A Retrospective Case–Control Study of the Relationship between the Gut Microbiota, Enteropathy, and Child Growth

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Abstract. The microbial communities residing in the child gut are thought to play an important role in child growth, although the relationship is not well understood. We examined a cohort of young children from Mirzapur, Bangladesh, prospectively over 18 months. Four fecal markers of environmental enteropathy (EE) (high levels of alpha-1-antitrypsin, calprotectin, myeloperoxidase, and neopterin) were examined and anthropometric measures obtained from a cohort of 68 children. The 16S rRNA gene of bacterial DNA was sequenced from stool samples and used to estimate amplicon sequence variants (ASVs). We age-matched children with poor growth to children with normal growth within 1 month and compared the change in abundance and diversity of ASVs over time. Elevated EE markers and poor linear growth in children were associated with changes in microbial communities in the gut. There were increased amounts of *Escherichia/Shigella* and Proteobacteria and decreased amounts of *Prevotella* associated with poorly growing children consistent with the mounting evidence supporting the relationship between intestinal inflammation, child growth, and changes in gut microbiota composition. Future research is needed to investigate this association among young children in low- and middle-income countries.

INTRODUCTION

Worldwide, 159 million young children are estimated to be stunted.¹ Stunting has been associated with poor cognitive outcomes and a higher risk for mortality.^{2,3} There is a growing literature demonstrating an association between environmental enteropathy (EE) and stunting among susceptible pediatric populations.^{4–8} Environmental enteropathy is defined by abnormal intestinal morphology, reduced intestinal barrier function, and increased inflammation.^{9–14} This disorder occurs from unhygienic environments leading to repeated pathogen exposure, enteric infections, inflammation, and reduced nutrient absorption.^{4,5,15} Fecal markers of EE, including higher levels of alpha-1-antitrypsin, calprotectin, myeloperoxidase, and neopterin, are easier to assess than the 5-hour period of urine collection often required for dual sugar permeability tests.¹³ These four markers have been associated with impaired growth in children.^{5,9,16}

Previous studies have found that the gut microbiota is an important determinant of diarrheal disease and impaired growth among pediatric populations.^{17,18} Pop et al.¹⁹ demonstrated major differences in the gut microbiota composition between those with diarrheal and non-diarrheal stool and identified *Escherichia/Shigella* and *Streptococcus* to be associated with diarrhea, whereas *Prevotella* was negatively associated with diarrhea. Growth faltering has been associated with reduced number and evenness of bacterial taxonomic units (i.e., alpha diversity).^{20,21} In Bangladesh, malnourished children were found to have a lower alpha diversity and a decreased number of taxonomic units in their gut compared to healthy children and had higher Proteobacteria and *Bacteroides*.²² Another study found that microbiota immaturity (marked by increased *Escherichia/Shigella* and *Streptococcus*), measured using a microbiota-for-age Z-

score, was associated with severe acute malnutrition.²³ However, the relationship between EE, the gut microbiota, and impaired growth¹⁸ must be better understood to develop therapeutic interventions to reduce EE and impaired growth among susceptible pediatric populations. Research in this area has been complicated by measurement issues around how children who are growing poorly can be identified because growth cannot necessarily be measured at a single point in time or with any single specific biomarker.²⁴ Measuring growth is further complicated by different underlying conditions that are thought to drive poor growth, which may include, but are not limited to, gut microbiome composition, nutritional intake, genetic differences between individuals, and chronic infection.²⁵ We aimed to describe the gut microbiota in young children with abnormally poor growth or EE identified by seven common criteria within a cohort of children in rural Bangladesh, and to compare their gut microbiota with similar children with normal or robust growth.

MATERIALS AND METHODS

A cohort of 68 children aged 6–31 months were followed up for a period of 18 months in Mirzapur, Bangladesh, at the site of the Global Enteric Multicenter Study demographic and surveillance system from 2014 to 2015.²⁶ Mirzapur is a primarily agricultural area roughly 60 km northwest of the capital city of Dhaka. Anthropometry of enrolled children was recorded at baseline and 18 months later by trained research associates, where each child's weight was measured once and their height (or length) was taken three times and averaged at each time point.

