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Characterization of the intestinal microbiome of Hirschsprung's disease with and without enterocolitis

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ABSTRACT

Hirschsprung's disease (HD) is a congenital malformation of the gastrointestinal tract characterized by the absence of the distal enteric nervous system. Hirschsprung-associated enterocolitis (HAEC) is severe life threatening complication of HD. The disease pathogenesis is still unclear, but evidences suggest that the intestinal microbiota may play important role in the development of HD and HAEC. Because microbial abundance and diversity might differ in HD patients with enterocolitis, we sought to generate comparative metagenomic signatures to characterize the structure of the microbiome in HD patients with and without enterocolitis. Our experimental design is to enroll four HD patients (two with enterocolitis and two without enterocolitis). The microbiome was characterized by 16S rRNA gene, and the data obtained will be used to taxonomically classify and compare community structure among different samples. We found that the structure of the microbiome within HAEC patients are differ from those without enterocolitis. This study helps us to understand microbial contributions to the etiology of Hirschsprung-associated enterocolitis.

1. Introduction

The human intestine contains a vast population of microbes which are essential for the control of intestinal epithelial homeostasis, mucosal inflammation, intestinal development and human health [1]. Given the importance of the microbiome in human health, understanding the role of microbial communities in human health is emerging as one of the most important and fascinating biomedical challenges of our times. The microbes are organized into complex communities adapted to inhabit niches of human body [2–4]. Such ecosystems carry a broad range of functions, and the rise of pathogens within such communities cause infection and inflammation, which causes a series of challenges in biomedical researches. Recent microbiome diversity works seem to point to a new perspective in which the etiology is attributed to a shift in the global balance of the microbial flora rather than to the specific appearance of individual pathogens [5–8].

Hirschsprung's disease (HD), usually diagnosed in newborns, is a congenital malformation of the gastrointestinal tract characterized by the absence of the distal enteric nervous system and is a birth defect that affects about 1 out of 5000 individuals [9]. Older infants and children with HD usually present chronic constipation. Hirschsprung-associated enterocolitis (HAEC) is severe life threatening complication of the congenital HD, which can lead to distension, diarrhea, fever and even death. The etiology of HAEC remains controversial, however, the role of microbiome in the development of HAEC has been proposed and HAEC is thought to be in part due to bacterial overgrowth caused by stool retention [10]. It has been documented that a loss of essential homeostatic balance can lead to pathologic infections, such as *Clostridium difficile*, which is associated with HD and HAEC [11]. However, the association of *Clostridium* species as a cause of HAEC remains controversial [12,13]. Recently, Shen et al. [14] quantify *Lactobacilli* and *Bifidobacteria* from 30 HD infants and 15 controls and found a statistically significant decrease in the levels of probiotic bacteria in the HD patients.

Recent advances in DNA sequencing technology enable scientists to generate billions of nucleotide bases at a relatively lower cost. This deep sequencing based method has revealed an unex-

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pectedly high diversity of human gut microbiome. Up to date, we still do not know how many of these microbes contribute to HAEC and how common or exclusive are the HD with and without enterocolitis. In this work, to better understand show microbiomes related to HAEC and HD patients, we have undertaken a comparative sequencing survey of the intestinal microbiomes of the HAEC and HD patients.

2. Materials and methods

2.1. Patients and samples

The subject population consisted of four patients (two patients with HAEC and two with HD). This study was performed on patients treated at Shanghai Children's Medical Center between April 20, 2012 and August 14, 2012. The study was approved by the joint committee of ethics of the Shanghai Children's Medical Center affiliated to school of medicine, Shanghai Jiaotong University (SCMCIRB-K2012022). Written informed consent with a signature was obtained from the parents of each patient. Study cohort is comprised of 4 patients: 2 with HAEC (No. 1 male, age 2 month; No. 2 male, 6 month) and 2 patients with HD (No. 3 female, age 7 month; No. 4 male, 12 month). Specimens of intestinal content were taken during the surgery from different sites along the intestine of patient No. 1 (ileum, appendix, transverse colon, and rectum), No. 2 (appendix, ascending colon, sigmoid colon, and, rectum), No. 3 (proximal and distal colon), No. 4 (appendix, transverse colon, and rectum). Specimens of intestinal content were taken during the surgery from different sites along the intestine (Table 1). Samples were cooled to 4 °C (dry ice) immediately after collection, and then frozen at −80 °C within 30 min. All patients had not been given probiotics and antibiotics at least 5 days before sample collection. All the patients were confirmed by the pathologic diagnosis.

