Enteric Parasites and Enteroaggregative *Escherichia coli* in Children from Cañazas County, Veraguas Province, Panama

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Abstract. This study was designed to examine the height-for-age *z*-scores (HAZ), and the prevalence of intestinal inflammation, gastrointestinal infections with parasites, and enteroaggregative *Escherichia coli* (EAEC) in rural Panamanian children. Stool microscopy and polymerase chain reaction (PCR) testing for EAEC detected *Giardia lamblia* (32%, 32 of 100) and EAEC (13%, 11 of 87) in the study participants, respectively. Anthropometric analyses showed that those children who were > 12 months of age had lower HAZ scores (mean of –1.449) than the reference population. As a group, the children in the study 1 to 5 years of age did not show recovery from the previously mentioned decline in terms of their HAZ. The HAZ means of the children infected with *G. lamblia*, EAEC, and *Ascaris lumbricoides* were –1.49, –1.67, and –2.11, respectively. Furthermore, the study participants with *A. lumbricoides* and EAEC infections in the presence of lactoferrin showed another decrease of 0.19 and 0.13, respectively, in their HAZ means.

INTRODUCTION

Preschool child growth retardation is often an anthropometric indicator of the accumulated, long-term effects of repeated infections and food scarcity.[1–3] Longitudinal studies in various parts of the world have convincingly shown that periods of growth faltering align with patterns of infection at least as much as changes in diet.[1–3] In particular, enteric infections represent prominent causes of growth failure. Data from a prospective study of 45 Guatemalan highland village children, who were examined every week from birth to 3 years of age for intestinal infections, found weight loss and height arrest to be concomitant with infectious disease.[4] The results from another investigation of 716 rural Guatemalan children ranging in age from 15 days to 7 years quantified an epidemiological association between diarrhea and reduced growth rates.[5] Longitudinal investigations of 152 children from Keneba, The Gambia, who were seen once a month until 3 years of age, indicated that children suffering from gastroenteritis for a whole month would have had a weight gain of ~750 gm/month less and an increase in height of slightly over 4 mm/month less than that of healthy children.[2,3] Giardiasis, in particular, showed a significant negative effect on growth rate in these children.[2,3] Furthermore, a surveillance study from 1989 to 1998 of 119 children in a Northeast Brazilian shantytown revealed that helminthiases at 0 to 2 years of age was significantly associated with linear growth faltering and a further 4.6 cm shortfall at age 7.[6]

In addition to possessing long-term effects on linear growth, enteric infections may also adversely affect cognitive development. A follow-up assessment of 143 Peruvian children, who had been previously examined longitudinally from birth to 2 years of age for anthropometric measurements, parasitic infections, and diarrheal illness, showed that the children with severe stunting by the second year of life scored 10 points lower on the Wechsler intelligence scale for children-revised (WISC-R) than children without severe stunting.[7] Children with more than one episode of *Giardia lamblia* per year also scored 4.1 points lower than children with one episode or fewer per year.[7] Another study of 46 children in a Northeast Brazilian shantytown showed that diarrhea in the first 2 years of life was associated with reduced cognitive function 4 to 7 years later as assessed by a battery of five cognitive tests.[8]

Of particular interest to this study are enteroaggregative *Escherichia coli* (EAEC) strains, which are increasingly recognized as a cause of watery diarrhea, occasionally with blood and mucus, in children and adults worldwide.[9] The basic strategy of EAEC infection seems to comprise the adherence of self-aggregating bacteria, in association with a thick mucus layer, to the mucosal surface of human ileal and colonic tissues, followed by secretion of enterotoxins and cytotoxins, and induction of mucosal damage and inflammation.[10,11] Several studies have suggested that persistent EAEC infection may produce an inflammatory enteritis in children that is associated with growth impairment and malnutrition.[12,13] Fecal leukocytes are found in diarrhea patients with diffuse colonic inflammation but absent in non-inflammatory cases, and are most commonly identified in infectious diarrheas of invasive bacterial origin.[14] Although it is not specific for a particular pathogen, the detection of elevated concentrations of fecal lactoferrin, an iron-based glycoprotein expressed by activated polymorphonuclear leukocytes, serves as a sensitive and specific diagnostic marker for the majority of inflammatory diarrheas.[14–16]

This investigation was undertaken to assess the prevalence of gastrointestinal infections with parasites and EAEC in children < 5 years of age in certain impoverished, rural communities within the Cañazas District of Veraguas Province, Panama. In addition, this study aimed to determine whether the children living in these villages experienced growth shortfalls in association with enteric infections and/or intestinal inflammation.

