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Etiology of Diarrhea, Nutritional Outcomes and Novel Intestinal Biomarkers in Tanzanian Infants: A Preliminary Study

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Abstract

Objective—Diarrheal diseases are a leading cause of morbidity and mortality worldwide, but the etiology of diarrhea and its relationship to nutritional outcomes in resource-limited settings is poorly defined. We sought to determine the etiology of community-acquired diarrhea in Tanzanian infants, and to assess the association with anthropometrics and novel intestinal biomarkers.

Methods—A convenience sample of infants in a trial of zinc and/or multivitamin supplementation in Tanzania was selected. Subjects were enrolled at age 6 weeks and followed for 18 months. Stool samples were obtained from children with acute diarrhea. A novel, PCR-based TaqMan array was used to screen stool for 15 enteropathogens. A subset of subjects had serum gastrointestinal biomarkers measured.

Results—123 subjects with diarrhea were enrolled. The mean $\pm$ SD age at stool sample collection was 12.4 ± 3.9 months. Thirty-five enteropathogens were identified in 34 (27.6%) subjects: 11 rotavirus, 9 *Cryptosporidium spp*, 7 *Shigella spp*, 3 *Campylobacter jejuni / coli*, 3 ST-enterotoxigenic *E. coli* and 2 enteropathogenic *E. coli*. Subjects with any identified enteropathogen had significantly lower weight-for-length z scores (-0.55 ± 1.10 vs. 0.03 ± 1.30 , $p=0.03$) at the final clinic visit, compared to those without an identified pathogen. Fifty of the 123 subjects (40.7%) had serum analyzed for antibodies to LPS and flagellin. Subjects with any identified

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enteropathogen had lower IgA antibodies to LPS (0.75 ± 0.27 vs. 1.13 ± 0.77 , $p=0.01$) and flagellin (0.52 ± 0.16 vs. 0.73 ± 0.47 , $p=0.02$), compared to those without an identified pathogen.

Conclusion—This quantitative PCR method may allow identification of enteropathogens that place children at higher risk for suboptimal growth. IgA anti-LPS and flagellin antibodies hold promise as emerging intestinal biomarkers.

Introduction

Diarrhea continues to be a leading cause of morbidity and mortality for children worldwide. In 2011, an estimated 700,000 child deaths were caused by diarrhea, with 72% of those deaths occurring in children under two years of age (1). Diarrhea and nutritional status are closely interrelated, as undernutrition increases susceptibility to diarrhea-causing infections (2), and childhood diarrhea predisposes to growth faltering. It is estimated that each episode of diarrhea before age 2 years increases a child's risk of stunting by 5% (3). The pathologic mechanisms linking diarrhea and undernutrition are complex, but likely involve alterations in immune function, increased intestinal permeability, impaired nutrient absorption, and repeated exposure to multiple enteropathogens (4).

An essential step in reducing diarrheal morbidity is the identification of causative infectious pathogens, but traditional methods of diagnosis including bacterial culture, stool microscopy, and antigen-based assays have poor sensitivity (5, 6). Newer polymerase chain reaction (PCR)-based methods to detect diarrhea-causing enteropathogens have recently been developed (7), but may detect pathogens that do not cause clinical symptoms of diarrhea. For example, in a longitudinal study that compared diarrheal samples to pre-diarrheal matched controls, PCR stool analysis was shown to correlate with diarrhea due to rotavirus, astrovirus and *Shigella/enteroinvasive Escherichia coli* only when the data were analyzed quantitatively (8). Similarly, a study of enteropathogens in diarrhea cases and non-diarrheal controls found high rates of detection even in asymptomatic controls; the specificity improved when pathogen quantity was assessed (9). In these ways, the calculation and interpretation of pathogen burden is of critical importance to the utility of these PCR methods.

In resource-limited settings, diarrhea-causing enteropathogens have most commonly been described in hospitalized children with acute/persistent severe diarrhea (10, 11). In hospitalized children under 5 years in Dar es Salaam, Tanzania, diarrheagenic *E. coli* was found by standard bacteriologic culture (12) in 22.9 % of patients (13). However, the etiology of more common, ambulatory diarrhea in Tanzania remains poorly reported. We sought to determine the etiology of community-acquired diarrhea in Tanzanian infants and young children, and hypothesized that identifiable diarrheal infections would correlate with a high pathogen burden and place children at risk for suboptimal growth. In addition, we evaluated the relationship of novel biomarkers of gastrointestinal function with outcomes in this cohort.

