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Pediatric Diarrhea in Southern Ghana: Etiology and Association with Intestinal Inflammation and Malnutrition

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Abstract. Diarrhea is a major public health problem that affects the development of children. Anthropometric data were collected from 274 children with (N = 170) and without (N = 104) diarrhea. Stool specimens were analyzed by conventional culture, polymerase chain reaction for enteroaggregative *Escherichia coli* (EAEC), *Shigella*, *Cryptosporidium*, *Entamoeba*, and *Giardia* species, and by enzyme-linked immunosorbent assay for fecal lactoferrin levels. About 50% of the study population was mildly to severely malnourished. Fecal lactoferrin levels were higher in children with diarrhea (P = 0.019). Children who had EAEC infection, with or without diarrhea, had high mean lactoferrin levels regardless of nutritional status. The EAEC and *Cryptosporidium* were associated with diarrhea (P = 0.048 and 0.011, respectively), and malnourished children who had diarrhea were often co-infected with both *Cryptosporidium* and EAEC. In conclusion, the use of DNA-biomarkers revealed that EAEC and *Cryptosporidium* were common intestinal pathogens in Accra, and that elevated lactoferrin was associated with diarrhea in this group of children.

INTRODUCTION

Diarrhea is a principal cause of morbidity and mortality in children < 5 years of age in developing countries, where acute watery diarrhea accounts for nearly two million diarrhea-related deaths annually in this age group.[1,2] In the last decades, however, while mortality caused by diarrhea has been decreasing worldwide mainly because of improved hygiene, morbidity attributable to diarrhea remains high.[2–4] A vicious cycle ensues between diarrhea and malnutrition, and studies have shown that malnutrition with frequent diarrheal episodes slows cognitive and physical development of children.[5,6] One mechanism for this is that diarrheagenic pathogens damage intestinal epithelium and reduce its absorptive function, leading to nutrient depletion and malnutrition.[6]

Obstacles to recognition of at-risk children are several. The plight of sub-optimally breast fed and malnourished children is often largely invisible because they are only mildly or moderately undernourished.[7] Additionally, anthropometric measurements are not routinely performed to identify malnourished children in most clinics and hospitals in Ghana, thereby missing the opportunity for diagnosis and appropriate management.[8] The most established method to identify those with malnutrition is by the use of z-scores, with the reference population defined for the study country or from the standard international reference chart of the National Center for Health and the World Health Organization (WHO).[9]

The agents capable of causing infectious diarrhea and the mechanisms responsible for disease pathogenesis are generally known, but the true prevalence of these agents in developing countries is poorly understood.[10] For example, in most sub-Saharan African countries including Ghana, microbiological methods for clinical investigation of diarrheal diseases are usually restricted to identifying conventional enteric bacteria such as *Salmonella* and *Shigella*, *Escherichia coli* (*E. coli*) isolates are often not fully characterized because of the lack of resources. Additional pathogens of potential importance include enteroaggregative *E. coli* (EAEC), which is associated with diarrhea in several contexts: traveler's diarrhea,[11,12] pediatric diarrhea,[13] foodborne outbreaks,[14] human immunodeficiency virus,[15] symptomatic and asymptomatic cases,[16] acute and persistent diarrhea,[5,17] among others; and *Cryptosporidium*, *Entamoeba histolytica*, and *Giardia*, which are parasitic causes of diarrhea.[18–20] Unfortunately, investigation of diarrhea caused by these parasites in most developing countries largely depends on expert microscopy, where technical competence is necessary.

The objective of this study was, first, to determine the prevalence of EAEC, *Shigella* spp., *Cryptosporidium* spp., *E. histolytica*, and *Giardia lamblia* in children < 5 years of age with and without diarrhea in southern Ghana. Second, this study aims to determine whether these enteropathogens were associated with intestinal inflammation in either nourished or malnourished children.

MATERIALS AND METHODS

Ethical clearance. The study was reviewed and approved by the Institutional Review Board of the University of Ghana Medical School, Ghana. Participation was voluntary and enrollment was subject to parents/guardians' approval, through signature or by thumb printing their names after the purpose of the study was explained to them.

Study design, population, and settings. This was a prospective cross-sectional study carried out between August 2007 and May 2008, of children ≤ 5 years of age consulting at the Princess Marie Louise Children's Hospital (PML), Accra, Ghana. Consecutive children from whom consent was given by their caregivers were included in the study. The group of children with diarrhea was recruited from the outpatient clinic and these children were brought to the hospital for acute health care. The control group of children without diarrhea was also from the outpatient clinic, but these children were visiting for routine child welfare care. No follow-up was done after the initial recruitment as a part of this study.

