over time with a trend toward increasing diversity.

Millar et al[26] was the first to use 16S rRNA gene PCR and evaluated the bowel flora of preterm infants with and without NEC. Fecal samples from 32 preterm infants, including samples from 10 infants with NEC, were examined by culture and PCR amplification of the 16S rRNA gene. In this study, uncultured bacteria detected by PCR-DGGE were no more frequent in the fecal samples of infants with NEC than in the samples of infants without NEC. Previous studies that utilized non-culture based techniques agree partially with our findings. Magne et al[13] also found a high inter-individual variability. The main groups encountered were the Enterobacteria family and the genera Enterococcus, Streptococcus, and Staphylococcus. Our study is in agreement with the finding of high inter-individual variability in the bands found on the gels and also 454 pyrosequencing, but in addition shows a rapid establishment of diversity, which after the first samples is not markedly different when comparisons are made under several environmental conditions.

In samples collected before onset of disease symptoms and analyzed by high throughput 16S rRNA sequencing we detected correlations between the presence/absence of specific bacterial groups and future disease status. Each preterm infant developed a microbiota composition that was clearly distinct from that in others, even in the samples collected from

twins. Another study using a combination of culture and molecular methods for analyzing bifidobacterial colonization[12] showed that colonization by bifidobacteria was affected neither by birth weight, mode of delivery, antibiotics given to the mother, and infants nor type of feeding, but gestational age at birth was a significant condition for colonization by bifidobacteria. However, in contrast to our study, which longitudinally evaluated infants at 23-32 weeks gestational age, this study didn't involve premature babies born at less than 30 weeks. These observations are suggestive of differences in the proportion of specific bacterial groups that should be evaluated in larger studies.

De la Cochetiere et al[27] previously reported the prospective detection of a temperature gradient gel electrophoresis (TGGE) band that corresponded to C. perfringens in 3 cases of NEC and none of 9 controls. We did not detect any particular band shared between cases only. Although we sequenced more than 10K 16S rRNA fragments, very few sequences had their closest match to the genus Clostridia and no sequences matched C. perfringens. Thus, our results do not support an association between C. perfringens and NEC.

We evaluated microbial DNA in the first stools of premature babies using non-culture based methods. It is likely that the microbes from the meconium of these infants are not of postnatal origin. Previous studies have demonstrated the presence of microbes in amniotic fluid without rupture of membranes using both culture and non culture based techniques.[28,29] In one of these, a correlation was found between extent of microbial colonization and length of gestation.[28] Our finding suggests that amniotic fluid containing microbes may have been swallowed in utero. The lower microbial diversity in the infants with gestational age < 30 weeks gestation appears to contradict the findings of DiGiulio,[27] If increasing degree of prematurity is associated with more amniotic fluid microbial colonization, then one would also expect to find more microbes in the first stools of the more premature infants. Additional studies are needed to further evaluate this apparent discrepancy.

Previous studies using primarily culture-based techniques have suggested differences in microbial colonization of breast versus formula fed infants, but this remains controversial.[30] Of interest in our study is the lower diversity of the first stools from babies of mothers who intended to breast feed. Most of these babies had received little or no feeding at the time of the first stool collection. Whether this is due to differences in antenatal care, socioeconomic differences, or other factors can only be speculated upon at this time.

We also found a trend toward less diversity in the first stools of babies whose mothers received antibiotics. This difference diminished over time. The effect of postnatal antibiotics could not be determined in this study because the majority of these premature babies received antibiotics in the first several days of postnatal life. Routine use of antibiotics in the premature has recently been associated with a higher likelihood of developing NEC.[31]

In conclusion, these data provide an important foundation for future studies. These include evaluations of the very early microbiota in premature infants with early onset of labor, the effects of pre- and postnatal antibiotics on the early intestinal microbial ecology and correlations with clinical effects and the effects of various nutrients and feeding regimens on the intestinal microbial environment and subsequent development of disease.

