

## Hirschsprung's Disease and the Intestinal Microbiome

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### Abstract

Hirschsprung-associated enterocolitis (HAEC) is the most common and serious complications of Hirschsprung's disease (HD), having mortality rate of 1-10%. Despite many proposed etiologies, the pathological mechanisms underlying HAEC are still unclear. The specific bacterial or viral infections are among the key factors to consider. The study of HAEC related intestinal flora was limited by the fact that 85% of intestinal microbiota cannot be cultured. The developments of modern next generation sequencing platforms and metagenomics methodology allowed to study and characterize the intestinal microbiota in infants with Hirschsprung's disease with a great precision. This article reviews the progress in study of Hirschsprung's disease and intestinal microbiota in patients with and without enterocolitis.

**Keywords** Hirschsprung-associated enterocolitis; Hirschsprung's disease; Metagenomics; Microbiome

### Hirschsprung's Disease Introduction

Hirschsprung's disease (HD), also known as congenital megacolon or Aganglionosis, was first described and named by Harald Hirschsprung in 1886. Hirschsprung's disease is a developmental disorder of the enteric nervous system, which affects approximately 1 in 5000 liveborn infants. This disease is characterized by the absence of ganglion cells in the myenteric and submucosal plexuses of the distal intestine, which results in lack of peristalsis and functional intestinal obstruction. In most cases, the aganglionosis involves the rectum or rectosigmoid, but it can extend for varying lengths, and 5-10% of cases can involve the entire colon or even a significant amount of the small intestine. The primary treatment of Hirschsprung's disease is surgery that involves resection of the aganglionic bowel and reconstruction [1]. Hirschsprung-associated enterocolitis (HAEC) is manifested by intestinal inflammation that is clinically characterized by fever, abdominal distention, diarrhea, and sepsis. HAEC mortality still reaches 1-10% [2,3]. Despite many proposed etiologies, the biological mechanisms underlying HAEC are poorly understood [4]. HAEC has a significant morbidity and potential mortality in the patient population and remains the most common cause of death in children with HD. HAEC has a reported incidence of 17% or even more than 40% during the postoperative course after pullthrough procedure. The rate may reach as high as 55% in patients with total colonic aganglionosis [5-8].

Currently, the pathogenesis of HAEC is still unclear. Recent studies have shown that injury to the intestinal mucosal barrier, abnormal immune responses in the intestinal tract and infections due to specific pathogens may play critical roles in the pathogenesis of HAEC [9]. Enteric pathogens can invade the bloodstream via damaged intestinal mucosal barrier and then induce a waterfall-like inflammatory response [10]. In addition, histological and immunological studies indicate that persistent inflammation and reduced immune function

are present in the intestinal tract of infants with recurrent HAEC [11]. In concordance with the pathogenesis of inflammatory bowel diseases, intestinal flora may also play a key role in the pathogenesis and development of HAEC [12,13].

### Search for Microbial Culprit

Until now, the specific pathogens involved in the development of HAEC have not been found. Several studies have shown that not only the increase of *Clostridium difficile* and rotavirus, but also a decrease in *Bifidobacteria* and *Lactobacillus* may play a role in the pathogenesis of HAEC. In 1982, Thomas et al., first reported increase in the level of *C. difficile* toxin in 4 cases among 6 HAEC patients [14]. In 1986, Thomas et al. found that 54% of infants with HAEC were positive for cytopathic toxin in stool. *C. difficile* was isolated from 18% and 30% of patients from HD-only and non-HD groups, respectively. In contrast, *C. difficile* was isolated from 77% of infants in the HAEC group. Thomas et al. also noticed that vancomycin treatment was beneficial for 63% of HAEC patients. In agreement to this observation Parsons et al. also reported more *C. difficile* and higher level of toxin in the intestinal contents of HAEC newborns [15]. However, Rietra and Slaterus [16] highlighted that *C. difficile* and the secreted toxin were also detected in the specimens of 90% of healthy newborns. These results suggested that pathogenic *C. difficile* might not be a single crucial determinant of HAEC. In 1990, Wilson-Storey et al. identified rotavirus in specimens from 77% of HAEC infants, suggesting a crucial role of rotavirus in the pathogenesis of HAEC [17]. They also proposed that HAEC is determined by multiple-factors including infections and IgA secretion. Since human intestinal secretory immunoglobulin (IgA) is an important component of the immune defense against infection, a disorder of IgA secretion could lead to a secondary rotaviral infection.

However, due to the complexity of intestinal microbiota, the pathogenesis related studies have encountered numerous challenges. Generally, regular microscopic examination fails to determine the state of intestinal flora and quantify precisely the species and number of

bacteria. Furthermore, the methodology is easily influenced by subjective factors. Although both the microbiota diversity and relative abundance can be determined using the colony count technique, it is currently widely accepted that specific selection of gut bacteria could not be accomplished in the culture medium. Moreover, we are not able to prepare fresh samples and provide a strictly anaerobic environment during sample handling, given that, no more than 15% of the intestinal bacterial species can be cultured [18]. Therefore, these two methods are still not sufficient for a comprehensive intestinal microbiome analysis.

In 2009, a real-time-PCR-based study reported a lower level of *Bifidobacteria* and *Lactobacillus* in the feces of HAEC infants in comparison to HD patients with no enterocolitis. In addition, the amount of these bacteria in HD patients without enterocolitis was similar to that in age-matched healthy children. These findings suggested that the low levels of *Bifidobacteria* and *Lactobacillus* might be directly correlated with the occurrence of HAEC [19].