2.2. DNA extractions

DNA from different samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Inc., Valencia, CA, USA) according to manufacturer's instructions. At first, a minimum of 2 ml of Buffer ASL and 300 mg of samples was used in the protocol; and then a ratio of 700 μl of Buffer ASL per 100 mg of sample weight was used for larger volumes using no more than 1500 mg of samples and 10.5 ml of Buffer ASL; next, following the addition of Buffer ASL to each sample, 0.70 mm Garnet Beads were added to the suspension and vortexed for 10 s; at last, a second bead-beating was performed following the heating of the suspension in 0.1 mm Glass Bead Tubes, and vortexed for 10 min.

2.3. 16S rDNA sequencing

Using the total DNA from the 13 samples as a template, we amplified the V1–V3 region of the bacterial 16S rRNA. PCR pools were sequenced using MiSeq Libraries that were constructed with the Illumina Nextera XT kit. It has been sequenced on an Illumina MiSeq using 2×250 bp paired-end sequencing according to the manufacturer's instructions. Sequencing reads with primer sequences were removed. All sequences are available upon request.

2.4. Data analysis

We next clustered the sequences using the CD-hit-est based clustering method [15]. Operational taxonomic units (OTUs) were defined using a 97% sequence similarity cutoff, corresponding to species-level groupings. Next, sequences were grouped into various OTUs using Felsentein-corrected similarity matrices and the sequences within an OUT share at least 97% similarity. The Ribosomal Database Project (RDP) classifier [16] was used to classify the 16S rDNA into distinct taxonomic category by aligning sequences to a curated database of taxonomically annotated sequences. All 16S rDNA sequences were mapped to the RDP database using BLASTN in order to achieve taxonomic assignments. Sequences greater than 97% identity were used to associate a group of OUTs to specific species, while those with less than 97% identity were considered novel reads. Statistical significance of factors potentially contributing to compositional differences between samples was examined using the non-parametric permutation analysis of similarity (ANOSIM), analogous to the univariate ANOVA test.

3. Results

3.1. A deep look at the microbiome in HD and HAEC patients

We compared the intestinal microbiomes of HD children (ages 7 and 12 months) to that of HAEC children (ages 2 and 6 months) by taking intestinal contents from different sites along the intestine during the surgery (Table 1 and Fig. 1). DNA was isolated from the samples of different sites along the intestines of HD and HAEC patients. The deep sequencing technology was used to produce a substantial genomic data for the human intestinal microbiome. Specifically, we generated 16S rDNA data from 13 samples of four

Table 1
Characteristics of the participants in this work.

Subject No.	Patients No.	Gender	Age (months)	Diagnosis	Site	Grouping
1	1	Male	2	HAEC	Transverse colon	Proximal
2	1				Appendix	Proximal
3	1				Ileum	Proximal
4	1				Rectum	Distal
5	2	Male	6	HAEC	Ascending colon	Proximal
6	2				Sigmoid colon	Distal
7	2				Appendix	Proximal
8	2				Rectum	Distal
9	3	Female	7	HD	Distal of the colon	Distal
10	3				Proximal of the colon	Proximal
11	4	Male	12	HD	Transverse colon	Proximal
12	4				Appendix	Proximal
13	4				Rectum	Distal