Acknowledgments

Abbreviations

NEC	necrotizing enterocolitis
GI	gastrointestinal
DGGE	denaturant gradient gel electrophoresis
SDI	Simpson diversity index
OTU	operational taxonomic unit
SIR	systemic inflammatory response
PROM	prolonged rupture of membranes
SIRS	systemic inflammatory response syndrome
CRP	C-reactive protein
MAB	maternal antibiotics

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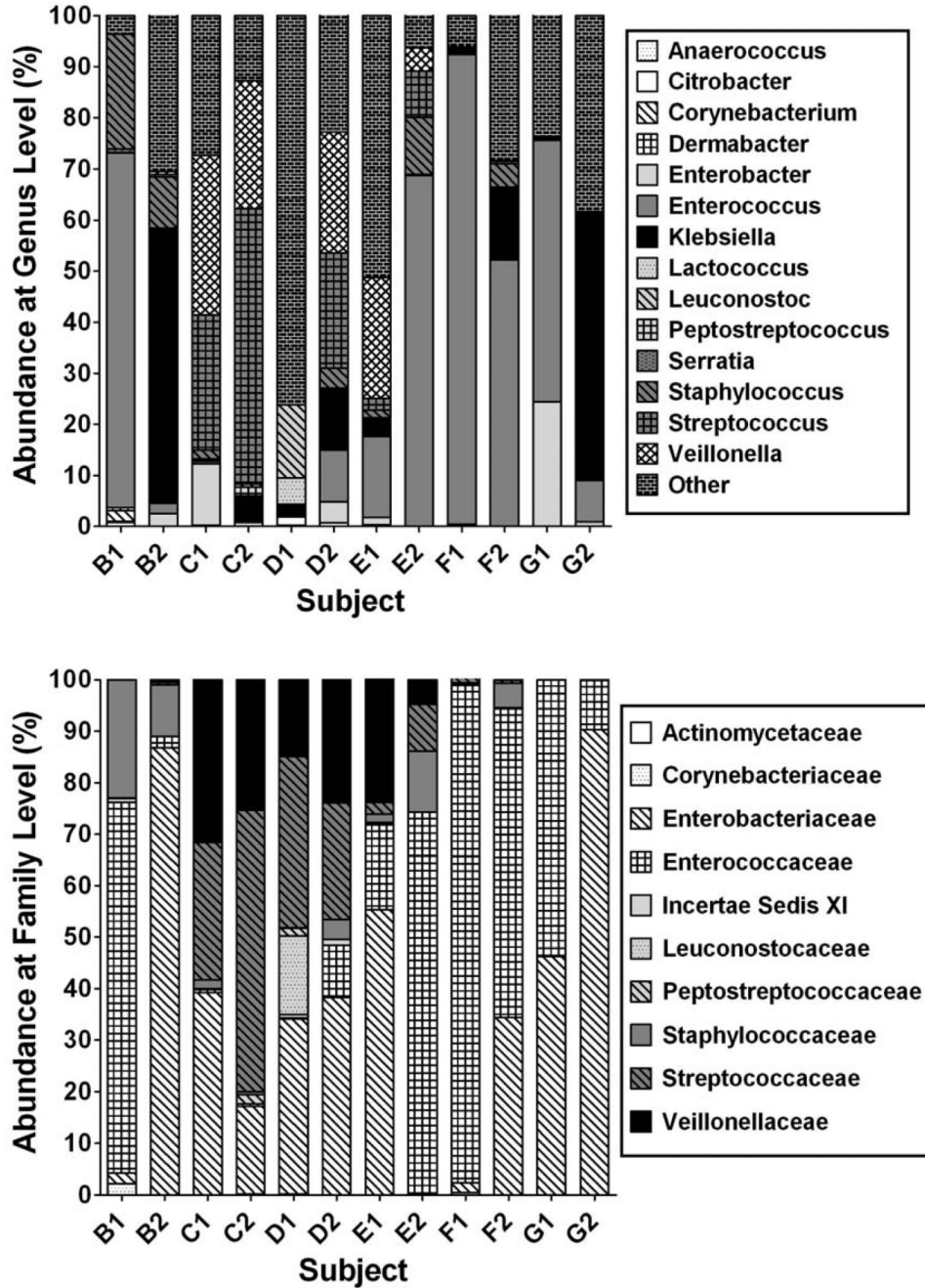
Figure.
Abundance of bacterial families & genera from Pyrosequencing Analysis Proportion of OTUs matching ten most frequently observed families. B1, C1, D1 and E1 represent cases with NEC, F1 and G1 SIRS, matched with controls B2, C2, D2, E2, F2, G2. Sequences matching other bacterial families represent < 2% in any of the samples are not shown. Subject characteristics and # of sequences are shown in Table II. "Other" represents minor and unclassified OTUs.