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Interviews and diarrhea definition. A structured questionnaire was used to obtain information on the children from their parents/guardians. Information that was obtained included demographic data, duration of diarrhea, residence/location, breast feeding status, and medication taken before visiting the hospital. Diarrhea was defined as the passage of three or more unformed stools within a 24-h period. Diarrhea duration lasting < 14 days was defined as acute and those lasting ≥ 14 days, persistent. The control (non-diarrhea) group consisted of children who have not passed three or more unformed stools at least within the 24-h period before enrollment.

Anthropometric data and nutritional status assessment. Height or length measurements in centimeter to the nearest one decimal were performed for children above or below 2 years of age, respectively. Weight measurements in kilogram to the nearest one decimal were performed using a 25 kg Salter hanging scale (CMS Weighing equipment, High Holborn, London, UK). The Z-score, weight-for-age (WAZ), height-for-age (HAZ), and weight-for-height (WHZ) were calculated by use of software designed for nutrition studies (EPINUT, World Health Organization, Geneva; Epi Info version 6.0, Centers for Disease Control and Prevention, Atlanta, GA). These anthropometric Z-scores are a measure of SD above or below the median for the international reference population. Z-score values were used to determine the nutritional status of children on the basis of the following definition: WAZ, well nourished (> –1), mild (–2 to –1), moderate (–3 to –2), and severe (< –3) malnutrition; HAZ, normal height (≥ –2), moderate stunting (–3 < –2), and severe stunting (< –3); WHZ, normal weight (≥ –2), moderate wasting (–3 < –2), and severe wasting (< –3).

Specimen processing and microbiological analysis. A stool specimen from each participating child was collected into a sterile container and processed within 2 h of collection. Routine enteric bacteria were cultured on MacConkey (MAC), *Salmonella-Shigella* (SS), and deoxychocolate (DCA) agars (Oxoid, Columbia, MD), using standard techniques. Selenite F broth was used as pre-enrichment for *Salmonella* before sub-culturing onto MAC, SS, and DCA. Bacterial colonies after an overnight incubation period at 37°C were identified by standard biochemical methods and stored on Muellar Hinton slopes for further analysis. No microscopy was performed for the detection of parasites. One aliquot of a fresh stool specimen from each child was kept frozen at –20°C in cryo-vials (deidentified) until sent to the Center for Global Health, University of Virginia for further analysis.

Fecal DNA extraction. We used the QIAamp stool kit (Qiagen, Valencia, CA) to extract genomic DNA from frozen stool specimens with some minor modifications. The modifications included the addition of dry beads (MO BIO Laboratory Inc., Carlsbad, CA) to weighed stool specimen before the addition of lysis buffer (ASL). The mixture was bead-beated for 2 minutes to make a uniform homogeneous mixture with the lysis buffer. Additionally, we incubated mixtures at 80°C instead of the 70°C recommended by the manufacture to lyse enteric pathogens. For each stool aliquot, between 15 and 20 μg or μL of stool was used depending on stool consistency. DNAs were also extracted from appropriate control organisms. All DNAs were kept frozen at –80°C until needed for analysis.

Quantitative real-time polymerase chain reaction (PCR). A single-plex quantitative PCR for each gene pair (Table 1) consisted of 5 μL template, 1 μL of each 6.2 μM primer, 12.5 μL of SYBR-Green –490 (Bio-Rad Laboratories, Beltsville, MD), and PCR grade water to a reaction volume of 25 μL. Reactions for each sample were performed using the Bio-Rad iQCycler Real-Time Detection System in Bio-Rad iCycler 96-well plates, where positive and negative controls were included with each reaction set. Table 1 shows the target genes, locations, and annealing temperature for each primer set. The results were analyzed with a user-defined threshold of 200 PCR baseline-subtracted curve-fit relative fluorescence units. Melt curve (ct) data collection and analysis was enabled at cycles 3 and 4, with an increase in set point temperatures after cycle 2 by 0.5°C.

We sought multiple loci for EAEC (*aap*, *aatA*, *aggR*, and *aaiC*) and single loci for *Shigella*, *Cryptosporidium*, and *Giardia* species. Standard cultures with known numbers of *E. coli* 042 and 17-2, *Shigella*, *Cryptosporidium*, and *Giardia* oocysts were used as reference and positive controls. Water and *E. coli* K-12 were used as negative controls. Melt curve analysis was used to determine positivity of samples using a user defined threshhold.