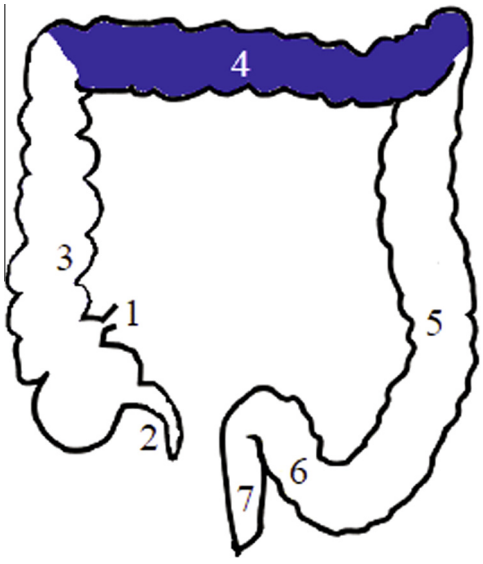

Fig. 1. Different regions of human intestine analyzed in this work. The regions–1. ileuma; 2. appendix; 3. ascending/proximal colon; 4. transverse colon; 5. descending colon; 6. sigmoid colon and 7. rectum.

patients. A total of 2,772,926 16S rDNA sequences were generated using the Illumina Miseq platform, 250 bp, paired-end run, yielding an average of 213,302 sequences per sample after removing low-quality sequences.

3.2. Bacterial diversity in HD and HAEC intestine samples

To determine the bacterial diversity, the entire trimmed 16S rDNA sequences were clustered into Operational Taxonomic Units (OTUs) using the program DNACLUST [17]. The total number of OTUs at 97% sequence similarity ranged from 581 to 3202. Based on the number of OTUs, the results showed that bacterial diversity varies significantly among the intestine samples of HD and HAEC patients included in this work. Overall, the HAEC patients exhibited greater diversity than the HD patients (P value <0.01). Notably, we found that the proximal samples with ganglion cells show bacterial diversity more variable than the distal samples without ganglion cells in HAEC patients, whereas the number of OTUs are significantly greater in the distal samples without ganglion cells in HD patients (Fig. 2). This result suggests that microbiota from HAEC and HD patients are quite different in the proximal and distal samples.

3.3. Microbiota communities are compositionally distinct in HD and HAEC patients

The principal coordinate analysis (PCoA) gives us a measure of bacterial genus community relatedness and the samples with similar bacterial communities are localized in similar positions in the diagram. In this work, Bray–Curtis dissimilarity was calculated based on taxonomic clustering of sequences at 97% similarity from each sample. We observed a substantial separation in bacterial at the genus level among different samples (Fig. 3A). In the Bray–

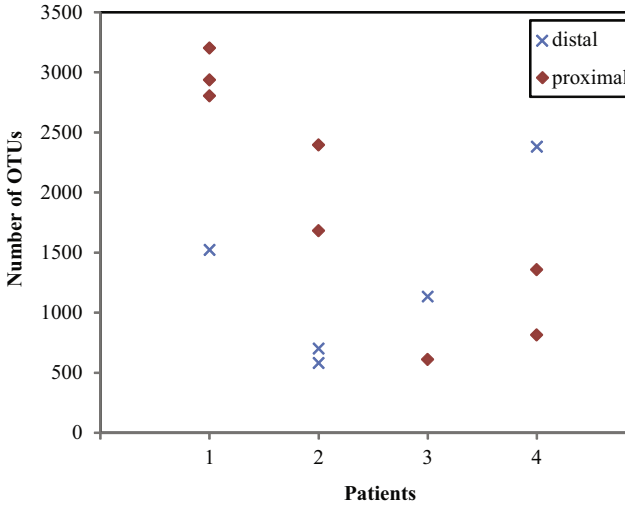

Fig. 2. Number of operational taxonomic units (OTUs) in different samples across different patients. OTUs are defined as 97% 16S rDNA sequences similarity.