Methods

A convenience sample of infants was selected from a prospective randomized trial of daily zinc and/or multivitamin supplementation in Dar es Salaam, Tanzania (14, 15) (clinicaltrials.gov identifier NCT00421668). Consenting mothers who were confirmed to be HIV-negative were enrolled into the study and their infants were randomized between 5–7 weeks of age. Infants of multiple births and infants with congenital anomalies or other conditions that would interfere with the study procedures were excluded. Infants were enrolled at 6 weeks of age and followed for 18 months. Demographic data was collected at enrollment, and morbidity and anthropometric data were collected at monthly outpatient follow-up visits. All children received a periodic dose of vitamin A every 6 months as per standard of care in Tanzania.

Mothers were asked to return to the study clinic with their infants every four weeks for data collection and standard clinical care, including growth monitoring, immunizations, and routine medical treatment for illnesses. During these visits, nurses interviewed caretakers about the child's morbidity history, and measured child's weight on a digital infant balance with 10-g precision (Tanita) and length with 1-mm precision using a rigid length board with a movable foot piece.

Stool samples were collected between January 2009 and January 2011 from children with acute diarrhea, defined as three or more watery stools in a 24 hour period. Stool from children with diarrhea was collected at the study site via fecal swab from either the diaper (napkin) or collection container (pot) and placed into vials containing transport media. Vials were placed into a cooler and transported to a central laboratory daily, where they were promptly frozen at -80°C . Blood was drawn from infants at 6 weeks and 6 months of age, and frozen at -80°C .

A PCR-based TaqMan array method was used to screen stool samples for 15 enteropathogens. Previously described pathogen-specific quantification cycle (Cq) cutoffs were used to define positive stool PCR results (7). The primary outcome was the prevalence of any identifiable stool enteropathogen. Secondary outcomes included weight-for-age Z score (WAZ), length-for-age Z score (LAZ) and weight-for-length Z score (WLZ, as measured by standard anthropometric measurements) and intestinal biomarkers (including antibodies to LPS and flagellin and the amino acid citrulline).

Flagellin- and LPS-specific IgA and IgG levels were quantitated by ELISA, as has been previously reported (16). Microtiter plates were coated overnight with purified *E. coli* flagellin (100 ng/well), or purified *E. coli* LPS (2 μg /well). Serum samples from study subjects diluted 1:200 were applied to wells coated with flagellin or LPS. After incubation and washing, the wells were incubated either with anti-human IgA (KPL) or IgG (GE Healthcare) coupled to horseradish peroxidase. Quantitation of total immunoglobulins was performed using the colorimetric peroxidase substrate tetramethylbenzidine, and optical density (OD) was read at 450 nm with an ELISA plate reader. Data are reported as OD corrected by subtracting background (determined by readings in samples lacking serum).

Underivatized citrulline (CIT) concentrations in serum specimens were evaluated in the Clinical and Epidemiological Research Laboratory in the Department of Laboratory Medicine, Boston Children's Hospital. Samples were ultracentrifuged to remove high-molecular-weight proteins. The separation selectivity of CIT was obtained using a C18 BEH column and the ion-pairing agent pentadecafluorooctanoic acid on a Waters Acquity UPLC System (Waters Corporation, Milford, MA). Positive electrospray ionization and a Waters Quattro premier triple quadrupole mass spectrometer were used to detect CIT by its characteristic 176–170 and 176–113 m/z transitions.

Data were double-entered using Microsoft Access software at the central study site, then converted to SAS datasets and uploaded to a secured UNIX-based server for analysis. T-tests were used to compare mean anthropometric measures, citrulline, flagellin, and LPS concentrations at 6 weeks, 6 months, and 12 months of age among children who tested positive for any pathogen vs. those who tested negative for all pathogens. T-tests used the pooled method when variances were equal and Satterthwaite method when variances were unequal. A scatter plot was constructed to assess the relationship between enteropathogen burden (as measured by PCR quantification cycle, Cq) and anthropometric outcomes. All analyses were performed using SAS software (version 9.2; SAS Institute). Institutional approval was granted by the Harvard School of Public Health Human Subjects Committee, the Muhimbili University of Health and Allied Science Committee of Research and Publications, and the Tanzanian National Institute of Medical Research.

Results

One hundred twenty three subjects with diarrhea were enrolled. Table 1 lists the baseline characteristics of the sub-study population, including both maternal and infant demographic and nutritional status. 87 (70.7%) of the mothers had been pregnant at least once previously. 120 (97.6%) of infants had a birth weight >2500 grams, and 8 (6.5%) were born prior to 37 weeks gestational age.