Stool samples were collected from children on enrollment and at 18 months after enrollment. After collection, the samples were placed in a cooler and transported to the International Center for Diarrheal Disease Research, Bangladesh (icddr,b) in Dhaka, Bangladesh, where they were stored at –80°C until analysis. Stool samples from baseline were

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analyzed for four fecal markers of EE: calprotectin, alpha-1-antitrypsin, myeloperoxidase, and neopterin using ELISA kits from ALPCO (Salem, NH), BioVendor (Asheville, NC), ALPCO, and GenWay (San Diego, CA), respectively. All assays were run according to the manufacturer's specifications. All dilutions were also performed according to the package inserts except for a 1:500 dilution used for initial runs for myeloperoxidase.

In addition to EE fecal marker analysis, DNA was extracted from each stool sample with a QIAGEN kit (Hilden, Germany) according to the manufacturer's instructions at baseline and 18-month follow-up.²⁷ This process included homogenization with iterative bead-beating steps for optimal disruption of cellular material, and the resulting concentration of DNA was measured by NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). Extracted DNAs were transferred to the University of Maryland for amplification of bacterial DNA with universal primers specific for the V1–V3 region of the 16S rRNA gene common to all bacteria. Amplified products were sequenced using Illumina® MiSeq® at the Institute for Genome Sciences (San Diego, CA). Raw reads were custom filtered to identify those with the best quality as described by Pop et al.¹⁹ The resulting high-quality sequences were used with DADA2 (v. 1.8.0, Stanford, CA) employing an algorithm to resolve sequencing errors for sample-specific resolution of amplicon sequence variants (ASVs).²⁸ Briefly, the sequences were dereplicated, sorted by abundance, and aggregated while accounting for the sequence-specific likelihood that the sequence was read from sample DNA or an artifact of the Illumina processing. The taxonomical annotation was performed on the resulting ASVs using the naïve Bayes classifier described by Wang et al.²⁹ based on the k-mer length 8 and the reference database SILVA (v. 123), Bremen, Germany.³⁰

Ethical approval. Written informed consent was received from the primary caregivers of participating children. This study was approved by the Institutional Review Board of icddr,b, and an exemption was granted from the Institutional Review Board of the Johns Hopkins Bloomberg School of Public Health.

Statistical methods. We defined growth by three anthropometric measures: height, weight, and weight for height standardized to Z-scores by age and gender for each child based on the WHO criteria (height for age Z-score (HAZ), weight for age Z-score (WAZ), and weight for height or length Z-score (WFLZ)),³¹ where a negative change in the Z-score over the 18-month follow-up period was assumed to indicate poor growth. We assumed children in the top tertile (largest increases in Z-score, defined as “control”) to be healthy children who were growing well and that those with the largest decreases in Z-score in the lowest or bottom tertile (defined as a “case”) were growing poorly. These criteria were applied separately, so that a child with a low change in HAZ would be defined as a “case” for HAZ but may have a normal change in WAZ and hence be grouped as a “control” for WAZ. We defined EE by four fecal EE markers, where high levels in the top tertile among all 68 children of each fecal marker (calprotectin, alpha-1-antitrypsin, myeloperoxidase, and neopterin) were defined as “case” and low levels in bottom tertile for each fecal marker were defined as “control.”

For each measure, child pairs were matched by age within one month in a retrospective case-control design, with potentially overlapping definitions of “case” as defined by HAZ, WAZ, WFLZ, and the four fecal markers alpha-1-antitrypsin,

calprotectin, myeloperoxidase, and neopterin. Age matching was performed given the known large variability in gut microbiota composition by age.¹⁹ Because we did not expect exact agreement between these different growth classifications, we also examined the Pearson correlation between measures. We included children whose stool samples had at least 2,000 reads of 16S RNA at baseline and follow-up in statistical analyses, discarding those with fewer than 2,000 reads at either time. We compared the proportional abundance of phyla and genera between cases and controls with nonparametric comparisons using the Wilcoxon rank sum test. We also compared the change over time in relative abundance with the rank sum test. For comparison, we also compared the relative abundance at baseline between age-matched children using the DESeq method and comparing the change in relative abundance over time, assuming a negative binomial distribution of sequence counts for each taxa.³² We did not adjust for multiple comparisons. In addition, we examined the Bray–Curtis distance between bacterial communities identified from stool samples for pairwise comparisons.³³ This distance was clustered hierarchically to identify similar subgroups among samples.³⁴

