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Changes of intestinal microflora in neonatal necrotizing enterocolitis: a single-center study

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Abstract

Objective: To investigate the changes in the diversity and dynamics of intestinal microflora in patients with neonatal necrotizing enterocolitis (NEC) in a single center in China.

Methods: In this prospective study conducted between 2016 and 2019, feces were collected from 28 neonates with NEC and 30 control neonates to analyze the species richness and Shannon's diversity index of the bands, which were also sequenced via PCR-denaturing gradient gel electrophoresis.

Results: The species richness and Shannon's diversity index were significantly lower in the NEC group than in the control group, indicating that the diversity of intestinal flora was reduced in NEC. The numbers of samples positive for *Bacteroides* and *Klebsiella* were higher in the NEC group, whereas *Escherichia coli*, *Bifidobacterium*, and *Lactobacillus* were less frequently detected in patients with NEC than in controls. The prevalence of intestinal bacteria normalized after the resolution of NEC.

Conclusion: The decrease of intestinal flora diversity in neonates with NEC, especially the decreased prevalence of probiotic bacteria, may be an important factor in the pathogenesis of NEC.

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Keywords
Neonatal necrotizing enterocolitis, pediatrics, intestinal microflora, probiotics, bacterial diversity, species richness, feces

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Introduction

Necrotizing enterocolitis (NEC) is a common and serious digestive disease in neonates that occurs mainly in preterm infants. The main clinical manifestations are vomiting, bloating, and blood in the stool, and its mortality rate is high.[1] At present, the detailed pathogenesis of NEC is unclear, but some studies attributed the condition to significant differences in intestinal flora between NEC and healthy newborns, and intestinal colonization by abnormal bacteria is an important risk factor for NEC.[2] Therefore, this study used PCR-denaturing gradient gel electrophoresis (DGGE) combined with DNA sequencing to analyze the diversity and dynamic changes of intestinal microflora in neonates with NEC.

Materials and methods

Participant characteristics

This prospective study was conducted in the neonatal intensive care unit of the First People's Hospital (Zunyi City, Guizhou Province, China), and neonates with NEC who were admitted between January 2016 and March 2019 were eligible for enrollment. All neonates with the following characteristics were eligible for recruitment into this study: (1) meeting the Bell NEC clinical diagnostic criteria and Bell's stage I; (2) neonates born at a gestational age (GA) $<$ 37 weeks; (3) no receipt of probiotics; and (4) provision of written informed consent by their parents. In addition, 30 premature neonates in the same age range without gastroenterology syndromes who had not received probiotics were included as a control group. After admission, the neonates in the two groups were treated with broad-spectrum antibiotics. This study was approved by the Ethics Committee of the First People's Hospital of Zunyi, Guizhou, China (Ethics Code No. 2015-0219).

Equipment and reagents

A PCR system was purchased from the United States MJ Research Company (Waltman, MA, USA). DGGE electrophoresis, gel imaging, grayscale scanning, and mutation detection systems were purchased from Bio-Rad (Hercules, CA, USA). A low-temperature high-speed centrifuge was purchased from Eppendorf (Hamburg, Germany). A QIAamp Stool Mini Kit was obtained from QIAGEN (Venlo, Netherlands), and Taq premix was acquired from Baosheng Co., Ltd. (Dalian, China). SYBR green I was purchased from Tektronix Biotechnology Co., Ltd. (Shanghai, China).

Sample collection and storage

Fecal samples were collected from neonates on days 1, 3, 5, 7, and 9 after admission using a sterilized stool collector and stored at −80°C. We additionally collected samples on the day of discharge to analyze the relationship between intestinal flora and outcomes.

DNA extraction and amplification

In total, 0.22 g of feces was collected in a 2-mL centrifuge tube followed by DNA extraction according to the instructions of the QIAamp Stool Mini Kit. Amplification was performed using universal 16S rDNA V3 hypervariable region-specific primers (forward, 357F [with a GC cap]; reverse, 518R) synthesized by Shanghai Biotechnology Bioengineering Co. (Shanghai, China). Each PCR reaction mixture contained 5 μL of the DNA template, 1 μL each of the forward and reverse primers (10 μmol/L), 25 μL of premix Taq, and ddH_2O to 50 μL. The PCR reaction conditions were as follows: denaturation at 94°C for 5 minutes; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 94°C 30 s; and extension at 72°C for 5 minutes. PCR amplification was performed using 2% agarose gel electrophoresis.

PCR-DGGE

DGGE was performed using 8% polyacrylamide gel, a deformation gradient in the range of 35% to 65%, a and a PCR product volume of 20 μL in 60°C 1× TAE electrophoresis buffer, and electrophoresis was performed at 16 hours at 85 V. After electrophoresis, samples were stained with SYBR green I dye for 30 minutes, and a gel imager was used to observe the tape and take pictures.

