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Evidence for Differentiation of Colon Tissue Microbiota in Patients with and without Postoperative Hirschsprung's Associated Enterocolitis: A Pilot Study

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ABSTRACT

Purpose: To investigate the differences in the colon microbiota composition of Hirschsprung's disease (HSCR) patients with and without a history of postoperative Hirschsprung's associated enterocolitis (HAEC).
Methods: Colon tissue microbiota was characterized by bacterial deoxyribonucleic acid (DNA) extraction and 16S rDNA sequencing for taxonomic classification and comparison.
Results: The sequence diversity richness within samples was significantly higher in samples from patients with a history of postoperative HAEC. We observed an increased relative abundance of the phyla *Bacteroidetes*, *Firmicutes* and *Cyanobacteria* in HAEC patients and *Fusobacteria*, *Actinobacteria* and *Proteobacteria* in HSCR patients and, an increased relative abundance of the genera *Dolosigranulum*, *Roseouria* and *Streptococcus* in HAEC patients and *Propionibacterium* and *Delftia* in HSCR patients.
Conclusion: Our findings provide evidence that the colon tissue microbiota composition is different in HSCR patients with and without postoperative HAEC.

Keywords: Microbiota; Microbiome; Hirschsprung's; Enterocolitis; Pediatrics; Children

INTRODUCTION

Hirschsprung's disease (HSCR) is a congenital disorder of the enteric nervous system characterized by absence of ganglion cells in the distal hindgut leading to a functional obstruction of the proximal bowel. Surgical correction during early childhood is required. Despite treatment, however, patients with HSCR are at increased risk of development of Hirschsprung's associated enterocolitis (HAEC), a common and life threatening complication with a reported incidence of 29% in the early and 21% in the late postoperative periods [1]. Most importantly, HAEC continues to be the leading cause of death in infants and children with HSCR [2].

Conflict of Interest
The corresponding author and co-authors have nothing to disclose. And the authors have no financial conflicts of interest.

HAEC is characterized by inflammation of the intestinal crypts, crypt dilation with mucin retention, abscess formation, mucosal ulceration and transmural necrosis of the bowel proximal to the involved segment [3]. Although the exact etiology of HAEC is largely unknown, bacterial translocation across the intestinal wall in combination with specific variations of the intestinal microbiota have been hypothesized as a contributing mechanism in the development of HAEC. Human and animal studies have reported microbiota variations by gene sequence analysis of fecal samples [4-7], but whether these changes are present in colon tissue of patients with HSCR is unknown. Therefore, this pilot study aimed to investigate the microbiota composition of biobanked colon tissue from HSCR patients with and without a history of postoperative HAEC.

MATERIALS AND METHODS

Study population

Approval was obtained from the Medical University of South Carolina Institutional Review Board (Study No. Pro00081103). A retrospective chart review was performed to identify patients with HSCR who were diagnosed, treated, and whose tissue specimens are biobanked at our institution. The patients were divided into a HSCR and HAEC group depending if they did or did not have a documented history of postoperative enterocolitis according to the scoring system developed by Pastor et al. [8]. Archived formalin-fixed paraffin-embedded (FFPE) colon tissue that was obtained at the time of surgical correction was pulled from storage and samples from the aganglionic segment were obtained. Each sample comprised of 4, 10 μm scrolls and stored at 4°C until analysis.

Colon tissue 16S rDNA analysis

Prior to deoxyribonucleic acid (DNA) extraction, paraffin was removed using deparaffinization solution from Qiagen (Hilden, Germany) followed by enzyme digestion and incubations at 56°C and 90°C. Genomic DNA was extracted from lysates using QIAamp (Qiagen) DNA FFPE Tissue Kit on the QIAcube (Qiagen) robotic workstation following manufacturer's instructions. Quality and purity of extracts were determined using the QIAxpert system (Qiagen). Bacteria 16S rDNA was sequenced utilizing the 16S protocol from Illumina (San Diego, CA, USA). Briefly, variable regions V3 and V4 of bacterial 16S rDNA were amplified by polymerase chain reaction (PCR). Following cleanup, the addition of Illumina sequencing adapters were added during a second PCR amplification step. Cleaned up sequencing products were clustered at 8pM followed by paired end sequence on an Illumina MiSeq for 2×300 cycles. Fastq files were processed using in house dada2-based bioinformatics pipeline in R statistical programming language [9]. SILVA release 128 database was used for taxonomic assignment of the resulting amplicon sequence variants [10].