Intestinal inflammation assessment. Intestinal inflammations were quantitatively assessed from frozen stool specimens using the IBD SCAN (TechLab, Blacksburg, VA) according

TABLE 1
Target genes screened from fecal DNA*

Strain	Gene target	Location	Primer sequence (5′–3′)	PCR size (bp)	Annealing temperature (°C)	Source/reference
EAEC	*aaiC*	Chromosome	CTTCTGCTCTTAGCAGGGAGTTTG AAGCGTGAAATGCCTGAGGA	123	47.5	Nataro's Laboratory
	aatA	Plasmid	CCTRTGTTGATGCTCGAGAGA CKTTCCTCCTCCTCAAGGACAT	118	55	Nataro's Laboratory
	aap	Plasmid	CTTGGGTATCAGCCTGAATG AACCCATTCGGTTAGAGCAC	310	45	Cerna and others[21]
	aggR	Plasmid	CTAATTGTACAATCGATGTA ATGAAGTAATTCTTGAAT	308	45	Czeczulin and others[22]
Shigella/EIEC	*ipaH*	Plasmid	GTTCCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC	619	60.5	Sethabutr and others[23]
Cryptosporidium	18s rRNA	Chromosome	CTCCACCAACTAAGAACGGCC TAGAGATTGGAGGTTGTTCCT	213	60	Gene ID cgd7_230[24]
E. histolytica	Eh	Chromosome	AACAGTAATAGTTTCTTTGGTTAGTAAAA CTTAGAATGTCATTTCTCAATTCAT	134	60	Haque and others[25]
Giardia lamblia	P241	Chromosome	CATCCGCGAGGAGGTCAA GCAGCCATGGTGTCGATCT	74	60	Guy and others[26]

* PCR = polymerase chain reaction; EAEC = enteroaggregative *Escherichia coli*; EIEC = enteroinvasive *E. coli*.

to the manufacturer's instructions. Stool specimens were allowed to thaw and were serially diluted 10-fold and analyzed by a polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) method. The detailed procedure is described elsewhere,[27] and absorbance of each assay well was measured spectrophotometrically at 450 and 620 nm ($A_{450/620}$). Fecal lactoferrin concentrations in μg/mL were determined by comparison with a standard curve using purified human lactoferrin and analyzed by linear regression in Microsoft excel (Redmond, WA). The lowest dilution of a specimen with an absorbance at 450/620 nm within the linear portion of the curve was used to determine the lactoferrin concentration. The final lactoferrin concentration was obtained by multiplying the dilution factor by the concentration. A positive control (purified human lactoferrin) and a negative control (washing buffer) was included in each batch of stools analyzed and linear regression was performed separately for each batch using standard controls. This assessment was performed only on subjects with adequate stool specimens and where necessary, the experiment was repeated. Among EAEC-infected malnourished children, two stool specimens (out of > 200 specimens analyzed) showed lactoferrin values that were 30–50 times more than the mean value for their subject group, were therefore designated as outliers, and not included in the statistical analysis. No satisfactory explanation for results on these two specimens was evident, because no diarrhea was present in one, and breast feeding was unlikely in both.

Statistical analysis. To avoid any experimental biases, stool specimens were coded before testing and only decoded for purposes of analysis. Statistical analyses were performed using SPSS software (version 17.0, SPSS, Chicago, IL) and Epi-Info. Statistical tests included χ^2 for associations of pathogens with age groups, diarrhea, and non-diarrhea and the paired *t* test for associations with lactoferrin level. Odds ratio (OR) and 95% confidence intervals (CI) are reported for all 2 × 2 comparisons. Two-tailed tests were used and $P < 0.05$ was considered statistically significant.

RESULTS

Study population and nutritional status. Within the 9-month study period, 287 children ≤ 5 years of age were recruited with only 13 excluded from analysis because of insufficient data. Of the 274 children included for analysis, 170 (62%) were with and 104 (38%) were without diarrhea; there were more males 156 (56.9%) than females. Acute and persistent diarrhea included 85.3% (145/170) and 7.6% (13/170) of total cases, respectively. Duration of symptoms in the remaining children with diarrhea (7.1%) was not recorded.

At least one anthropometric measurement was taken for 269 out of the 274 children analyzed. Of 269 children from whom weight measurements were recorded, 134 (49.8%) showed mild to severe malnutrition (WAZ < –1) (93/168 [55.4%] in children with and 41/101 [40.6%] without diarrhea, OR = 1.82 [95% CI, 1.102–2.988], $P = 0.023$). Of 170 children from whom height or length measurements were recorded, 61 (35%) showed moderate to severe stunting (HAZ < –2) (38/86 [44.2%] in children with and 23/84 [27.4%] without diarrhea, OR = 2.10 [95% CI, 1.110–3.972], $P = 0.026$). Of 161 children from whom both weight and height measurements were recorded, 37 (22.9%) showed moderate to severe wasting (WHZ < –2) (24/82 [29.3%] in children with and 13/79 [16.5%] without diarrhea, OR = 2.101 [95% CI, 0.989–4.454], $P = 0.062$). The mean age, weight, and height were 15.1/14.6 months, 9.8/9.5 kg, and 88.0/84.0 cm (diarrhea/non-diarrhea, respectively). Table 2 shows the baseline characteristics of the study population.