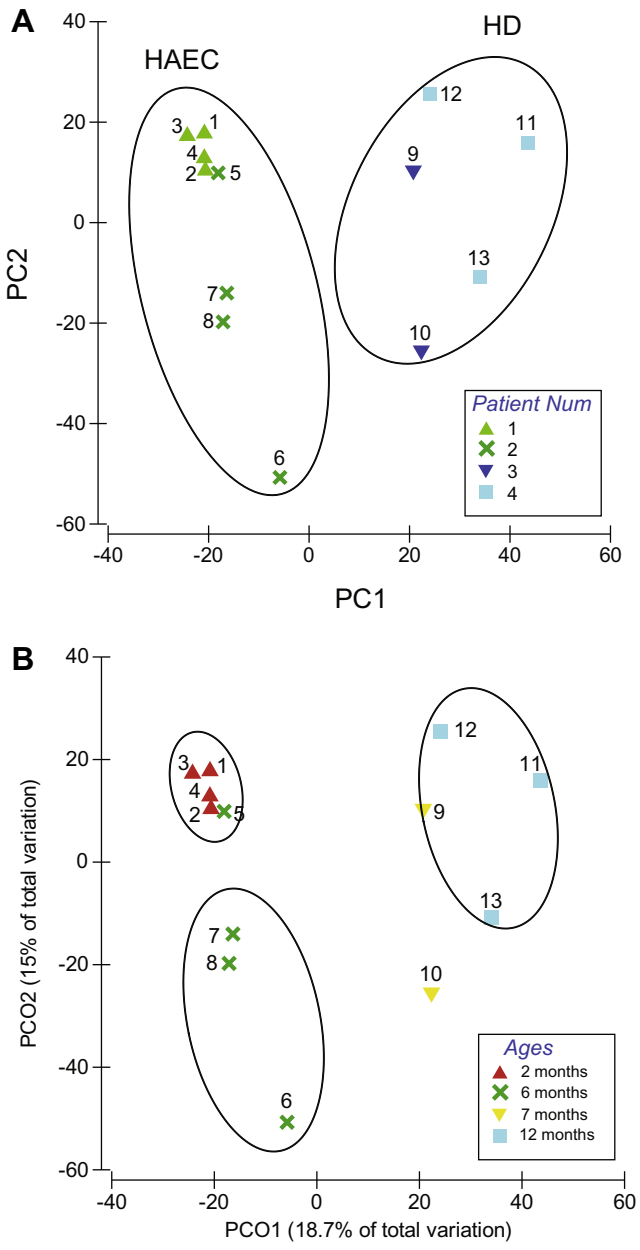

Fig. 3. Principal coordinates analysis of unweighted unifrac distances. Beta diversity patterns were explored using principal coordinates analysis (PCoA).

Curtis dissimilarity based PCoA, the first principal coordinate (PC1) and PC2 accounts for 30% and 14% of the intersample variance, respectively. The result showed a sharp distinction between the intestinal microbiota of HAEC and HD patients. This difference was significant with a non-parametric permutation analysis of similarity (ANOSIM) test, analogous to the univariate ANOVA (analysis of variance) test, where $R = 0.78$ (P value <0.01), revealing statistically significant influence of enterocolitis to microbiota formation. Furthermore, PC1 (30% of variance) and PC2 (14% of variance) separated samples based on the age subjects, and varying levels of clustering of ages occurred in all children (Fig. 3B). Different age subjects were distinct from each other (ANOSIM comparisons generated an $R > 0.6$, P value <0.01). Taken together, the results suggest the possibility that intestinal microbiota of HAEC and HD children is distinct, and that enterocolitis contribute to the microbiota diversity.

3.4. Taxonomic differences

To obtain the taxonomic composition, the Ribosomal Database Project (RDP) Classifier [16] was used to classify bacterial 16S rRNA at 70% Bayesian bootstrap cutoff. These sequencing reads were assigned to 14 phyla. We observed a different pattern of genera distribution for every diverse communities across different subjects (Fig. 4A). Bacteroidetes occupied the largest portion (46%) of the genomic sequences in HD patients, followed by Proteobacteria (21%); In contrast, Proteobacteria occupied the largest portion (55%) in HAEC patients, followed by Firmicutes (18%). In addition to the phylum level analysis, the relative abundance of genera in each samples was examined using RDP classifier at 70% Bayesian bootstrap cutoff. At the genus level, pronounced differences were observed between HAEC and HD patients. Fig. 4B shows that the genus *Enterobacteriaceae* (56%) was the most prevalent genus in