Statistical analysis

Descriptive statistics are reported as mean values with standard deviations for continuous variables and frequencies for categorical variables. Clinical data was compared between the HSCR and HAEC groups using student *t*-test for continuous variables and Chi-squared or Fisher's exact test for categorical variables. The *p*-values less than 0.05 were considered statistically significant. Alpha diversity measurement was compared using two-sample Welch *t*-tests. Beta diversity was compared using robust distance-based multivariate analysis $W_d^\star$ test. Univariate analysis at the taxon level was performed with Mann-Whitney rank sum test on the relative abundances and Welch *t*-test on centered log-ration transformed abundances

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with false discovery rate (FDR) adjustment for multiple comparison. Comparisons with FDR values of less than 0.05 were considered significant.

RESULTS

A total of 8 colon tissue samples were analyzed. Seven were from male patients. A total of 4 patients had a history of postoperative HAEC with a mean of 3.00±0.81 documented episodes until last follow-up visit. The mean time at first HAEC episode was 150.0±153.2 days after definite corrective surgery. None of the HSCR subjects had a documented postoperative HAEC episode. All patients received antibiotics after birth and perioperatively. The histopathology report of all of the subjects in the HSCR and HAEC groups were reviewed and the proximal bowel segment at the time of definitive corrective surgery demonstrated presence of submucosal normal ganglion cells without hypertrophic nerve trunks. Other demographic and clinical data is shown in **Table 1**.

Table 1. Demographic and clinical data

Variable	HSCR	HAEC	*p*-value
Sex			0.28
Male	3	4	
Female	1	0	
Race			0.47
Black	1	2	
White	3	2	
Birth history			
Gestational age (wk)	37.75±1.50	38.25±0.96	0.13
Birth weight (kg)	3.05±0.79	3.27±0.51	0.48
Delivery mode			0.43
Vaginal	2	0	
Cesarean	2	4	
Antibiotics after birth	2	2	0.76
Clinical history			
Age at presentation (d)	9.25±11.79	1.75±0.50	0.055
Age at diagnosis (d)	13.0±10.30	4.75±1.89	0.74
Genetic syndrome*	1	0	0.50
HSCR type			0.29
Short segment	3	4	
Long segment	1	0	
Surgical history			
Initial ostomy	3	1	0.24
Time lapse diagnosis-ostomy (d)	2.67±1.53	13	0.02
Time with ostomy (d)	183.00±15.72	85	0.03
Time lapse diagnosis-definitive surgery (d)	139.50±91.98	31.25±44.96	0.08
Weight at definitive surgery (kg)	6.82±1.98	4.13±1.04	0.053
Surgery type			0.22
Soave	0	1	
Swenson	4	3	
Follow-up time after Definitive Surgery (mo)	23.72±3.65	37.55±22.30	0.13
Time lapse from definitive surgery to sample analysis (mo)	15.81±6.14	26.95±20.90	0.37
Postoperative complication			0.21
Ileus	0	1	
Stricture	0	1	

Values are presented as number only or mean±standard deviation.
HSCR: Hirschsprung's disease, HAEC: Hirschsprung's associated enterocolitis.
*Mowat-Wilson Syndrome.