Microbiological studies. In only 1 of 170 diarrhea stool specimens was *Shigella* recovered as an enteric bacterial pathogen from culture. This strain was serotyped with *Shigella* polyvalent anti-sera (Mast Group Ltd., Merseyside, UK) and was *Shigella flexneri*. In the entire study population, *E. coli* was the predominant bacterium obtained from culture (79.6%), followed by *Klebsiella* spp. (5.1%). Other commensals included 9.8% of the total and no bacteria grew in 5.5% of the total stool specimens cultured.

Pathogen detection by real-time PCR. Table 3 shows bacterial and parasitic agents detected from fecal DNA by real-time PCR in children with and without diarrhea. The EAEC was defined as positivity for any of the four EAEC virulence genes sought (*aap*, *aatA*, *aggR*, and *aaiC*). Although EAEC was significantly associated with diarrhea (147/170 versus 80/104, OR 1.917 [95% CI = 1.024–3.592], $P = 0.048$), it was also found in similar frequencies in both nourished and malnourished children.

In 6 out of 170 diarrheal stool specimens, the *ipaH* gene, which is expressed by both *Shigella* and enteroinvasive *E. coli* (EIEC) was detected. The *ipaH* gene was not detected in fecal DNA from any of the children without diarrhea. The numbers were, however, too small to assess statistical significance. Five out of 6 of the children in whom the *ipaH* gene was detected were well-nourished (Table 3).

Cryptosporidium spp. was the most frequently detected protozoan parasite in fecal DNA and was associated with diarrhea (14/170 versus 1/104, OR = 9.244 [95% CI 1.197–71.371], $P = 0.011$). Cryptosporidiosis was also primarily (10 out of 14)

TABLE 2
Baseline characteristics of study population*

Characteristic	Diarrhea *N* (%)	Non-diarrhea *N* (%)
Age/months	*N* = 170	*N* = 104
0–6	26 (15.3)	35 (33.7)
7–12	54 (31.8)	30 (28.8)
13–24	77 (45.3)	24 (23.1)
25–60	13 (7.6)	15 (14.4)
Sex	*N* = 170	*N* = 104
Male	97 (57.1)	73 (42.9)
Female	59 (56.7)	45 (43.3)
Weight/kg	*N* = 170	*N* = 104
2.5–4.9	10 (5.9)	4 (3.8)
5.0–9.9	121 (71.2)	75 (72.1)
10–19.9	34 (20.0)	22 (21.2)
20–86.0	5 (2.9)	3 (2.9)
WAZ	*N* = 168	*N* = 101
Normal (> –1)	75 (44.6)	60 (59.4)
Mild (–1 to –2)	33 (19.6)	19 (18.8)
Moderate (–2 to –3)	32 (19.0)	12 (11.9)
Severe (< –3)	28 (16.7)	10 (9.9)
HAZ	*N* = 86	*N* = 84
Normal (≥ –2)	48 (55.8)	61 (72.6)
Moderate (–2 to –3)	6 (7.0)	11 (13.1)
Severe (< –3)	32 (37.2)	12 (14.3)
WHZ	*N* = 82	*N* = 79
Normal (≥ –2)	58 (70.3)	66 (83.5)
Moderate (–2 to –3)	6 (7.3)	8 (10.1)
Severe (< –3)	18 (22.0)	5 (6.3)

*WAZ = weight-for-age; HAZ = height-for-age; WHZ = weight-for-height.

TABLE 3
Organisms detected by real-time PCR from fecal DNA*

	Diarrhea (N = 170)			Non-diarrhea (N = 104)			
	No. (%)	WN (N = 75)	MN (N = 95)	No. (%)	WN (N = 60)	MN (N = 41)	Odds ratio [95% CI] P value†
Any infection							
EAEC	147 (86.5)	66	79	80 (76.9)	49	30	1.917 (1.018–3.612) 0.048
Shigella/EIEC	6 (3.5)	5	1	0 (0)	–	–	n/a
Cryptosporidium spp.	14 (8.7)	4	10	1 (1.0)	1	0	9.244 (1.197–71.371) 0.011
E. histolytica	5 (3.0)	2	3	0 (0)	–	–	n/a
*Giardi*a spp.	0 (0)	–	–	0 (0)	–	–	–

*PCR = polymerase chain reaction; WN = well nourished (WAZ > 1); MN = malnourished (WAZ < 1); EAEC = enteroaggregative *Escherichia coli*; EIEC = enteroinvasive *E. coli*; n/a = not applicable.
† P value is between diarrheal and non-diarrheal stool specimen.

detected in children who were malnourished and had diarrhea (Table 3). *Entamoeba histolytica* was only detected in children with diarrhea 5 out of 170 (2.9%) and *Giardia* was not detected in either sub-populations.