MATERIALS AND METHODS

Study area and population. On June 30, 2010, every household with young children in four rural communities, Alto y Bala, Calle Lourdes, Polo Sur, and San José, in Cañazas

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County (Cañazas District, Veraguas Province) was visited (Figure 1). All eligible members of each existing dwelling were invited to participate in the study, given a plastic container to collect a stool sample, and asked to deliver the stool specimen to the San Francisco Javier Hospital, a rural hospital in Cañazas District, by the next day. Information concerning age, sex, anthropometry, clinical symptoms, defecation habits, hygiene customs, and dwelling characteristics were collected with the use of a questionnaire administered by trained medical technologists and biologists. From the 174 children invited to join the study, 120 provided stool specimens. Of these, 100 were < 5 years of age and were selected to be the study population. Informed consent was obtained from all parents or legal guardians of minors, with the name of the appropriate institutional review board having approved the project. This study was approved by the National Review Board, Comité Nacional de Bioética de la Investigación, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panamá City, Panamá (1985/CNBI/ICGES/).

Sample collection. At the time of collection, 87 fresh samples contained enough stool to divide each sample into two portions. The first portion was frozen at –20°C without any additives in a small plastic container. The second portion was stored with a 5% formalin solution in preparation for the fecal parasite concentration processing. The remaining 13 samples were processed with a 5% formalin solution only.

Immunochromatography. An immunochromatographic assay for the detection of *Cryptosporidium* spp. and *G. lamblia* was performed on all of the fresh samples using CERTEST CRYPTO-*GIARDIA* (Biotec S.L., Zaragoza, Spain), according to the manufacturer's instructions.

Fecal parasite concentration. Within a week of collection, the 100 fresh samples were processed by means of a 5% formalin-ethyl acetate sedimentation procedure at the recommended centrifugation speed and time of $500 \times g$ and 10 minutes, respectively.[17]

Kinyoun stain. The dry slides were initially fixed and stained with the modified Kinyoun's acid-fast stain (cold method).[17] Each slide was then left to air dry before being examined under the oil immersion objective lens at $100\times$ magnification. This procedure stained the oocysts of coccidia pink to red to dark purple over a blue background.[17]

DNA extraction. The DNA was extracted with QIAmp DNA Stool Minikit (Qiagen Inc., Germantown, MD) from 200 mg of each frozen stool sample, according to the manufacturer's instructions, with minor modifications as follows. Initially 650 µL of Buffer ASL were added to each sample, before vortexing and incubating it at 82.5°C for 5 minutes. After inhibitors had adsorbed to the InhibitEX matrix, 200 µL of nuclease-free water were added to each sample, and the samples were centrifuged for 2 minutes. The supernatant was pipetted into a new tube with 30 µL of proteinase K, to which 400 µL of Buffer AL were added. Each sample was vortexed and incubated according to the manufacturer's instructions. The 400 µL of ethanol (100%) were then added to each lysate. Subsequently, Qiagen's "Protocol: Isolation of DNA from Stool for Pathogen Detection" was followed to completion.