Diversity. In addition to comparing proportional abundance at baseline and the change in proportional abundance, the diversity of bacterial communities at each observation time was also compared using alpha diversity described by the Shannon index, with 0 corresponding to total dominance from one ASV and larger values indicating more diversity.^{20,35} We also examined the turnover of bacterial ASVs over time between children with poor or normal growth and children with low or high EE markers with beta diversity³⁶ using the metric β_w as defined by Whittaker,³⁷ ranging from 0 (no change) to 1 (complete turnover). Diversity was estimated using the vegan statistical package in R.³⁸ We compared these measures of diversity and change in bacterial diversity between cases and controls using Student's *t*-test.

RESULTS

Of the 68 children included in this analysis, 50% were male (34 of 68), with an average age of 17 months at baseline. Baseline anthropometry and fecal EE markers are shown in Table 1. Relative to the standards defined by the WHO for child growth, children in this cohort were below average height (average Z-score −1.4), weight (average −1.1), and weight for height (average −0.6). In addition, on average, standardized height and weight were declining over the 18-month follow-up period (average decline of 0.5 SD in HAZ and 0.5 SD in WAZ). Further details for this study cohort are available in George et al.⁵ Levels of fecal markers and change in anthropometric measures that were used to define child pairs for classification of cases and controls are shown in Table 1. Complete distributions of anthropometric factors are available in Supplemental Appendix S1.

The correlations between the four EE and three growth measures are shown in Table 2. Baseline fecal markers were not significantly correlated with each other, with the exception of calprotectin and myeloperoxidase, which have a moderate association at 0.41 ($P < 0.001$). Baseline myeloperoxidase was positively correlated with changes in WFLZ (correlation 0.29, $P = 0.021$), unexpectedly indicating higher myeloperoxidase being related to a positive change in WFLZ. Among changes in

TABLE 1
Description of cohort of 68 children from Mirzapur, Bangladesh, followed up for 18 months

Factor	Mean (SD) or N (%)	Control classification	Case classification
Gender			
Male	34 (50%)	—	—
Female	34 (50%)	—	—
Age at baseline (months)	16.8 (3.6)	—	—
Baseline			
Alpha-1-antitrypsin (g/L)*	0.4 (0.4)	< 0.20	> 0.36
Calprotectin (μg/g)*	897.1 (928.6)	< 230.4	> 1,041
Myeloperoxidase (ng/mL)*	6,380 (6,224)	< 2,643	> 6,003
Neopterin (mmol/mL)*	2,002 (2,395)	< 590.0	> 1,999
HAZ	-1.4 (1.1)	—	—
WAZ	-1.1 (1.1)	—	—
WFLZ	-0.6 (1.2)	—	—
18-month follow-up			
HAZ	-1.8 (1.0)	—	—
WAZ	-1.4 (1.1)	—	—
WFLZ	-0.5 (1.1)	—	—
Change from baseline to 18 months			
HAZ**	-0.5 (0.7)	> -0.32	< -0.73
WAZ**	-0.3 (0.8)	> -0.05	< -0.65
WFLZ**	0.1 (1.0)	> 0.23	< -0.38

HAZ = height for age Z-score; WAZ = weight for age Z-score; WFLZ = weight for length or height Z-score.

* Controls defined by the tertile of highest growth defined by low fecal markers, and cases were defined in the lowest tertile of growth (highest fecal markers).

** Controls defined by the tertile of highest growth, and cases were defined in the lowest tertile of growth.

anthropometry, change in HAZ was correlated with change in WAZ (0.35, $P = 0.005$).