We observed children who were co-infected, with two or more pathogens detected in the stool, predominantly in children who had diarrhea. The EAEC-*Cryptosporidium* was the most prevalent (7.6%, 13/170), followed by EAEC-*Shigella*/EIEC (2.9%, 5/170), and EAEC-*E. histolytica* (2.4%, 4/170). *Cryptosporidium-E. histolytica* and *Cryptosporidium-Shigella*/EIEC co-infection each formed 0.6% (1/170), and one child who had diarrhea was co-infected with EAEC-*Cryptosporidium-Shigella*/EIEC (0.06%, 1/170). There was no obvious trend in the distribution of pathogens by age in the two sub-populations, especially for EAEC (Table 4).

EAEC virulence genes distribution. Of the four genes associated with EAEC, *aatA* was the most frequently detected (67.2%) of all fecal DNA, followed by *aap* (59.9%), *aggR* (42.7%), and *aaiC* (33.6%) (Table 5). The EAEC's plasmid gene *aap* was significantly associated with diarrhea (OR = 2.506 [95% CI, 1.516–4.144], $P < 0.001$) and the chromosomal gene *aaiC* was not (OR = 1.639 [95% CI, 0.0962–2.792], $P = 0.086$) (Table 5). Multiple gene combinations were also observed in EAEC infections in our study population, and the presence of any three genes was associated with diarrhea (OR = 2.101 [95% CI, 1.261–3.502], $P = 0.006$). We did not find any of the EAEC virulence genes associated with malnutrition (WAZ < –1) ($P > 0.05$).

Fecal lactoferrin levels. Figure 1 shows enteric pathogens detected in children with/without diarrhea and the distribution of fecal lactoferrin levels. Generally, the mean lactoferrin levels were lower in children who had diarrhea and were also malnourished compared with those with diarrhea but were nourished. Regardless of the enteric pathogen detected, fecal lactoferrin levels were relatively high (manufacturer's cut-off value = 7.24 μg/mL). Especially for EAEC infection, both controls and patients had a wide range of lactoferrin levels, regardless of whether they were nourished or malnourished (Figure 1).

Children with diarrhea had significantly higher fecal lactoferrin levels (N = 143; 1658.9 ± 204.2 μg/mL) compared with those without diarrhea (N = 84; 935.5 ± 194.4 μg/mL) ($P = 0.019$). The *aatA* gene and the presence of any one or two genes of EAEC were also significantly ($P < 0.05$) associated with elevated fecal lactoferrin levels (Table 6). In comparing diarrhea with non-diarrhea stool specimens, EAEC's chromosomal gene *aaiC* showed the highest fold-increase in fecal lactoferrin level (2.7) followed by the *aap* gene (2.5). Additionally, detection of a multiple virulence gene of EAEC in a stool was associated with an increased fold-rise in the mean fecal lactoferrin level between children with and without diarrhea (Table 6).

DISCUSSION

Nutritional shortfalls among our study population. Mild to severe malnutrition (WAZ < –1, which reflects both acute and chronic types of malnutrition) was identified in 49.8% of our study population. Severe growth shortfalls occurred in both children with and without diarrhea in the entire study population. Growth faltering in infants from developing countries has been reported to occur as early as 2–3 months of age[28,29] in contrast to developed countries,[28] a fact attributable to timing of complementary feeding. For example, in the DARLING (Davis Area Research on Lactation, Infant Nutrition and Growth) study, although breast milk intakes were similar, the amount and nutrient density of food consumed after 6 months were lower in Peru than in the United States.[28] In 2005, Antwi[8] noted a 21.2% incidence of wasting (acute malnutrition) among children seen in Kumasi, Ghana, similar to an overall incidence of 22.9% (37/161) in the current study—both consistent with the 22.1% estimate for Ghana by the World Food Program.[30]

TABLE 4
Distribution of pathogens by age*

	Diarrhea (N = 170)				Non-diarrhea (N = 104)			
	Age category/months				Age category/months			
Any infection	0–6 (N = 26)	7–12 (N = 54)	13–24 (N = 77)	25–60 (N = 13)	0–6 (N = 35)	7–12 (N = 30)	13–24 (N = 24)	25–60 (N = 15)
EAEC	24 (92.3)	42 (77.7)	69 (89.6)	12 (92.3)	25 (71.4)	24 (80)	18 (75)	13 (86.7)
Cryptosporidium	3 (11.5)	4 (7.4)	6 (7.8)	13 (7.7)	–	–	1 (4.2)	–
E. histolytica	–	2 (3.7)	3 (3.9)	–	–	–	–	–
Shigella/EIEC	1 (3.8)	1 (1.8)	3 (3.9)	1 (7.7)	–	–	–	–
Giardia spp.	–	–	–	–	–	–	–	–

*EAEC = enteroaggregative *Escherichia coli*; EIEC = enteroinvasive *E. coli*.