EAEC polymerase chain reaction (PCR) analysis. One PCR method amplified *aggR* gene using the appropriate forward (5′-CTAATTGTACAATCGATGTA-3′) and reverse (5′-ATGAAGTAATTCTTGAAT-3′) primers.[18] The other PCR assay targeted the *aatA* gene using the corresponding forward (5′-CTGGCGAAAGACTGTATCAT-3′) and reverse (5′-CAATGTATAGAAATCCGCTGTT-3′) primers.[18] These two plasmid-borne loci are highly specific for EAEC and have been validated in several studies.[18,19] Both PCR tests were performed in a final reaction volume of 25 µL. Each reaction tube was prepared by adding 4.3 µL of nuclease-free water, 1.6 µL (6.2 µM) of the forward primer, 1.6 µL (6.2 µM) of the reverse primer, 12.5 µL of Master Mix 2X (Promega M7502, Madison, WI), and 5 µL of DNA template. For *aggR*, the amplification was performed with an initial denaturation at
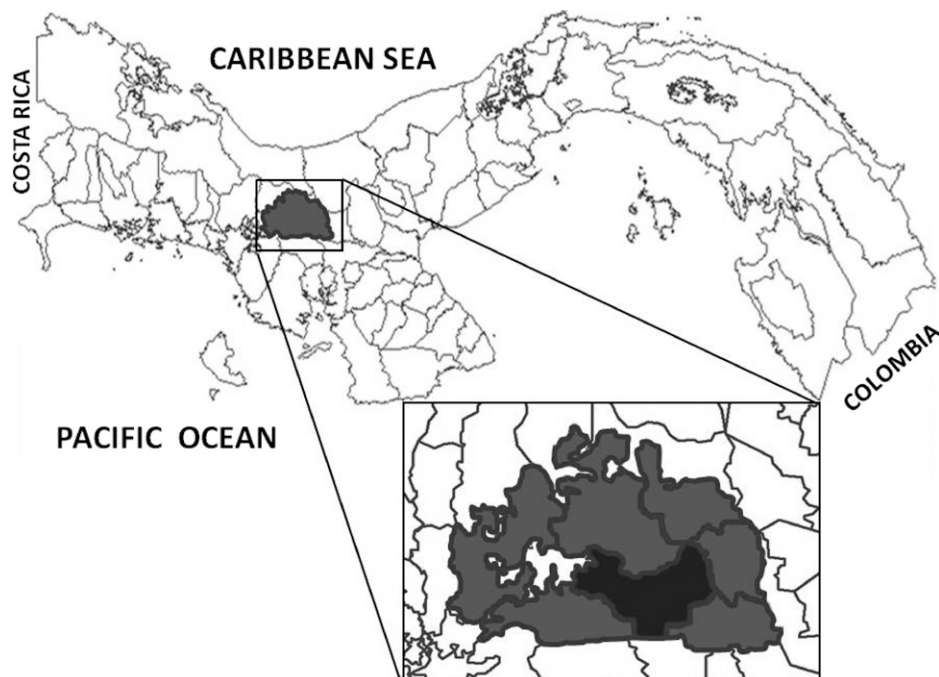

FIGURE 1. Map of the Cañazas District (projected region) showing the geographic location of the Cañazas County (darker zone).

95°C for 5 minutes followed by 30 cycles of 95°C for 30 seconds, 43.6°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The cycling conditions were the same for *aatA* with the exception of an annealing temperature of 53°C. An EAEC prototype strain 042 was included in each test as a positive control. Electrophoresis on 1.5% agarose gels and staining with 5 μL of ethidium bromide in 100 mL was used for visualization of amplified targets. A negative control, which consisted of the PCR mixture without the DNA template, was included in every run.

Fecal lactoferrin testing. An enzyme-linked immunosorbent assay (ELISA) test for the qualitative detection of lactoferrin, a marker of fecal neutrophils and an indicator of intestinal inflammation, was performed on the previously frozen stool samples using IBD-CHECK (Techlab, Blacksburg, VA), according to the manufacturer's instructions. The samples with positive results for the original ELISA were then assayed using IBD-SCAN (Techlab), a quantitative ELISA for measuring concentrations of fecal lactoferrin, according to the manufacturer's instructions. The qualitative and quantitative assays included positive and negative controls as per the manufacturer's instructions. Because the presence of breast milk is considered to give false positive results for fecal lactoferrin tests,[20,21] breastfeeding children who had positive lactoferrin tests were excluded from the data sets regarding presence or absence of intestinal inflammation.

Height-for-age *z*-scores (HAZ). Algorithms provided by the World Health Organization (WHO) by an SPSS version (SPSS, Inc., Chicago, IL) were used to calculate the corresponding indicators of attained growth standards. These algorithms were based on the 2006 WHO international growth reference.

Statistical analysis. The study participants' HAZ were grouped according to the presence or absence of fecal lactoferrin and enteric pathogens, and the detection of the three most common enteric pathogens, *Giardia lamblia*, *Ascaris lumbricoides*, and EAEC. To compare the HAZ means of the eight groups and test the null hypothesis that samples in these eight groups were drawn from populations with the same mean values, a one-way analysis of variance by GraphPad Prism (Charlottesville, VA), a two-dimensional graphing and statistics software published by GraphPad Software, Inc. (San Diego, CA), was used. A *P* value of less than or equal to 0.05 indicated statistical significance.