Each child contributing to case-control pairs had at least 2,000 reads of DNA at each observation time for analysis. For some EE and growth measures, children classified as having poor growth were dissimilar in age (not within 1 month) to all children classified as having normal growth, and 26 stool samples were excluded because of an insufficient number of reads. The resulting number of pairs for growth status defined by high/low alpha-1-antitrypsin, high/low calprotectin, high/low myeloperoxidase, high/low neopterin, short/tall height for age, light/heavy weight for age, and small/large weight for height/length was 10, 8, 4, 8, 7, 8, and 9, respectively.

Using DADA2 to incorporate quality scores from the Illumina MiSeq sequencer for resolving errors in the corresponding read of 16S RNA, we identified 10,676 unique bacterial taxa among all 68 children in this cohort over the observation period. Study children contributed a total of 136 stool samples; however, there were 29 samples with fewer than 2,000 reads of DNA, leaving 107 samples for analysis from 56 children. On average, 26,000 sequences of DNA were read from these 107 samples, with a median of 14,000 sequences.

A summary of common phyla is shown in Figure 1 both for cases and controls at each observation time, excluding phyla with 1% or lower average proportional abundance. Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, and Proteobacteria are included. The proportional abundance of bacterial phylum did not appear different at baseline between case and control children for any case/control definition; however, sensitivity analysis with DESeq identified lower Cyanobacteria at baseline among the case children defined by the HAZ as well as the alpha-1-antitrypsin criteria (see Supplemental Appendix S2). Comparing the change over time between child pairs, Proteobacteria increased more among those with high calprotectin than low calprotectin (10% increase versus 15% decrease, $P = 0.005$).

The 10 most commonly observed genera are shown in Figure 2, including *Bifidobacterium*, *Escherichia/Shigella*, *Streptococcus*, *Lactobacillus*, *Catenibacterium*, *Bacteroides*, *Faecalibacterium*, *Collinsella*, and *Prevotella*. At baseline, the proportional abundance of genera was different among *Bifidobacterium* (25% among those with high alpha-1-antitrypsin versus 17% among those with low alpha-1-antitrypsin, $P = 0.035$). There were also changes in the microbiota from

TABLE 2

Correlation among different measures defining criteria for poor growth among 68 children from the Mirzapur cohort for measurements at baseline and 18-month follow-up

	AA	CAL	MPO	NEO	Delta HAZ	Delta WAZ	Delta WFLZ
Baseline							
AA	1.00	—	—	—	—	—	—
CAL	-0.03	1.00	—	—	—	—	—
MPO	0.10	0.41*	1.00	—	—	—	—
NEO	-0.07	0.06	0.18	1.00	—	—	—
Change from baseline to 18 months							
HAZ	0.18	-0.12	-0.21	-0.11	1.00	—	—
WAZ	-0.02	-0.01	0.16	-0.10	0.35†	1.00	—
WFLZ	-0.15	0.07	0.29**	-0.03	-0.18	0.84*	1.00

AA = alpha-1-antitrypsin; CAL = calprotectin; MPO = myeloperoxidase; NEO = neopterin; HAZ = height for age Z-score; WAZ = weight for age Z-score; WFLZ = weight for length or height Z-score.

† $P = 0.005$.

* $P < 0.001$.

** $P = 0.021$.

FIGURE 1. Average proportional abundance of phyla in stool samples at baseline and 18-month follow-up among Mirzapur, Bangladesh, age-matched growth pairs, for seven separate growth criteria. Growth pairs were constructed so that those in the tertile of highest growth were matched to a child in the lowest growth tertile whose age at baseline was within 1 month. This figure appears in color at www.ajtmh.org.

baseline to 18 months, *Escherichia/Shigella* increased more for those with high calprotectin than among those with low calprotectin (3% increase versus no change, $P = 0.016$). *Prevotella* declined more among those with large decline in WAZ (5% increase versus no change, $P = 0.014$). The proportion of unidentifiable bacteria increased more among those with large declines in HAZ (8% increase versus no change, $P = 0.017$). The normalized abundance of the 40 most common genera in those with low change in HAZ and their respective age-matched children with normal or robust change over time in HAZ is shown in Figure 3. Hierarchical clustering does not appear to identify the HAZ growth classification, although many of the ASVs measured at a similar follow-up time are grouped together, either at baseline or at 18 months. Heterogeneity across age may be better identified, not unexpectedly given the strong relationship between age and gut microbiota.¹⁹

The Bray–Curtis distance among sample bacterial communities based on genus for child pairs with large decline versus small changes in HAZ is shown in Figure 4. The Bray–Curtis distance between sample communities was similar between those with low change in HAZ and high change in HAZ (average 0.66) at baseline to that within cases (0.69) and within controls (0.64). At 18-month follow-up, the distance between cases and controls was also similar (0.52)

and maintained similarity with the average distance within cases (0.54) and within controls (0.54).