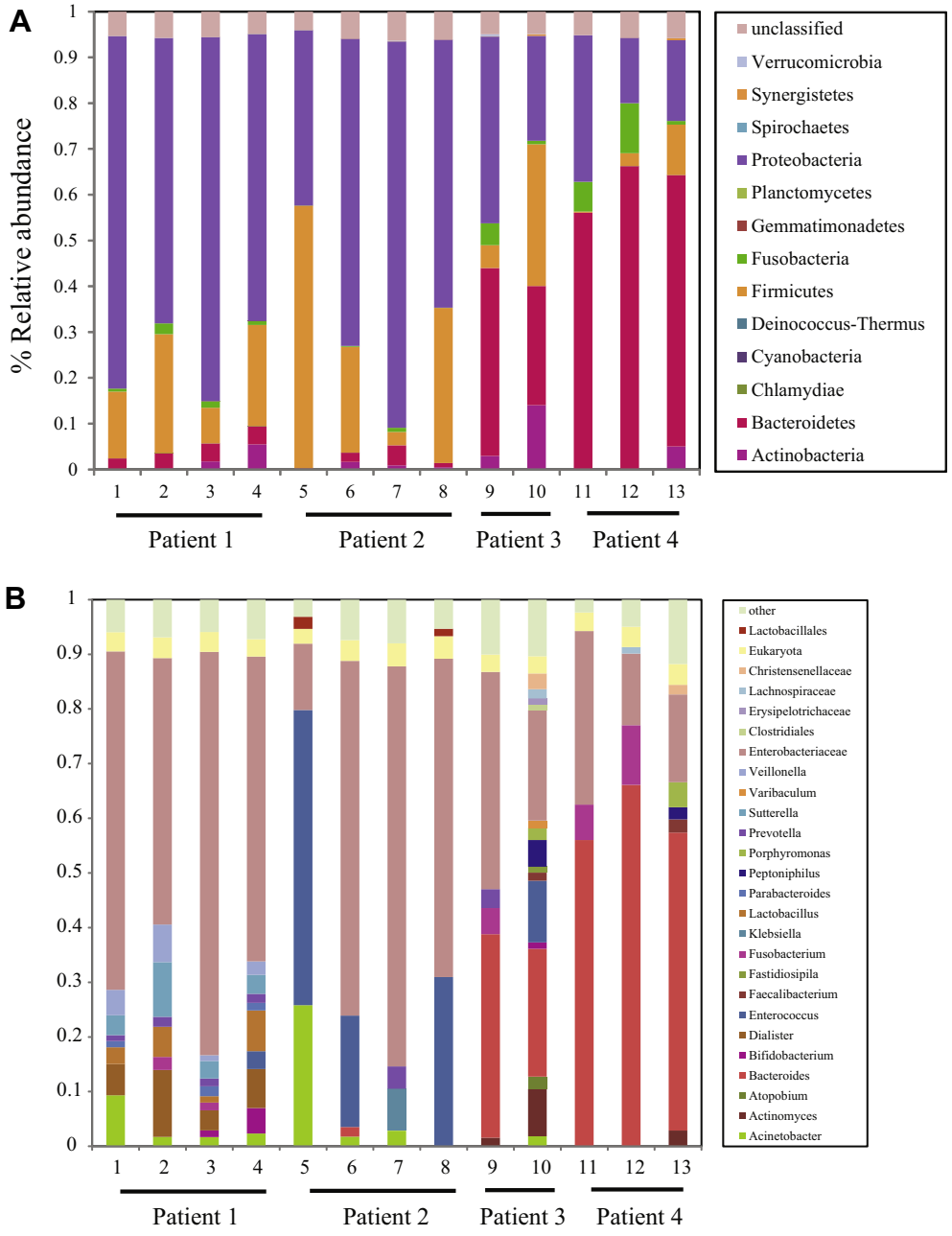

Fig. 4. Relative abundance of (A) phylum and (B) genera members of bacteria from 13 subjects.