RESULTS

From the 174 children surveyed and invited to join the study, 120 provided stool specimens. Out of these, 100 were < 5 years of age and were considered to be the study population. Nonetheless, only 87 specimens were frozen and used for detection of fecal lactoferrin and EAEC. Our study population included 52 female and 48 male children ranging from 4.4 to 59.4 months of age with a median age of 35.1 months. On questioning, 47% (47 of 100) of the participants' parents reported living in a house with an earth floor and 52% (52 of 100) indicated that they had no electricity in their homes. The survey revealed that 93% (93 of 100) of the participants had access to a latrine and only 10% (10 of 100) had a private toilet. The region's aqueduct with unchlorinated water served as the sole water source for 86% (86 of 100) of the participants. There was an average of six people per household.

On further questioning, 37% (37 of 100) of the parents reported having been aware of prior intestinal parasitic infections in their children. Out of these 37 children, 65% (24 of 37) had received medical treatment of their illness at the time. A health care provider, in the form of a nurse, a nutritionist, or a physician, had informed the parents of 42% (42 of 100) of the participants that these children were underweight. Overall, high numbers of gastrointestinal symptoms, such as diarrhea, abdominal pain, and vomiting, were noted among these children. According to the parents, 50% (50 of 100) of the participants had one or more of the previously mentioned symptoms: 39% (39 of 100) of the participants were suffering diarrhea, 39% (39 of 100) had abdominal pain, and 23% (23 of 100) experienced vomiting close to the time of the survey conduction. Intestinal pathogens were detected in 61% (61 of 100) of the participating children, *G. lamblia* in 32% (32 of 100), *A. lumbricoides* in 14% (14 of 100), EAEC in 12.6% (11 of 87), *E. coli* in 12% (12 of 100), *Iodamoeba büstchlii* in 8% (8 of 100), *Entamoeba histolytica/Entamoeba dispar* complex in 4% (4 of 100), and *Ancylostoma duodenale/Necator americanus*, *Cryptosporidium* spp., *Cyclospora cayetanensis*, *Endolimax nana*, and *Hymenolepis nana* in 1% (1 of 100) each.

After analyzing the anthropometric measurements of the participants, their HAZ were grouped according to the presence or absence of fecal lactoferrin, as a marker of intestinal inflammation, and the most common enteric pathogens. The following information summarizes the number of children (in parentheses) in each of the eight resulted groups: children with no enteric pathogens (39); children with no enteric pathogens and no fecal lactoferrin (15); children with *G. lamblia* (32); children with *G. lamblia* and fecal lactoferrin (12); children with *A. lumbricoides* (14); children with *A. lumbricoides* and fecal lactoferrin (8); children with enteroaggregative *E. coli* (11). Study participants with no enteric pathogens and
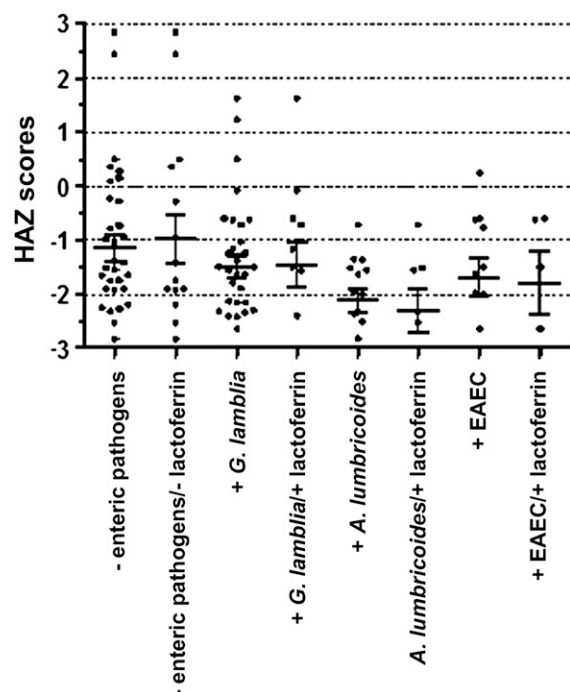