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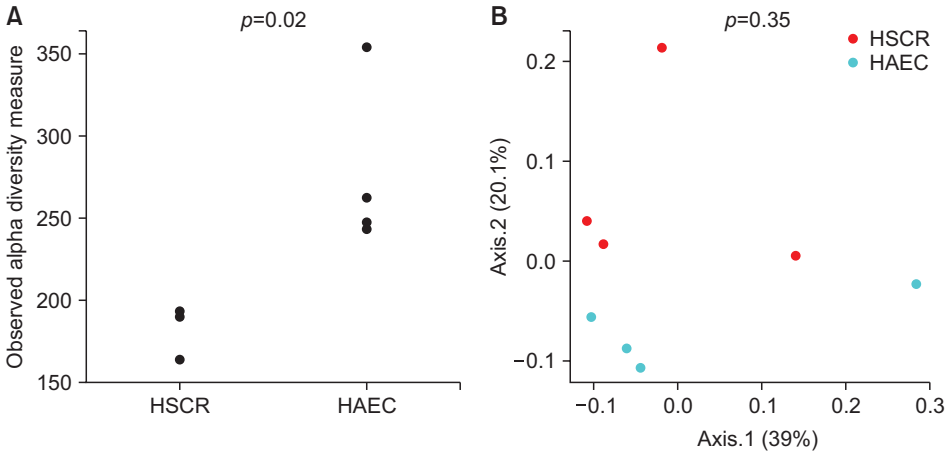

Fig. 1. (A) Alpha diversity using observed richness metric is higher in HAEC group relative to HSCR. (B) Beta diversity principal coordinate analysis of the Jensen-Shannon divergence distances plot shows visual separation of the HSCR and HAEC samples on Axis.2.
HAEC: Hirschsprung's associated enterocolitis, HSCR: Hirschsprung's disease.

The sequence diversity richness within samples (observed alpha diversity) was significantly higher (Welch *t*-test, p=0.02) in the HAEC group compared to the HSCR group (**Fig. 1A**). Although clustering of microbial communities by HAEC status is visible in the principal coordinates (beta diversity based on Jensen-Shannon divergence distances) analysis plot, the effect is not statistically significant (W_d^*, p=0.35) (**Fig. 1B**).

Taxonomic analysis revealed trends in the colon tissue microbiota composition between the groups but did not produce any significant results after multiple comparison correction using FDR method. We observed a predominance of the phyla *Actinobacteria*, *Firmicutes*, *Proteobacteria* and *Bacteroidetes* in both groups. Univariate comparisons analysis demonstrated an increased relative abundance of *Bacteroidetes*, *Firmicutes* and *Cyanobacteria* in the HAEC group and of *Fusobacteria*, *Actinobacteria* and *Proteobacteria* in the HSCR group (**Fig. 2A**). The genera *Propionibacterium*, *Escherichia* and *Enterococcus* predominated on both groups. By univariate comparisons, we observed an increased relative abundance of *Dolosigranulum*, *Roseouria* and *Streptococcus* in the HAEC group and an increased relative abundance of *Propionibacterium* and *Delftia* in the HSCR group (**Fig. 2B**).

DISCUSSION

To our knowledge, this is the first study to investigate the colon tissue microbiota composition in patients with HSCR with and without a history of postoperative HAEC. Previous studies have reported significant differences in the microbiota composition in HSCR patients with and without HAEC. However, these studies performed gene sequencing analysis of fecal samples or colonic aspirates which has potential disadvantages: 1) it provides only a proxy for the gut microbiota; 2) it might contain dead bacteria and bacteria from unspecified gastrointestinal tract compartments; and, 3) could be affected by bowel cleansing that is routinely performed prior to surgery or colonoscopy [11]. Intestinal dysbiosis has been proposed as a contributing factor in the pathogenesis of HAEC and our approach investigated the microbiota composition directly from colon tissue and found taxonomic differences between the two groups that could explain the histopathologic