Diversity. Diversity at each observation time for 56 children with samples having at least 2,000 sequences at both observation times was quantified. Both Simpson and Shannon diversity, as well as richness and evenness are all increasing over time, as expected for children in this period of rapid gut microbiota maturation, having $P < 0.001$ when compared with a paired t-test. Diversity at each observation time and for turnover in ASVs during the study period are shown in Table 3. Follow-up ASV Shannon diversity was significantly higher in children identified in the top tertile of change in WAZ than those with poor growth for change in WAZ (5.47 versus 5.34, $P = 0.036$). Baseline Shannon diversity was also higher among controls defined by low neopterin concentration than in children with high neopterin (2.48 versus 2.11, $P = 0.016$).

DISCUSSION

We examined the association between markers of EE, growth, and gut microbial communities among a cohort of children from a demographic surveillance area in rural Bangladesh over an 18-month period. Elevated EE markers and poor linear growth in children were associated with changes in

FIGURE 2. Average proportional abundance of genera in stool samples at baseline and 18-month follow-up among Mirzapur, Bangladesh, age-matched growth pairs, for seven separate growth criteria. Pairs were constructed so that those in the tertile of highest growth were matched to a child in the lowest growth tertile whose age at baseline was within 1 month. This figure appears in color at www.ajtmh.org.

microbial communities in the gut. There were increased amounts of *Escherichia/Shigella* and Proteobacteria and decreased amounts of *Prevotella* consistent with the growing evidence demonstrating the relationship between intestinal inflammation, child growth, and changes in gut microbiota composition.

Our findings are consistent with previous observations. Segata et al.³⁹ found higher bacteria in the Proteobacteria phylum in children who were stunted. In the study by Dinh et al.,⁴⁰ children who were not stunted had a gut microbiome with higher *Bifidobacterium longum* than children who were stunted. In addition, we previously found that in this cohort, children with enterotoxigenic *Escherichia coli* infections had elevated fecal calprotectin concentrations, and children with *Shigella* infections were at significantly higher odds of being stunted.⁴¹

We observed low diversity in ASVs among children with higher markers for EE at study enrollment. This is consistent with previous studies that have found an association between elevated EE markers and low diversity in gut microbial communities in young children.^{18,21,42} In the study by Subramanian et al.²³ in Bangladesh, severe acute malnutrition was also associated with reduced bacterial diversity. Low ASV diversity in children with poor growth may indicate dominance by a few bacterial species, when more species are thought to be beneficial.¹⁶

Our findings are also consistent with the theory that younger aged microbiota containing more *Escherichia/Shigella* are associated with poor growth. *Escherichia/Shigella* may be contributing to EE as part of a vicious cycle. This genera contains numerous strains known to be associated with inflammation and have enzymatic pathways that detoxify nitric oxide (NO).^{43–45} Nitric oxide is released by human colonic epithelial cells as part of the normal inflammatory response. Although NO can kill most bacteria without a pathway that can detoxify it, when NO is produced *Escherichia/Shigella* can actually thrive and potentially gain a selective advantage. By producing inflammatory biomolecules that cause human cells to produce NO, *Escherichia/Shigella* enters into a cycle in the human intestine that inhibits other bacteria and allows *Escherichia/Shigella* to proliferate, reinforcing the pattern of enteric infection, inflammation, and poor growth.⁴¹

Our study has limitations. First, we only measured fecal markers of EE at study enrollment, so we were unable to quantify their changes or consistency over time. We did not observe strong agreement between fecal markers and change in anthropometry. We also observed a tendency for children in our sample with high myeloperoxidase to have higher change in WFLZ over time, indicating more weight gain, although the expectation is that high myeloperoxidase would correspond to less weight gain. This counterintuitive result may also

FIGURE 3. Abundance of 40 most common genera in stool samples at baseline and 18-month follow-up among Mirzapur, Bangladesh, age-matched pairs, for the height/length for age growth criteria. Pairs were constructed so that those in the tertile of highest growth were matched to a child in the lowest growth tertile whose age at baseline was within one month. Genera are shown in the decreasing order of overall average abundance. This figure appears in color at www.ajtmh.org.