The mean $\pm$ SD age of diarrheal illness that resulted in a stool sample was 12.4 ± 3.9 months (range 2.9–19.9 months). One stool sample was collected from each of the n=123 subjects. Table 2 lists the enteric pathogens detected. Thirty five pathogens were identified in 34 (27.6%) subjects: 11 rotavirus, 9 *Cryptosporidium spp*, 7 *Shigella spp*, 3 *Campylobacter jejuni / coli*, 3 ST-enterotoxigenic *E. coli* and 2 enteropathogenic *E. coli*. The quantification cycle (Cq) thresholds used to determine a positive sample were < 30 for rotavirus, < 25 for ST-ETEC, <20 for EPEC eae, < 25 for EPEC bfpa, <30 for shigella/EIEC, <30 for campylobacter, and <30 for cryptosporidium. These are quantities previously associated with diarrhea in similar settings (7). There was no difference in pathogen detection in children receiving zinc (n=14 vs. n=47 in the group with any pathogen vs. no pathogen detected, respectively; p=0.25) or multivitamin supplementation (n=17 vs. n=51; p=0.47). One subject had two pathogens detected simultaneously (EPEC and *Campylobacter*). Compared to children without an identified pathogen, subjects with any identified enteropathogen had significantly lower WLZ (-0.55 ± 1.10 vs. 0.03 ± 1.30 , p=0.03) at the final clinic visit. There were no significant differences in WAZ or LAZ between the two groups.

The mean $\pm$ SD age at the final clinic visit was 18.2 ± 2.5 months. The mean $\pm$ SD time of follow up after stool collection was 5.9 ± 4.0 months. Figure 1 shows the relationship between PCR Cq and LAZ at the final clinic visit ($R=0.329$, $p=0.062$) in those subjects with any positive enteropathogen, suggesting a possible trend between lower Cq (indicating higher pathogen burden) and linear growth deficits.

Fifty of the 123 subjects (40.7%) underwent serum analysis for antibodies to the bacterial products LPS and flagellin, which are emerging intestinal biomarkers that may correlate with intestinal permeability (16, 17). Table 3 reports the antibody levels to LPS and flagellin at 6 months of age, in those with any identified enteropathogen vs. no detected enteropathogen. Subjects with any identified diarrheal enteropathogen had lower IgA antibodies to LPS (0.75 ± 0.27 vs. 1.13 ± 0.77 , $p=0.01$) and flagellin (0.52 ± 0.16 vs. 0.73 ± 0.47 , $p=0.02$) at age 6 months, compared to those without an identified pathogen. These differences were not present at age 6 weeks. There was no significant difference between IgG antibodies to LPS and flagellin between the two groups.

Citrulline, a non-protein amino acid produced by enterocytes, is a biomarker of intestinal mass and function (18–20). Subjects with any identified diarrheal enteropathogen had a non-significantly lower citrulline concentration (17.2 ± 1.6 vs. 21.0 ± 8.8 , $p=0.06$) at age 6 weeks, compared to those without an identified pathogen. This trend did not persist at age 6 months of age (20.8 ± 5.1 vs. 20.7 ± 6.5 , $p=0.99$).

Discussion

The identification of diarrhea causing enteropathogens worldwide is an important factor in reducing global child morbidity and mortality, and our understanding of enteropathogens in various geographic settings is growing. The Global Enteric Multicenter Study (GEMS) (21) identified 4 causative enteropathogens in non-hospitalized children in Africa and Asia with moderate-to-severe diarrhea, including rotavirus, *Cryptosporidium*, enterotoxigenic *Escherichia coli* and *Shigella spp.* This study used traditional stool assays (22) (conventional bacterial culture, enzyme-linked immunosorbent assays and RT-PCR for viruses.) In contrast to conventional methods, multi-target PCR stool analysis allows for a single stool sample to be screened for multiple bacterial, viral and parasitic enteropathogens. Previous studies using qualitative PCR stool analysis have reported high levels of pathogen detection in control, non-diarrheal stool samples (79% in the study by Kabayiza et al (23), 84% in the study by Elfving et al(9)). Platts-Mills et al. (8) reported a median of 3 positive enteropathogens detected in each pre-diarrheal stool sample. In this way, the high sensitivity of PCR makes it difficult to differentiate between intestinal microbial colonization versus pathogenic infection. One solution to this problem is utilization of the PCR Cq, which allows for quantification of pathogen burden and helps differentiate between colonizing pathogens and those causing diarrheal disease. Lower Cq (indicating higher pathogen burden) has been shown to correlate with an increased diarrheal risk (7, 9) and in the case of rotavirus, ETEC and *Shigella*, more severe diarrhea. (23). Figure 1 shows the relationship between lower Cq and LAZ at the final clinic visit, and suggests that higher pathogen burden may be associated with the development of linear growth faltering.