TABLE 5
Enteroaggregative *Escherichia coli* (EAEC) virulence factor-positive in stool samples*

Characteristic	Diarrhea ($N = 170$) N (%)	Non-diarrhea ($N = 104$) N (%)	Total ($N = 274$) N (%)	OR [95% CI]	χ^2	P value
EAEC virulence-related gene						
aaiC	64 (37.6)	28 (26.9)	92 (33.6)	1.639 [0.962–2.792]	3.327	0.086
aggR	79 (46.5)	38 (36.5)	117 (42.7)	1.508 [0.914–2.486]	2.602	0.131
aatA	118 (69.4)	66 (63.5)	184 (67.2)	1.307 [0.780–2.188]	1.036	0.354
aap	116 (68.2)	48 (46.2)	164 (59.9)	2.506 [1.516–4.144]	13.093	< 0.001
EAEC gene combination						
Any 1 gene	147 (86.5)	80 (76.9)	227 (82.8)	1.917 [1.018–3.612]	4.139	0.048
Any 2 genes	130 (76.5)	73 (70.2)	203 (74.1)	1.380 [0.797–2.391]	1.325	0.259
Any 3 genes	84 (49.4)	33 (31.7)	117 (42.7)	2.101 [1.261–3.502]	8.224	0.006
All 4 genes	34 (20.0)	13 (12.5)	47 (17.2)	1.750 [0.876–3.496]	2.554	0.110

* OR = odds ratio; 95% CI = confidence interval; $P < 0.05$ is significant.

Approximately 14% (38/269) of the children we studied were severely malnourished (WAZ < –3), and, out of this number, 73.7% had diarrhea. A self-perpetuating vicious cycle in which malnutrition and diarrhea are synergistic is suggested, and may explain their effect on cognitive development of children, especially their semantic fluency.[6] Our data supports this link between malnutrition and diarrhea, placing these groups of children at a higher risk of morbidity over time.

Bacterial culture versus real-time PCR for detection of pathogens. The PCR methodology is more sensitive in screening for pathogens. In sub-Saharan Africa as in many settings, however, the cost involved cannot be passed on to patients and therefore this tool is used mainly in research facilities. In this study, routine bacterial culture detected only one stool positive for *S. flexneri* and none for *Salmonella* spp. However, PCR detected six *ipaH* genes in diarrheal stool specimens. Although, the *ipaH* gene is expressed by both *Shigella* and enteroinvasive (EIEC), EIEC is not often detected in Ghana,[31] and we speculate that the *ipaH* gene detected most likely reflects the presence of *Shigella* spp. that was missed on the culture. The sero-group identified by culture and serology is predominant in Ghana[32] as it is in many developing countries.

Cryptosporidium spp. was not only the most prevalent parasite detected, but it is also significantly associated with diarrhea (8.7% versus 1.0%, $P = 0.011$). Children who were malnourished and had diarrhea often had cryptosporidiosis. Prevalence rates of *Cryptosporidium* spp. using presumably less sensitive methods like microscopy and ELISA in Ghana,[33,34] Liberia,[35] Mexico,[36] and Guinea Bissau[37] reported ranges between 7.7% and 29% in symptomatic children. The current study recorded 8.2% in symptomatic and 1.0% in non-symptomatic patients.

Entamoeba histolytica causes amebic colitis, amebic dysentery, and liver abscess. Modern diagnostic tools like PCR are able to discriminate *E. histolytica* from the non-pathogenic