FIGURE 4. Dissimilarity among and between cases and controls for seven pairs matched by age and for differential growth based on the change in height for age Z-score over an 18-month follow-up period. Dissimilarity estimated with Bray–Curtis distance, where darker blue indicates more difference in the community structure defined by genera. This figure appears in color at www.ajtmh.org.

indicate poorer gains in height for those with high myeloperoxidase, which would similarly result in more increases in WFLZ. Second, we did not measure the nutritional intake for children in this study and, therefore, were unable to examine how child diet was directly related to gut microbiota composition in this population or to account for diet when comparing ASVs across groups of children. We also did not measure micronutrient deficiencies nor whether children were receiving supplementary nutrition. Third, we quantified ASVs from DNA isolated from stool samples, not in biopsy samples from endoscopy directly from colon ileum. Endoscopy is challenging to perform in low-resource settings such as rural Bangladesh. Fourth, we were constrained by our sample size, increasing the likelihood of differences due to sequencing depth or other technical artifacts. We did not rarefy sampled sequences as rarefaction may result in the loss of useful sample data.^{46,47} Fifth, although gut bacteria populations are highly variable, we were not able to measure all variability in gut microbiota over the study period, since we only measured gut microbiota composition at two time points for each child.

Our study contributes to building hypotheses and informing future studies relating to the gastrointestinal microbiota of young children and how to prevent poor growth in this and

TABLE 3

Diversity of bacterial ASVs at baseline and 18-month follow-up among age-matched cases and controls for seven separate growth criteria in the Mirzapur, Bangladesh cohort with at least 2,000 reads

Case/Control Classification	Number of children	Mean among cases	Mean among controls	P-value*
Shannon (alpha) diversity at baseline				
ΔHAZ	14	2.31	2.24	0.710
ΔWAZ	16	2.46	2.27	0.257
ΔWFLZ	18	2.42	2.26	0.328
Shannon (alpha) diversity at 18 months				
ΔHAZ	14	5.34	5.47	0.314
ΔWAZ	16	5.45	5.59	0.036
ΔWFLZ	18	5.41	5.48	0.473
Beta diversity for ASV turnover from baseline to 18 months				
ΔHAZ	14	1.00	1.00	0.172
ΔWAZ	16	1.00	1.00	0.430
ΔWFLZ	18	1.00	1.00	0.632
Shannon (alpha) diversity at baseline				
Alpha-1-antitrypsin	20	2.30	2.32	0.901
Calprotectin	16	2.28	2.12	0.340
Myeloperoxidase	8	2.26	2.30	0.885
Neopterin	16	2.11	2.48	0.016
Shannon (alpha) diversity at 18 months				
Alpha-1-antitrypsin	20	5.41	5.50	0.442
Calprotectin	16	5.33	5.40	0.521
Myeloperoxidase	8	5.35	5.37	0.918
Neopterin	16	5.36	5.44	0.618
Beta diversity for ASV turnover from baseline to 18 months				
Alpha-1-antitrypsin	20	1.00	1.00	0.162
Calprotectin	16	1.00	1.00	0.447
Myeloperoxidase	8	1.00	0.99	0.289
Neopterin	16	1.00	1.00	0.692

ASV = amplicon sequence variant; HAZ = height for age Z-score; WAZ = weight for age Z score; WFLZ = weight length or age Z-score.

* Difference in mean diversity determined by Student's t-test.

other similarly vulnerable populations in low- and middle-income countries.

Received October 14, 2019. Accepted for publication April 8, 2020.

Published online May 18, 2020.

Note: Supplemental appendices appear at www.ajtmh.org.

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