Rotavirus was the most commonly identified enteropathogen in our cohort. Rotavirus is a known global cause of moderate to severe diarrhea in young children (21), and oral rotavirus vaccination has proven to be safe and effective in African infants (24, 25). In our cohort, after applying a PCR Cq threshold of <30 cycles, 8.9% of subjects with diarrhea were positive for rotavirus. In a previous study of Tanzanian children age 0–60 months hospitalized for diarrhea, 18% were found positive for rotavirus by enzyme-linked immunosorbent assay (26). One likely explanation for this difference is the expected higher incidence of severe diarrhea due to rotavirus in the hospital setting as compared to the community. The MAL-ED study, which used ELISA methodology to test for rotavirus (27), reported an adjusted attributable fraction of diarrhea due to rotavirus of 14.3% in Tanzania during the second year of life (28). Of note, our sample collection was completed before Tanzania initiated routine oral rotavirus vaccination in 2012, and therefore would not reflect widespread oral rotavirus vaccination within the cohort.

We found that any identified diarrheal pathogen was associated with lower subsequent weight-for-length, indicating acute or sub-acute undernutrition. Many enteropathogens have been correlated with suboptimal child growth. Cryptosporidium, which was found in 7.3% of subjects in our cohort, has been associated with persistent diarrhea (29) and childhood malnutrition (30). Nearly 5% of subjects in our cohort had diarrhea due to ETEC or EPEC, which have been associated with severe diarrhea and child malnutrition (31). In a study of infants with diarrhea in Bangladesh, Qadri et al. (32) reported that infants with ETEC-positive diarrhea were more likely to become stunted and wasted at 12 months, compared to those with ETEC-negative diarrhea, and that growth faltering persisted through age 24 months. Shigella/EIEC was detected in 5.7% of our cohort, and has previously been associated with prolonged diarrhea and growth faltering (29). Stool protein loss followed by inadequate post-diarrhea protein consumption has been proposed as a mechanism for growth faltering in the setting of Shigella infection (33).

The extent to which intestinal permeability influences diarrheal disease and child growth is controversial. Environmental enteric dysfunction has been hypothesized to be associated with small intestinal exposure to pathogenic organisms, and has been proposed as more proximal cause of suboptimal childhood growth than diarrheal illness (34, 35). To investigate this possibility we examined levels of serum antibodies to LPS and flagellin. Based on the original descriptions of such antibodies as indirect markers of gut permeability, one might have postulated that stunting would correlate with increased levels of these bacterially-directed antibodies. In fact, we found that lower IgA antibody levels to LPS and flagellin at age 6 months correlated with the identification of at least one stool pathogen. When considering more recent studies that mucosal antibodies, particularly IgA, play a critical role in regulating flagellated bacteria both by altering microbiota composition and flagellin gene expression (36, 37), a possible explanation for our observations herein is that those subjects with elevated anti-flagellin and anti-LPS antibodies could have more robust immune function. This increased immunity, whether endogenous or maternal, might help to contain the quantity and/or activity of potentially diarrheagenic microbes. Currently, this assay cannot reliably distinguish between passively-transferred maternal antibodies and infant antibodies, which is of particular importance given that the measurements were taken at age 6 months. Therefore, as these intestinal biomarkers become further refined, it will be

important to understand whether and/or how maternal immunity affects anti-LPS and anti-flagellin antibodies. Additionally, testing at later timepoints, including after passively transferred maternal immunity wanes, will be important. Further study in this area is needed to elucidate the complex relationship between intestinal permeability, bacterial translocation, infant diet, diarrhea, and growth.

There were several limitations to our study. First, our sample size was relatively small, and may have limited our ability to detect significant relationships between diarrhea etiology and linear growth. Secondly, despite our efforts to do so, we were unable to collect pre-diarrheal control stools, which likely limited our ability to better differentiate colonized pathogens from disease-causing pathogens. Fortunately, enteropathogen detection in pre-diarrheal control samples has been well described previously (7, 19). Finally, only a small proportion of our cohort was able to undergo intestinal biomarker testing. Addressing these limitations in future studies will be important.