In 2010, a new technique, amplified ribosomal DNA restriction analysis (ARDRA), was implemented in HAEC microbiota research. De Filippotua and colleagues assessed 15 stool specimens of a 3-year-old patient with HAEC, which were harvested during HAEC episodes and remission phases. They found that the incidence of HAEC was correlated with the specific distribution pattern of intestinal bacteria and was influenced by antibiotics administration [20]. The authors also suggested that omics technology, as an efficient tool, could be used for analysis of recurrent HAEC episodes.

### Promises of Modern Sequencing Techniques

The recent development of high-throughput sequencing techniques provides a new approach for the study of intestinal microflora [21,22]. High-throughput sequencing, also known as "next-generation" sequencing technology, is conceptually "sequencing by synthesis" technique with the ability to simultaneously determine sequences of a large amount of DNA with overall read length of tens to hundreds base pairs. For example, one run at Illumina MiSeq machine produces almost 25 million sequences with a read length of 2×300 bp. In the conventional method, the bacterial isolate is cultured, followed by DNA or cDNA insertion into a cloning vector and sequencing. However, for high-throughput sequencing, total bacterial DNA (metagenomics approach) or RNA (metatranscriptomics approach) can be directly extracted from an environmental sample and sequenced avoiding culture and cloning steps. These sequencing results not only contain information about uncultivable microorganisms but also reflect a real time unbiased community structure and provide an insight into community functional capabilities [23-27]. Therefore, via high-throughput sequencing, researchers gained a direct and minimally biased access to physiologically active microbial communities and generated an emporium of information about genetic diversity and molecular ecology of intestinal flora. Currently, high-throughput sequencing, in association with metagenomic methodology, is widely adopted in gastro-intestinal studies. The metagenomic methodology is especially productive in studies of infants and newborns due to relative simplicity of neonatal microbiota [28-31]. Originally, less than 20 bacterial genera have been reported residing in the gut of the newborn infant in the first few weeks after birth [32-34]. The modern data concur with the magnitude of that estimation [28-31]. For example, only 16 complete or near complete bacterial genomes were assembled from 9 stool samples collected from a preterm infant during the third

week of life [29]. The simplistic nature of neonatal microbiota is greatly favorable for metagenomic analysis. Consequently, a comprehensive and precise insight into HAEC etiology appears highly achievable via studies driven by next generation sequencing.

### From Single Microorganism to Microbial Communities in HAEC

In 2012, a murine HD and HAEC model was constructed and successfully maintained in the University of Wyoming (USA). This model was based on endothelin receptor type B (*Ednrb*<sup>-/-</sup>) knockout line. For the first time, next generation sequencing (454 pyrosequencing) was used to assess intestinal flora in HAEC model using colonic samples of *Ednrb*<sup>-/-</sup> mice after 7, 20, and 24 days of life. An increase of *Bacteroidetes* and a decrease of *Firmicutes* phyla were observed in the intestinal tract of *Ednrb*<sup>-/-</sup> mice as compared to wild type animals. Moreover, this variation of microbial composition was age-dependent, indicating a correlation between the particular intestinal microflora in the early postnatal period and the pathogenesis of HAEC [35].

Our group used Illumina-MiSeq high-throughput sequencing to study intestinal microflora in clinical samples of HAEC patients [36]. The study cohort comprised of 4 patients: 2 with HAEC (2 males, aged 2 and 6 months) and 2 patients with HD (1 female, aged 7 months and 1 male, aged 12 months). A total of 13 samples were collected for further study [36]. The 16s RNA gene sequencing results demonstrated that *Bacteroidetes* accounted for the highest proportion (46%) among the intestinal flora in HD infants, followed by *Proteobacteria* (21%). In contrast, *Proteobacteria* occupied the largest portion, 55% in HAEC patients, followed by *Firmicutes* (18%). A marked difference was observed between HAEC and HD patients at the genus level. *Enterobacter* was the most prevalent genus in HAEC patients (56%), followed by the genera *Enterococcus* (13%), *Acinetobacter* (6%) and *Eukaryota* (4%). *Bacteroides* had the highest proportion in the intestinal tract of the HD infants (47%), followed by *Enterobacteriaceae* (24%) and *Fusobacterium* (4%). Seven genera were found to be unique to HAEC patients and 11 were exclusive to HD patients. These results suggested that there was a significant difference in the intestinal microbiota between HAEC and HD patients. Therefore, the colonization by specific type of intestinal microbiota might be responsible for the development of HAEC. In addition, no significant difference was found in the intestinal flora of the distal aganglionic intestine and the proximal ganglionic intestine, indicating that the presence or absence of ganglion cells was not the main determinant in intestinal microbiota composition. The intestinal microbial community differed in different patients, which was in accordance with the transitory dynamics of the microbiota in the digestive tract from newborns to two-year olds and to adults. However, due to the limited number of samples only a cross sectional study design was utilized, a longitudinal study must be performed in future with larger number of samples.

### Conclusion

HAEC is the most common complication in HD patients, and is characterized by increased mortality and decreased quality of life. Recently, bacteria and viruses have been shown to be involved in the pathogenesis of HAEC. The infection and colonization by specific intestinal bacteria may be harmful to the intestinal barrier, microenvironment and the immune responses, leading to recurrent

HAEC. Metagenomics, metatranscriptomics and 16s-RNA taxonomic profiling provide comprehensive and precise data describing properties of intestinal microbiome. Therefore, this technology could be exploited in clinical studies of HAEC. The specific intestinal microbiomes and the time course of intestinal microbiota development should be identified at different ages in HAEC patients. In turn, the delineation of the role of intestinal microbiome in the pathogenesis of HAEC may provide a new therapeutic approach in HAEC treatment.

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