PCR-DGGE map analysis

The number of bands detected using Quantity One software (Bio-Rad, Hercules, CA, USA) denoted the richness of the DNA sequence (S). Shannon's diversity index was calculated using Biodiversity Data Analysis Package Biodap software (Gordon Thomas, Vancouver, Canada). The index was positively proportional to the number of bands (reflecting the type of bacteria) and density (reflecting the number of bacteria).

The recovery, amplification, and sequencing of the desired bands

After the target strip was selected, excised, and rinsed, the sample was immersed in 30 μL of ddH_2O overnight. The leaching solution was used as the DNA template for PCR amplification. The primers were 357F (without a GC cap) and 518R. PCR amplification products were sent to Shanghai Health and Biotechnology Engineering Co., Ltd. (Shanghai, China), and the sequencing results were blasted (http://blast.ncbi nlm.nih.gov/Blast).

Statistical analysis

SPSS 22.0 (IBM Corp, Armonk, NY, USA) software was used for data analysis. Continuous variables were presented as the mean ± SD and compared using a t-test. Categorical variables were expressed as percentages and compared using the χ^2 test. $P < 0.05$ denoted statistical significance.

Results

Cohort demographics

The NEC group included 28 neonates, whereas the control group included 30 neonates. There were no significant differences in gender, gestational age, birth weight, delivery mode, feeding, and age at the time of sample collection between the two groups ($P > 0.05$). The NEC group included 16, 11, and 1 neonate with Bell stage I, Bell stage II, and Bell stage III, respectively (Table 1).

Intestinal microflora diversity

Fecal samples were collected from the study group and the control group. All samples

Table 1. Demographic and clinical characteristics.

Characteristics	Controls (n = 30)	NEC (n =28)	*t*-value/χ^2	*P*
Gender (males/females)	14/16	15/13	0.294	>0.05
Gestational age (week)	34.7 ± 1.6	34.2 ± 1.3	0.839	>0.05
Birth weight (Kg)	2.4 ± 0.5	2.2 ± 0.4	0.427	>0.05
Cesarean section (number of cases)	15	14	–	–
Feeding (breastfeeding/formula feeding)	17/13	16/12	0.316	>0.05
Age at feces collection (days)	5.9 ± 1.7	5.8 ± 1.4	0.285	>0.05
Bell's stage (I, II, III)	NA	16, 11, 1	–	NA

NEC, necrotizing enterocolitis.

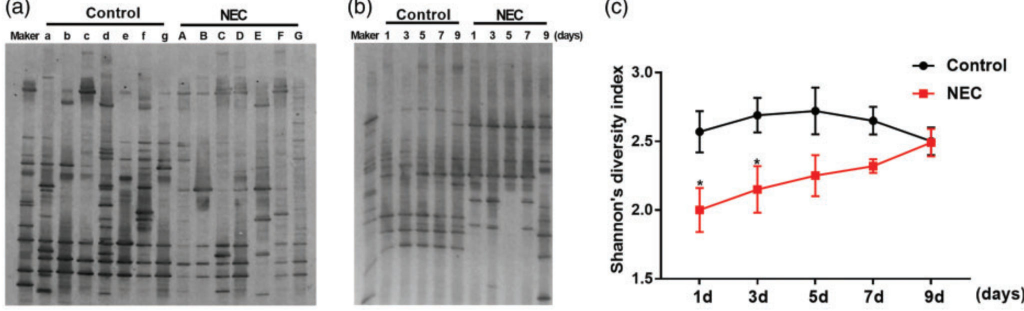

Figure 1. a. PCR-denaturing gradient gel electrophoresis for intestinal flora in the two groups (a–g, controls; A–G, necrotizing enterocolitis [NEC] group). b. PCR-DGGE of fecal bacteria in one patient in each group over time. c. Changes in Shannon's diversity index in the two groups (*$P < 0.05$ vs. control).

were isolated from the denaturing gradient gel. Figure 1a presents the PCR-DGGE results of the two groups. Table 2 reveals that the number of bands was significantly lower in the NEC group than in the control group ($P < 0.05$), indicating that the diversity of the intestinal flora was significantly lower in the NEC group.

Composition of bacterial species

Twenty-one strains belonging to 11 genera were identified in the intestinal flora with 95% to 100% similarity via sequencing on the first day of admission in both groups, indicating no significant differences in the types of bacteria. However, the numbers of samples positive for *Bacteroides* and *Klebsiella* were higher in the NEC group than in the control group, whereas the numbers of samples positive for *Escherichia coli*, *Bifidobacterium*, and *Lactobacillus* were lower.

Relationships among NEC-associated bacteria over time

Samples were collected from all neonates in both groups at all five time points. The richness of bands in the NEC group tended to increase with prolonged hospitalization (Figure 1b). In addition, Shannon's diversity index gradually increased over time in the NEC group, whereas it was relatively stable in the control group (Figure 1c).

Table 2. Species richness and Shannon's diversity index.