In conclusion, quantitative PCR stool analysis methods allow for the rapid screening of stool for multiple causative enteropathogens, and may help identify those children with diarrhea who are most at risk for subsequent suboptimal growth. Applying PCR Cq cutoffs allows for assessment of diarrheal pathogen burden, and will likely become more clinically important as the pathogen-specific Cqs become further refined. Novel intestinal biomarkers may hold promise in identifying those infants most at risk for diarrheal illness.

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List of abbreviations

ST-ETEC	heat stable toxin producing enterotoxigenic <i>Escherichia coli</i>
EPEC	enteropathogenic <i>Escherichia coli</i>
LPS	Lipopolysaccharide
LAZ	length-for-age Z score
WAZ	weight-for-age Z score
WLZ	weight-for-length Z score
PCR	polymerase chain reaction
Cq	PCR quantification cycle

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What is known

- Diarrhea is a leading cause of child morbidity and mortality worldwide
- Traditional methods to identify enteric pathogens in low-resource settings (including bacterial culture, stool microscopy and antigen-based assays) lack specificity and sensitivity
- Childhood diarrhea is associated with suboptimal child growth

What is new

- Quantitative PCR stool analysis methods allow for the rapid, sensitive screening of stool for multiple causative enteropathogens, and may help identify those children with diarrhea who are most at risk for suboptimal growth.
- Serum antibodies to the bacterial products LPS and flagellin hold promise as emerging intestinal biomarkers

Figure 1. scatter plot of PCR cycle time (Cq, y axis) versus length-for-age Z-score (LAZ, x- axis) at the final clinic visit in the N=33 subjects with positive enteropathogens (1 subject with missing anthropometrics)

Table 1

Baseline characteristics of n=123 subjects with diarrhea

	Mean $\pm$ SD or N(%)
<i>Child characteristics</i>	
Age at sample collection (mths)	12.4 (3.9)
Male sex	61 (49.6)
Low birth weight, <2500 g	3 (2.4)
Length-for-age Z score	-1.03 $\pm$ 1.14
Weight-for-length Z score	0.08 $\pm$ 1.21
Weight-for-age Z score	-0.51 $\pm$ 1.11
<i>Maternal characteristics</i>	
Age (y)	26.7 $\pm$ 4.9
Formal education	
None	4 (3.3)
1–7 y	87 (71.9)
8 y	30 (24.8)
Employment	
Housewife without income	69 (56.6)
Housewife with income	42 (34.4)
Other	11 (9.0)
Marital status	
Married or cohabitating with partner	110 (90.2)
Prior pregnancies	
None	34 (28.1)
1–4	84 (69.4)
5	3 (2.5)
Mid-upper arm circumference (cm)	26.8 $\pm$ 3.9
<i>Socioeconomic characteristics</i>	
Daily food expenditure per person in household is < 1000 TSh	31 (26.7)
Household possessions ^I	
None	37 (30.3)
1–3	69 (56.6)
3	16 (13.1)

^IFrom a list that includes sofa; television; radio; refrigerator; and fan

Table 2

Prevalence of enteric pathogens among Tanzanian infants and young children with diarrhea

Stool samples analyzed	n=123	%
Any positive pathogen *	35	28.4
Rotavirus	11	8.9
Cryptosporidium	9	7.3
Shigella spp/EIEC	7	5.7
Campylobacter jejuni / coli	3	2.4
E. coli species		
ST-EPEC	3	2.4
EPEC	2	1.6
Dual infection		
EPEC + Campylobacter spp	1	0.8

* After applying diarrhea-associated Cq thresholds

Table 3

Comparison of IgA and IgG antibodies to LPS and flagellin at 6 months of age by diarrheal enteropathogen status

	Enteropathogen identified	No enteropathogen identified	p ^I
LPS IgA			
N	11	39	
Mean±SD	0.75 ± 0.27	1.13 ± 0.77	0.01
LPS IgG			
N	11	39	
Mean±SD	1.10 ± 0.47	1.44 ± 0.74	0.16
Flagellin IgA			
N	11	39	
Mean±SD	0.52 ± 0.16	0.73 ± 0.47	0.02
Flagellin IgG			
N	11	39	
Mean±SD	0.73 ± 0.42	0.93 ± 0.55	0.26

^IObtained from a t-test using the pooled method when variances are equal and Satterthwaite method when variances are unequal