HAEC patients, while the *Bacteroides* genus (47%) was the most prevalent in HD patients. After the *Enterobacteriaceae* (56%), the most abundance taxa in HAEC patients included the genera *Enterococcus* (13%), *Acinetobacter* (6%) and the *Eukaryota* (4%). After the *Bacteroides* genus (47%), the most prominent taxa in HD patients include *Enterobacteriaceae* (24%) and *Fusobacterium* (4%). Seven taxa were found only in HAEC patients and 11 taxa were exclusive to HD patients.

4. Discussion

HAEC is the most common cause of morbidity and death in HD patients, which is characterized by an acute inflammatory infiltrate into the crypts and mucosa of the intestinal epithelium in ganglionic segments [18,19]. In recent years, increasing attention has been paid at the role of the intestinal microbiota in various diseases, and HAEC is considered to be in part due to bacteria overgrowth caused by stool retention [10]. Some previous works have identified potential microbial changes associated with Hirschsprung's disease, however, microbiome comparison has not been comprehensively performed [14,20]. In this work, we have undertaken a comparative sequencing survey of the intestinal microbiomes of Hirschsprung's children with (HAEC) and without enterocolitis (HD).

Here, the microbiomes were characterized by 16S rDNA high-throughput sequencing method. The data obtained were used to taxonomically classify and compare community structure between samples. The most striking observation from our current study was the dramatically different microbiomes in HAEC and HD patients. We found that the OTU numbers are quite different between the distal and proximal samples with ganglion cells, however, there's no significant difference microbiomes between the distal and proximal samples with ganglion cells (Fig. S1). It suggests that presence or absence of ganglion cells is not a significant determinant in microbiota assembly. Our preliminary study demonstrated strong difference in microbial community composition between cases of Hirschsprung's disease with and without entercolitis. The results suggest that microbial profiling can be a basis of the diagnostic tool to control for development of enterocolitis in Hirschsprung disease patients. Recently, it has been reported that bacteria and viruses have been associated with the enterocolitis, and HAEC is thought to be in part due to bacterial overgrowth [10,21]. Previous works have reported that *Clostridium* species are associated with HAEC and HD [11]. It was found that the isolation rates for *C. difficile* were significantly higher in HAEC patients than those HD patients without enterocolitis. However, some works did not found such association, so the association of *Clostridium* species as a cause of HAEC is still controversial [12,13]. Here, we identified *Clostridium* genera in the transverse colon, sigmoid colon and rectum segments in HAEC and HD patients. However, we did not find *Clostridium* genera in ileum and appendix segments. It suggested that the association of *Clostridium* species as a cause of HAEC might only confine to specific intestinal regions.

To date, most of the human gut microbiota has been characterized in depth using the fecal samples, and it suggested that the utility of fecal microbiomes as proxies for the colonic microbiome. However, the differences between fecal samples and intestinal content samples contribute substantial variation in microbiome. To our knowledge, this study is the first work to compare the intestinal microbiota of HAEC and HD patients using the specimens of intestinal contents which were taken during the surgery. These samples provide us opportunities to more comprehensively and precisely detect the microbiome diversity between HAEC and HD patients.

In conclusion, we have demonstrated that the microbiomes of HAEC and HD patients show diverse taxonomic composition. Identifying the microbiome differences between HAEC and HD patients provides us a framework for future researches of determining the role of specific bacteria in inducing inflammation in HAEC. This work will provide a basis for early intervention of HAEC risk in Hirschsprung's disease.

Author contributions

Conceived and designed the experiments: L.H. Performed the experiments: Z.L.Y, V.P., L.H., S.G., L.Y.P., J.W. Analyzed the data: V.P., Z.Z., N.B., L.H. Wrote the paper: V.P., L.H.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.104.

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