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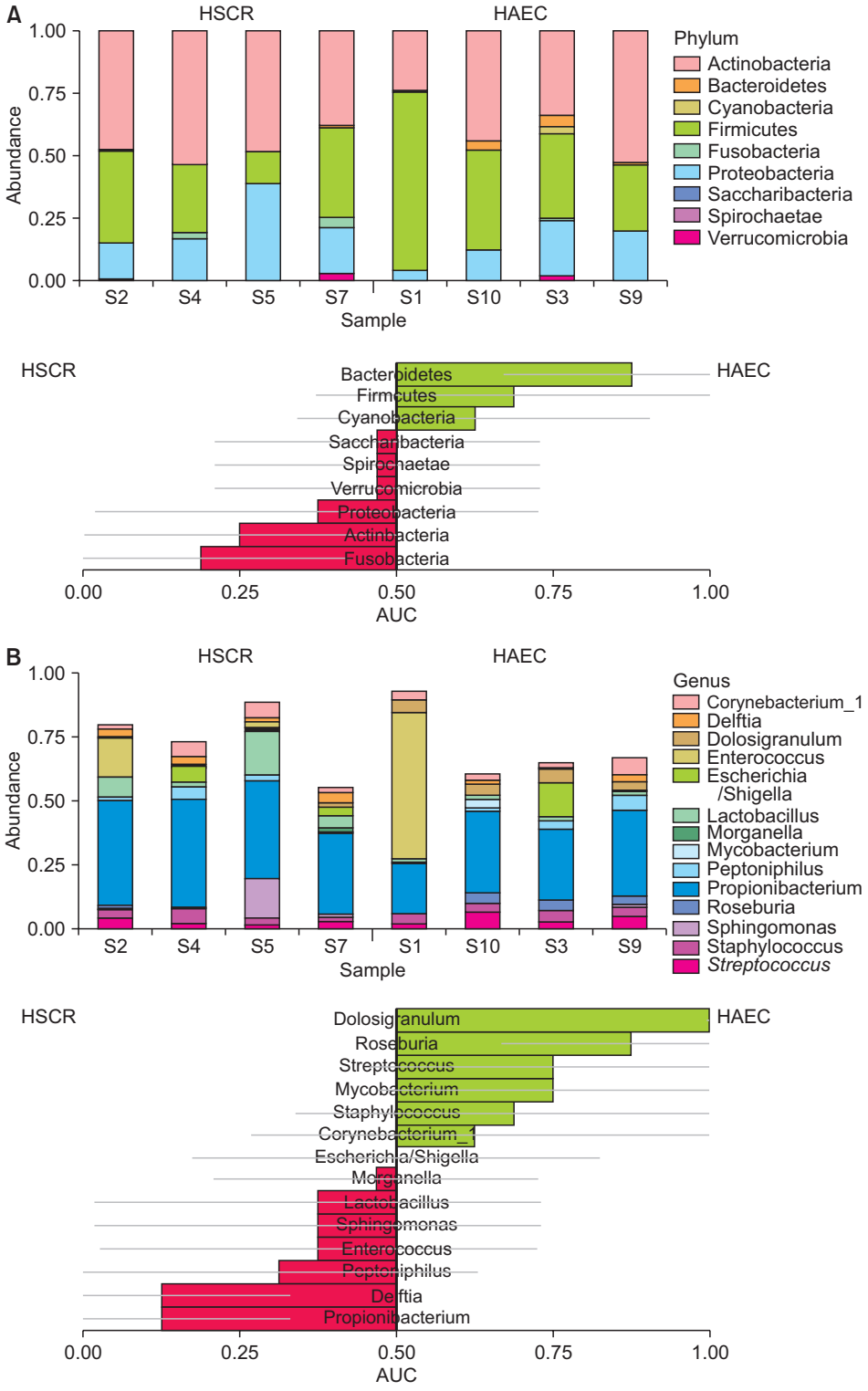

Fig. 2. Colon tissue bacterial (A) phyla and (B) genera relative abundance analysis in HSCR and HAEC patients. Left column shows bar plots of the composition of major phyla and genera, right columns show the AUC for each taxon in distinguishing HSCR vs. HAEC patients.
HSCR: Hirschsprung's disease, HAEC: Hirschsprung's associated enterocolitis, AUC: area under receiver operating characteristic curve.

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inflammatory findings encountered in HAEC. Although it was not feasible for us to compare the microbiota composition in colon tissue and stool from the same subject, or if tissue formalin and paraffin fixation might affect results, other studies have demonstrated similar bacterial profiles at the phylum level by analyzing FFPE intestinal tissue or fecal samples of preterm infants with necrotizing enterocolitis [12,13].