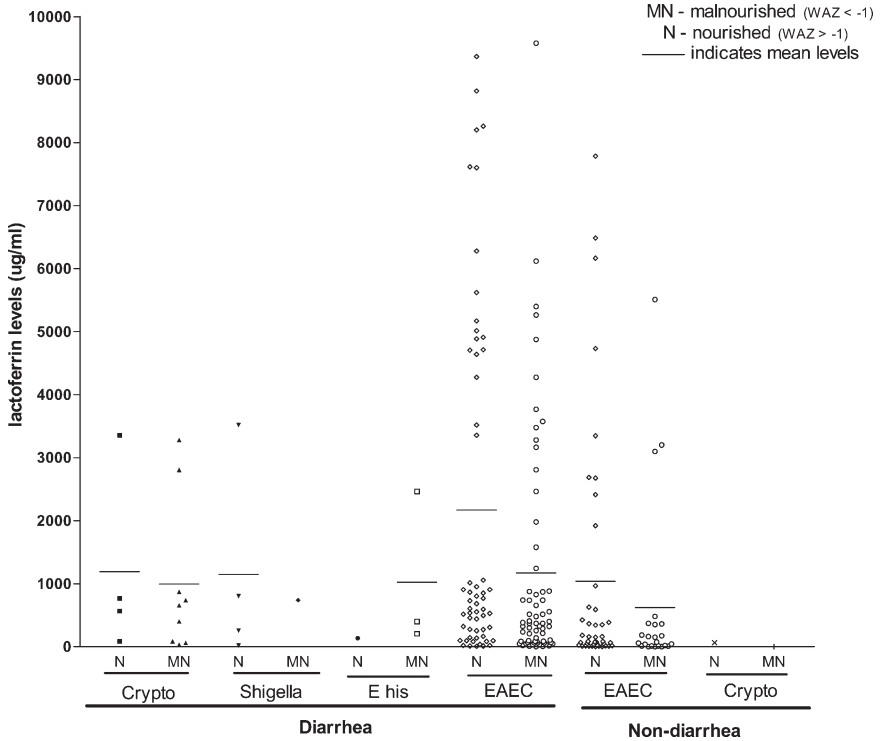

FIGURE 1. Enteric pathogens detected and fecal lactoferrin (LF) levels. Crypto = *Cryptosporidium* spp.; E his = *E. hiostolytica*; EAEC = enteroaggregative *Escherichia coli*. *Breast-feeding may cause moderately (15 ≤ 120 μg/mL) increased LF (Lima and others, unpublished observation).

TABLE 6
EAEC genes detected and fecal lactoferrin levels*

Characteristic	Diarrheal stool n (mean ± SE) µg/mL	Non-diarrheal stool n (mean ± SE) µg/mL	Fold-rise†	*P* value
EAEC gene				
aaiC	56 (1021.0 ± 251.6)	25 (821.2 ± 402.1)	1.2	0.667
aggR	66 (1713.9 ± 292.5)	33 (934.1 ± 329.2)	1.8	0.104
aatA	98 (1706.1 ± 244.1)	54 (927.1 ± 259.1)	1.8	0.044
aap	101 (1656.7 ± 245.9)	39 (901.2 ± 261.6)	1.8	0.080
EAEC gene combinations				
Any 1 gene	123 (1633.5 ± 215.4)	66 (1175.4 ± 356.8)	1.4	0.029
Any 2 gene	107 (1679.8 ± 231.1)	60 (892.6 ± 234.3)	1.9	0.028
Any 3 gene	73 (1504.9 ± 269.7)	30 (1132.8 ± 394.8)	1.3	0.451
Any 4 gene	30 (1319.5 ± 400.4)	11 (370.5 ± 285.5)	3.6	0.175
All specimens	143 (1658.9 ± 204.2)	84 (935.5 ± 194.4)	1.8	0.019

* EAEC = enteroaggregative *Escherichia coli*.
† Ratio of diarrhea and non-diarrhea mean lactoferrin levels.

E. dispar.[25] This study detected *E. histolytica* in 2.9% (5/170) of the children who had diarrhea. No visible blood was observed in any of the stools from these children. All the *E. histolytica* detected in the current study were from children who had diarrhea. In Bangladesh and elsewhere, relatively few numbers of people infected with *E. histolytica* develop symptomatic disease.[18,38,39]

This study did not detect any *Giardia* in the stool specimens screened for the p241 gene of *Giardia* spp. The p241 gene target we used is well validated[26] and our positive control, which was included in each PCR run was amplified while the negative control was not. Addy and others[40] in 2004, found a 3.7% prevalence rate of *Giardia* in Kumasi, Ghana. In a 7-year study of diarrhea caused by parasites in Guinea Bissau, the most prevalent parasite was *Giardia lamblia* (14.8%) followed by *Cryptosporidium* (7.7%).[37] Although seasonality, study duration, or geographical location may influence parasite prevalence, we cannot pinpoint additional reasons for the zero prevalence of *Giardia* in the current study.

Children who had diarrhea were often co-infected, with two or more pathogens detected in the stool, and EAEC-*Cryptosporidium* was the most prevalent (13/170) in the current study. Among human immunodeficiency virus (HIV)-infected children in South Africa, Samie and others[15] identified children who were co-infected with as many as six different species of pathogens. This study detected a child who had diarrhea with EAEC-*Cryptosporidium*-*Shigella*/EIEC co-infection. The HIV status of this child is however not known. Relatively fewer children who are > 2 years of age were sampled in this study. However, cryptosporidiosis seemed to be more common in children < 2 years of age, and this is in agreement with an earlier study in Ghana.[34]

Prevalence of EAEC virulence-associated genes. Among the EAEC plasmid genes (*aap*, *aatA*, and *aggR*) we tested, only *aap* was significantly associated with diarrhea ($P = 0.0003$). A recent publication, which compared molecular probes to the "gold standard" (aggregation of cultured epithelial cells), however, suggested that the *aap* gene is not restricted to EAEC, but is also detected in diffusely adherent *E. coli* (DAEC) and in non-pathogenic *E. coli*.[41] We did not observe any significant statistical association between any individual EAEC gene (whether plasmid or chromosome borne) and malnutrition (WAZ < –1).