Table I

Clinical Characteristics of infants

Characteristic	DGGE Group (n=23) frequency or mean (SD)
Male/Female	15/8
Gestational Age (weeks)	29.9 (2.5)
Birth Weight (grams)	1394 (420)
Total Number of Stools	5.2 (3.3)
Delivery: C-section/Vaginal	13/10
Feeding: Breast Milk/Formula[‡]	15/8
Infant: Antibiotics Y/N[†]	19/4
Mother: Antibiotics Y/N[*]	7/16
Prolonged Rupture of Membranes	Y/N 6/17

[‡]intent to breast milk feed and actual breast milk feed group vs Formula

[†]infants antibiotic therapy immediate after birth

[*]Mother's intrapartum antibiotic therapy

Table II

16S rRNA analysis prior the development inflammatory conditions

Subject	Status	Gestational Age (weeks)	Birth Weight (grams)	Sex	Delivery Mode[‡]	Diet[†]	DOL[*]	Number of Sequences
B1	NEC	28	1000	M	CS	B	12	794
B2	Control	32	1437	M	CS	B/F	13	1002
C1	NEC	29	1148	M	CS	F	24	830
C2	Control	29	1134	F	CS	F	22	971
D1	NEC	31	1711	M	CS	F	4	755
D2	Control	31	1395	M	CS	F	4	825
E1	NEC	29	1496	M	VD	B/F	13	963
E2	Control	31	1377	M	CS	F	12	605
F1	SIRS	24	583	M	VD	B	24	881
F2	Control	28	1026	M	VD	F	20	933
G1	SIRS	23	520	F	VD	B	58	1012
G2	Control	27	705	M	CS	F	58	1085

VD=Vaginal Delivery

F-formula

[‡]CS=C-section

[†]B=actual breast milk feed

[*]DOL=Day of life of the stool sample collection.

Table III

Simpson Diversity Index in Meconium and > 7 Days Postnatal Age

Variable		Simpson Diversity Index Mean (SD) <n>	
		Meconium	>7 Days Postnatal Age
Maternal intrapartum Antibiotics	Yes	6.76 (2.90) <6>	9.27 (2.52) <7>
	No	9.37 (2.55) <15>	8.97 (1.73) <15>
	P-Value	0.06	0.75
Feeding type	Breast Milk	[‡]7.60 (2.58) <13>	[†]9.09 (2.03) <14>
	Formula	10.28 (2.60) <8>	9.04 (2.13) <7>
	P-Value	0.03	0.96
Gestational Age	⪖30	9.38 (2.54) <16>	9.31 (1.78) <15>
	<30	6.20 (2.62) <5>	8.56 (2.37) <7>
	P-Value	0.03	0.42
Delivery	C-section	8.99 (3.33) <12>	9.64 (1.86) <12>
	Vaginal	8.13 (2.14) <9>	8.39 (1.95) <10>
	P-Value	0.50	0.14
Prolonged Rupture of Membranes	Yes	7.11 (2.80) <7>	8.90 (2.25) <7>
	No	9.38 (2.65) <14>	9·15 (1.89) <15>
	P-Value	0.09	0.79

[‡] intent to breast milk feed

[†] type of breast milk feed