Characteristic	Control (n = 30)	NEC (n = 28)	*t*-value	*P*
Species richness (S)	15.47 ± 2.62	7.68 ± 0.73	6.218	<0.05
Shannon's diversity index	2.68 ± 0.31	1.97 ± 0.54	5.726	<0.05

NEC, necrotizing enterocolitis.

Table 3. Comparison of bacteria in the two groups.

	Bacteria	Control (n = 23)	NEC group (n = 23)
Gram-positive bacteria	*Lactococcus lactis*	4	4
	Clostridium	1	2
	Enterococcus	15	14
Gram-negative bacteria	*Klebsiella pneumoniae*	9	10
	Escherichia coli	16	13

NEC, necrotizing enterocolitis.

Intestinal flora and prognosis

After treatment, 46 patients (including the 28 patients who fully met the inclusion criteria) with NEC were cured and discharged after a mean length of hospital stay of 12.3 ± 2.7 days. We collected 23 samples from each group to analyze the relationship between intestinal flora and prognosis. The sequencing results illustrated that the intestinal bacteria species were similar at discharge in the two groups, indicating that the intestinal flora composition had normalized after recovery (Table 3).

Discussion

The health of the neonatal gastrointestinal tract is closely related to the balance among host cells, immune cells, and intestinal flora.[3] It has been reported that the anomalous colonization of intestinal flora and intestinal flora disorders can cause various gastrointestinal diseases.[4] NEC is one of the most common and life-threatening gastrointestinal emergencies in neonates.[5] Interestingly, studies have revealed that the use of intestinal probiotics can significantly reduce the incidence of neonatal NEC and severity of NEC, shorten the duration of the disease, and effectively reduce its mortality.[2,6] Thus, it is widely believed that the gastrointestinal flora and anomalous colonization of pathogenic bacteria are closely related to the incidence of NEC.[7] In recent years, it has been widely agreed that the diversity and dynamics of the intestinal microflora are altered in neonatal NEC, which is consistent with our research. However, different reports have described the specific changes of intestinal bacteria.[8–10] In our study, the number of bands was significantly lower in the NEC group than in the control group, indicating that the diversity of intestinal flora was significantly lower in neonates with NEC. In addition, Shannon's diversity index remained stable of the control group with prolonged hospitalization, whereas it gradually increased in the NEC group. Shannon's diversity index was similar between the two groups on day 9 after admission. The results revealed that reductions in the diversity and counts of intestinal bacteria were important factors in the pathogenesis of NEC.

Neonatal intestinal probiotics mainly include *Bifidobacterium* and *Lactobacillus*,[11]

and they are beneficial to human health. There probiotics can promote the growth of normal intestinal flora, effectively prevent the excessive growth of pathogens, and regulate the diversity of intestinal flora.[12] Decreased numbers of intestinal probiotics will lead to intestinal flora disorders and rapid pathogen growth, which cause gastrointestinal diseases.[13] Notably, DNA sequencing revealed no significant differences in the bacterial species between the two groups. However, the proportions of samples positive for *Bacteroides* and *Klebsiella* were higher in the NEC group than in the control group, whereas the proportions positive for *Escherichia coli*, *Bifidobacterium*, and *Lactobacillus* were lower. This result indicated that intestinal probiotic counts (*Bifidobacterium*, *Lactobacillus*) were reduced after the onset of NEC, whereas those of pathogens (*Klebsiella*) were increased. *Klebsiella pneumoniae* is an opportunistic pathogen and common intestinal bacterium under normal physiological conditions. It is not a causative bacterium in healthy children, but under certain abnormal conditions, it can lead to some diseases.[14] In our study, we found that the proportion of samples positive for *Klebsiella pneumoniae* was increased in the NEC group compared with that in the control group, and thus, we speculated that this bacterium may be involved in the incidence of NEC. However, this requires further exploration for confirmation. In addition, the prevalence of *Bacteroides* was also higher in the NEC group, but its total incidence was lower than that of atypical bacteria. Therefore, the effect of *Bacteroides* on NEC requires further study. Notably, intestinal bacteria were similar between the groups at the time of discharge.

In summary, our study used the PCR-DGGE technique combined with DNA sequencing to analyze the fecal bacteria of neonates with NEC in a Chinese hospital. The changes of the diversity and dynamics of intestinal bacteria in patients with NEC were confirmed, indicating that reduced intestinal bacteria counts may be important factors in the pathogenesis of NEC. Further sequencing results suggested that the decreases in probiotic counts resulted in a relative advantage for *Klebsiella*, which may be involved in the pathogenesis of NEC. However, we did not perform a sample size calculation, and the limited number of samples may affect the statistical significance of the results. We will conduct more in-depth research in the future to provide a basis for the further study of probiotics in neonatal NEC.

Author contributions

MD and NQH wrote the manuscript, MD and ZHH performed the statistical analysis, and MD and NQH supervised all protocols. All authors read the manuscript, provided feedback, and approved the final version.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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