Information on the presence of EAEC virulence-associated genes in body fluids of persons in Africa is limited, as is the prevalence and distribution of this organism in Ghana. The *aggR* is known to regulate its own expression and that of several plasmid genes and chromosomal genes of EAEC,[42] including the aggregative adherence fimbriae, a dispersin (aap), a dispersin translocator apparatus called aat, and several chromosomal loci including the *aaiC*.[43] In a different population, Huang and others failed to show any association between four EAEC virulence-associated genes (*aggA*, *aspU*, *aafA* and *aggR*) and clinical illness in travelers from the United States to Mexico.[43] Furthermore, in that study, *aspU* (now designated *aatA*) was the least prevalent gene among the four EAEC virulence genes studied,[43] whereas this same gene (*aatA*) was the most prevalent (67.2%) in the current study. In South Africa, Samie and others[15] found the *aap* gene to be the predominant among others and also associated with diarrhea. These disparate findings on the relative distribution and importance of EAEC genes in diarrheal illnesses suggest that geographical location, type of exposure, and/or host factors may dictate the nature of EAEC infection. For example, among the risks of contracting traveler's diarrhea, the country of destination was the most important determining factor as reported by Cabada and White.[12]

Although *aatA* was the most prevalent gene observed in this study, it was not associated with diarrhea ($P > 0.05$) (Table 5). Some studies have shown that the novel protein aatA, which is encoded on EAEC virulence plasmid pAA2, localizes to the outer membrane of this bacterium and facilitates export of the dispersin *aap* across the outer membrane.[45,46] Our results may support this notion because *aatA* and *aap* were detected in greater than 68% of patients with diarrhea. Further study is required for a firm conclusion. Of interest, the chromosomal gene *aaiC* did not show any significant differential association between the diarrhea and the non-diarrhea group in the current study ($P = 0.068$). About 12% (13/104) of children without diarrhea in the current study were positive for all four EAEC genes tested. The presence of 2, 3, or 4 genes in stool specimens of study children suggests a strong association. For example, the presence of three of these genes is associated with a higher OR than one gene alone (Table 5).

One explanation for the high frequency of EAEC-associated virulence genes in symptomatic and asymptomatic patients and the heterogeneity of the different gene assortments found is that EAEC is endemic in our study population. In support of this notion, almost all prior studies recover EAEC from controls[47–49] and from individuals with diarrhea.[17,50] Pathogenic factors may influence the initiation of a symptomatic phase,

determined by and include distinct mechanisms as reviewed by Kaper and others.[51] For example, Huang and others suggested that a first exposure to EAEC infection "primes" the immune system to prevent a second infection.[44] Furthermore, in their study of traveler's diarrhea, after the initial EAEC infection, only 4 (11%) of the subjects had a subsequent symptomatic EAEC infection.[44]

Lactoferrin levels. Lactoferrin is bactericidal to enteric pathogens, modulates the intestinal immune response, and is released by neutrophils into stool in response to infection.[52] Of interest in the current study, the mean lactoferrin levels were lower in children who were malnourished and had diarrhea compared with those without malnutrition who had diarrhea. We speculate that the lactoferrin assay may be marginally less sensitive in the setting of malnutrition. Perhaps, the enterocytes are less able to synthesize lactoferrin in children who are malnourished and have diarrhea. This observation needs further investigation for a firm conclusion. In earlier studies, the mean lactoferrin levels for healthy controls were less than 12.8 μg/mL[27,52] compared with healthy controls (298.8 μg/mL) in the current study. We do not have immediate explanation for this observation. However, unpublished observations by Lima and others suggest that breast milk may contribute to moderately (15 to 120 μg/mL) increased fecal lactoferrin levels. Some studies have demonstrated an association of EAEC infection with inflammatory cytokines[53,54] and several others have associated EAEC with elevated lactoferrin levels.[15,55] In children with diarrhea, we found a significant statistical association of elevated fecal lactoferrin with the *aap* gene, and with the detection of any one or two of the EAEC genes tested ($P < 0.05$). Between diarrhea and non-diarrhea stool specimens, EAEC's chromosomal gene *aaiC* was associated with the highest fold-rise in fecal lactoferrin level (2.7) followed by the *aap* gene (2.5). Additionally, more virulence EAEC genes detected corresponded to a rise in the fecal lactoferrin level (Table 6). The protective function of lactoferrin on infections with enteropathogens have been acknowledged,[56] and colonization/infection, particularly by EAEC in the current study probably contributed to the high lactoferrin levels (Table 6).

The current study had some limitations. Accurate height measurements were the most difficult to obtain on an outpatient basis, and this limited two of the three z-scores of malnutrition (WHZ and HAZ). Knowledge of history of antimicrobial exposure is important, however, this information was obtainable in only a few of the patients. Only selected pathogens were assayed using PCR and thus, we may have missed other less common pathogens. That is, no stool analyses for intestinal parasites other than *Cryptosporidium*, *Giardia*, and *E. histolytica* were performed, therefore, missing helmintic infections.

In conclusion, through the use of specific DNA-biomarkers, we were able to determine that EAEC and *Cryptosporidium* were common intestinal pathogens and that elevated fecal lactoferrin levels were associated with diarrhea in this group of children from southern Ghana.

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