Early Empiric Antibiotic Use in Preterm Infants Is Associated with Lower Bacterial Diversity and Higher Relative Abundance of *Enterobacter*

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Objectives To determine the impact of empiric ampicillin and gentamicin use in the first week of life on microbial colonization and diversity in preterm infants.

Study design The 16s ribosomal DNA community profiling was used to compare the microbiota of 74 infants born ≤32 weeks gestational age by degree of antibiotic use in the first week of life. The degree of antibiotic use was classified as 0 days, 1-4 days, and 5-7 days of antibiotic administration. All of the antibiotic use was empiric, defined as treatment based solely on clinical suspicion of infection without a positive culture result.

Results Infants who received 5-7 days of empiric antimicrobial agents in the first week had increased relative abundance of *Enterobacter* (P = .016) and lower bacterial diversity in the second and third weeks of life. Infants receiving early antibiotics also experienced more cases of necrotizing enterocolitis, sepsis, or death than those not exposed to antibiotics.

Conclusions Early empiric antibiotics have sustained effects on the intestinal microbiota of preterm infants. Intestinal dysbiosis in this population has been found to be associated with elevated risk of necrotizing enterocolitis, sepsis, or death. *(J Pediatr 2014;165:23-9)*.

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Antibiotics are the most commonly prescribed medications administered to infants in neonatal intensive care units (NICUs).[1] Even though the incidence of early-onset sepsis is low, preterm infants often receive empiric antibiotic treatment in the first few days of life when infection is suspected.[2,3] Concerns about occult intrauterine infection precipitating spontaneous premature labor, premature rupture of membranes, and chorioamnionitis often prompt initiation of empiric antibiotic treatment.[4] Although initiation of antibiotic treatment for premature infants may be prudent under these circumstances, the duration of treatment is often arbitrary, based not on positive culture results but on the clinician's perception of risk.[5] When empiric antibiotic treatment is clinically warranted, many clinicians limit such treatment to 2 days as the standard. Nevertheless, empiric antibiotic treatment is sometimes continued for 5 days or more based on antepartum factors, such as prolonged rupture of membranes or suspected chorioamnionitis, or for nonspecific postnatal signs of infection including need for resuscitation at delivery, respiratory distress, or feeding intolerance. Unfortunately, early antibiotic therapy has the potential to cause harm as well as benefit to the preterm infant by impeding initial microbial colonization.[6,7] The microbial community of preterm infants is known to consist of dramatically fewer beneficial species, lower bacterial diversity, and more pathogens than observed in healthy term infants,[8-11] but it is not known to what extent this observation can be attributed to the high use of antimicrobial agents. Even though recent advances in culture-independent sequencing methods have revolutionized our understanding of the microbial communities that live on and within the human body, studies of preterm infants have not yet attempted to examine the impact of antimicrobial use on the gut microbiome.

Unrestricted antimicrobial use can have persistent, unintended consequences, including reduced diversity of the microbial community, and after use is discontinued, recovery of a healthy microbiome is not assured.[12] Early empiric antibiotic use in preterm infants is associated with increased risk of necrotizing enterocolitis (NEC), sepsis, and death.[13-15] Although aberrant early intestinal microbial colonization is thought to contribute to the pathobiology of NEC,[8,11,16]

GEE	Generalized estimating equations
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit

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Funded by the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health (HD059140, HD13021, and HD27853), National Institute of Environmental Health Sciences (T32 ES010957), National Center for Research Resources, National Institutes of Health (U01 RR026314), National Human Genome Research Institute, National Institutes of Health (HG005969), Danone Research (PLF-5972-GD), and the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services (contract HHSN272200900018C). The authors declare no conflicts of interest.

Sequence data generated for this work is deposited under the NCBI bioproject ID 63661.

http://dx.doi.org/10.1016/j.jpeds.2014.01.010

the impact of early empiric antibiotic use on the preterm microbial community is not well studied.

We sought to test the hypothesis that early intensive antibiotic use in preterm infants alters the ontogeny of the microbiota and decrease microbial diversity in a longitudinal study of intestinal colonization in 74 preterm infants. Serial stool samples were collected from days 4-23 of life to test any differences in the early establishment of the intestinal microbiome by early and intensive empiric antibiotic therapy.