We found taxonomic differences between the HSCR and HAEC groups. In the HAEC group the phyla *Bacteroidetes* and *Firmicutes* predominated compared to *Actinobacteria* and *Proteobacteria* in the HSCR group. Previous studies have demonstrated that the intestinal microbiota alpha diversity increases throughout time in infancy with the introduction of different foods resulting in the replacement of *Proteobacteria* and *Actinobacteria* by *Firmicutes* and *Bacteroidetes* [14,15]. All of our subjects underwent surgical treatment during infancy and the mean age at the time of definitive surgery was not statistically different between the two groups indicating that their colon tissue microbiota is in fact different. We found that within patients that had a two stage surgery, the mean time from diagnosis to ostomy creation was significantly higher in the HAEC group and, the mean time having an ostomy was higher in the HSCR group. Generally, an ostomy generates a diversion that relieves intestinal distension and fecal matter stasis from the functional obstruction encountered in HSCR patients. The presence of an ostomy may provide a protective effect and potentially explain the taxonomic differences in our analysis that could predispose patients for postoperative HAEC.

In contrast to the fecal microbiota composition described elsewhere our findings demonstrate that the colon tissue microbiota differs between HSCR patient with and without a history of postoperative HAEC. A study by Frykman et al. [4] found a lower proportion of *Firmicutes* and *Verrucomicrobia* and an increased proportion of *Bacteroidetes* and *Proteobacteria* in fecal samples of the group of subjects with HAEC when compared to the HSCR group. In our study we found that the proportion of *Firmicutes* was increased in the HAEC group followed by *Actinobacteria* and *Proteobacteria*. A study by Yan et al. [6] reported that *Enterobacteriaceae* and *Enterococcus* were the most prevalent genus in fecal samples of HAEC patients while we found an increased proportion of the genus *Propionibacterium* followed by *Enterococcus*. These differences, however, are not unusual as it has been previously demonstrated that the fecal and rectal mucosal microbiota diversity is not similar, even on the same individual [16]. Another possible explanation for the observed differences in bacterial taxa in our study compared to others is that we specifically analyzed tissue samples from the surgically resected aganglionic bowel and we identified particular bacterial profiles as possible predictors for postoperative HAEC. In contrast, other studies analyzed fecal samples from the remaining ganglionic bowel of variable lengths at different time points after definitive corrective surgery that could lead to intestinal dysmotility and fecal matter stasis, all variables that have been shown to alter the diversity of the intestinal microbiota [17]. In addition, the discrepancies between the microbiota profiles from our study and even across other studies could be explained by the heterogenicity of the patient population, ethnicity, geographic location, disease state, antibiotic exposure and age at the time of definitive corrective surgery.

A limitation to our pilot study is the small number of samples in each group limiting the power to detect statistically significant differences. Also, patients did not have a history of enterocolitis at the time of surgery and it is unknown if the tissue microbiota differs on the same patient at the time of HAEC. Antibiotics were given to all patients prior to surgery which may confound the interpretation of our results. Another limitation is that analysis was performed only on aganglionic colon samples. However, a previous study suggested that the

presence or absence of ganglion cells is not a significant determinant in microbiota assembly [6]. Nevertheless, extrapolation of our findings to other segments of the gastrointestinal tract should be made with caution as the microbiota composition may vary along the gut. Finally, tissue aerobic exposure during the formalin and paraffin-embedding process performed after surgical correction may cause alteration in the microbiota composition. Nevertheless, despite that this process may introduce biases relative to fresh tissue, we observe interesting signals differentiating HAEC vs HSCR associated microbiota. Since we have no reasons to suspect of systematic biases in preparation of the tissues of either type, we are reassured that upon replication in larger cohorts and possibly with fresh tissues with more control over variability, these signals will replicate.

In conclusion, our data provide pilot support to the hypothesis that the microbiota composition in colon tissue differs between HSCR patients who develop postoperative HAEC and those free of this complication. We hypothesize that these differences are potential contributors in the pathogenesis of HAEC and that there may be identifiable bacterial community variations in HSCR that puts patients at risk for developing HAEC. Our approach is feasible and provides a basis for future prospective studies to determine a causative role of specific microbiota communities in patients with HSCR and as practical translatable predictors of HAEC.

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