Methods

Study infants were enrolled from 3 level III NICUs in Cincinnati, Ohio as part of an ongoing cohort study of novel biomarkers for cases of NEC, sepsis, or death in infants ≤32 weeks gestational age. All infants remained free of NEC, sepsis, or death in the first postnatal week and had no identified congenital anomalies. The Institutional Review Boards at the 3 participating hospitals approved the study. Early empiric antibiotic exposure was defined as antibiotic treatment initiated within the first postnatal day.[14] The duration of early empiric antibiotic therapy was defined as the total number of continuous days of administration of antibiotics with sterile culture results. Sepsis was defined as a positive blood, cerebrospinal fluid, urine, or sterile site culture. NEC was defined using modified Bell stage II or III criteria.[17]

In this cohort, early empiric antibiotic use consisted of ampicillin and gentamicin, using standard dosing. Antibiotic exposure groups were defined as no antibiotics (0 days), brief antibiotics (1-4 days of empiric antibiotic therapy), and intensive antibiotics (5-7 days of antibiotic therapy).

Serial stool samples were collected from infants during the first 3 weeks of life: at week 1 (4-7 days), week 2 (10-16 days), and week 3 (20-23 days of age). Samples were collected from soiled diapers, immediately refrigerated in the NICU, and transported to the laboratory where they remained in the refrigerator until processing with thioglycollate and storage at −80°C.

As previously described,[16] bacterial DNA was extracted from infant stool samples using 1 of 2 methods: phenol-chloroform or the QiaAmp DNA stool kit (Qiagen, Valencia, California). Bacterial 16S ribosomal DNA sequences (**Table I**) were produced by the Broad Institute (Boston, Massachusetts) using production protocols established for the Human Microbiome Project.[18] The V3 to V5 window of the 16S ribosomal RNA gene was amplified and the sequences were determined using the 454 FLX Titanium platform (Life Sciences, Branford, Connecticut). A total of 1.3 M resulting sequences were processed using a data curation pipeline implemented in mothur for operational taxonomic unit clustering,[19] complemented by abundant operational taxonomic unit,[20] UCHIME for chimera detection,[21] and NEWBLER for assembly-based error reduction.[22,23]

Table I. Definitions for metagenomic sequencing and analyses

- **16S ribosomal DNA (or 16S rDNA)** – The gene coding for the 16S component of the 30S subunit of prokaryotic ribosomes. 16S rDNA, contains hypervariable regions that can provide species- specific signature sequences useful for bacterial identification
- **Diversity index** – Also known as alpha-diversity, this is a quantitative measure of the number of different species in the population, also accounting for how evenly species are distributed. (The diversity index increases both when the number of species increases and when the evenness increases.)
- **Operational Taxonomic Unit (OTU)** – sequence reads clustered by relatedness to provide a phylogenetic description of the microbial community. In this study, an OTU is defined as sequence reads clustered by at least 97% similarity.
- **Relative abundance** – proportion of a microbial operational taxonomic unit represented in a microbial community

Statistical Analyses

Sequence data was generated for 256 samples from 81 infants. Samples collected following NEC or sepsis were excluded from analysis. Five infants with only week 1 samples were also excluded. Thus, a total of 74 infants with 239 samples were available for study. Differences among groups were tested using Fisher exact test for categorical variables and ANOVA for continuous variables. The nonparametric Kruskal–Wallis test was used to compare differences in diversity between antibiotic groups.

An important metric to describe a microbiome is the diversity of bacterial species identified in that microbiome. We used the Quantitative Insights into Microbial Ecology program[24] to calculate the Simpson diversity index, which measures both species richness (number of species present) and evenness of abundance. The entire data set was rarefied to 2200 reads per sample before alpha-diversity was calculated. This procedure was repeated 5 times. Alpha-diversity metrics were then averaged per sample across these 5 iterations.

To account for multiple samples per infant, generalized estimating equations (GEE) models of alpha diversity were used to analyze differences in Simpson diversity index between infants with differing levels of antibiotic use. GEE models were run with an exchangeable correlation structure using the *geeglm* function in the *geepack* package in R.[25]

The association between early antibiotic use and the relative abundance of the 2 most common operational taxonomic units were examined using a linear regression model. These 2 most abundant operational taxonomic units were classified, respectively, as an *Enterobacter* and a *Staphylococcus*, and were selected based on initial evidence of change in relation to antibiotic use groups, and being sufficiently abundant to allow robust modeling. The much lower abundance of other operational taxonomic units disallowed modeling due to zero inflation and limited statistical power. Because data from the same individuals tend to be positively correlated, the regression model was modified using GEE. Because the distribution of the antibiotic use was different for different sampling time, we stratified the data related to sample collection time, either week 2 or week 3 of life. We also categorized the duration of antibiotic use into 3 categories: none (0 days); brief (1-4 days); or intensive (5-7 days). Prior to analysis, raw operational taxonomic unit