FIGURE 2. Height-for-age *z*-scores (HAZ) of study participants according to presence or absence of enteric pathogens and fecal lactoferrin, as a marker of intestinal inflammation.
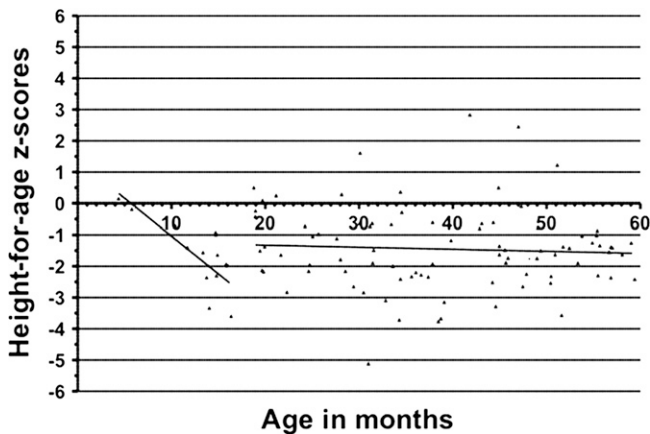
FIGURE 3.   Height-for-age z-scores (HAZ) of all study participants according to age in months. Out of 100 subjects, two were excluded from this analysis because anthropometric data were not collected from them.

no fecal lactoferrin had the highest HAZ mean of −0.97, closely followed by those children who presented no enteric pathogens with a HAZ mean of −1.14 (Figure 2). The rest of the data sets had lower means of HAZ, −1.49, −1.67, and −2.11 for *G. lamblia*, EAEC, and *A. lumbricoides* infections, respectively. Except for the HAZ mean of children infected with *G. lamblia*, which showed an increase of 0.03 when the presence of fecal lactoferrin was taken into account, the study participants with *A. lumbricoides* and *EAEC* infections showed a decrease of 0.19 and 0.13, respectively, in their HAZ means when the presence of intestinal inflammation was included as a factor. Overall, the HAZ means of the children with any of the three most common enteric infections and of those with fecal lactoferrin, but no intestinal pathogens, were all more than one standard deviation below the international reference value. Of note, even the study participants with no enteric pathogens and no fecal lactoferrin possessed a HAZ mean ~1 standard deviation below the mean of the reference population.

As shown in Figure 3, the HAZ of the study participants > 12 months of age experienced a decline (HAZ mean of −1.449) from the reference population. As a group, the children in the study 1 to 5 years of age did not show recovery from the previously mentioned decline in terms of their HAZ, suggesting that growth impairment may occur in early childhood and may persist over the first 5 years of life.

DISCUSSION

According to the Panamanian Ministry of Health, 76,417 new cases (21,779.5 of 100,000 children) of diarrheal illness occurred in children < 5 years of age in the Republic of Panama in 2007, and 76,032 new cases (22,009 of 100,000 children) ensued in the same population of young children in 2008.[22] Over a 16-month period in 2008 and 2009, a study on diarrhea, intestinal protozoan infection, and stunting in 373 preschool children of the comarca Ngäbe-Buglé, an impoverished, rural indigenous area within the Besiko District in western Panama, identified chronic protozoan infections (primarily *G. lamblia*) as a risk factor for low height-for-age.[23] Thus, it highlighted the link between chronic intestinal infection and stunting in a region in which 91% of the inhabitants

live in extreme poverty.[23] Our investigation assessed the prevalence of gastrointestinal infections with parasites and EAEC in children < 5 years of age in four rural communities within Cañazas County of Veraguas Province in central Panama. Given that the incidence of poverty in Panama's rural non-indigenous areas comprises ~50.7%, in contrast to 17.7% in urban areas, and Veraguas possesses a poverty level of 52%, the study participants were also among the poorest inhabitants of Panama.[24] Overall, 61% (61 of 100) of the children in the study presented with at least one enteric pathogen, mainly *G. lamblia* (32 of 100), *A. lumbricoides* (14 of 100), and EAEC (11 of 87), at the time of stool collection. Similarly, the investigation in the comarca Ngäbe-Buglé detected *Giardia* spp. in 18–34% of the collected fecal samples.[23] Our survey by direct observation and formally obtained data from the participants' parents indicated that the environmental hygienic conditions were poor in the study population. Houses with unchlorinated water supply and deficiencies in physical infrastructure, such as earth floor and inappropriate traditional latrines, were commonly registered. This information, which is suggestive of a widespread lack of clean water and sanitation in these communities, may explain the high prevalence of enteric infections in the study participants.

Furthermore, this research study intended to determine whether these young children presented arrested linear growth in association with enteric infections and/or intestinal inflammation. As mentioned previously, study participants with no enteric pathogens and no fecal lactoferrin, a marker of intestinal inflammation, had the highest HAZ mean of −0.97, closely followed by those children who presented no enteric pathogens with a HAZ mean of −1.14. The means of the HAZ of the children infected with *G. lamblia*, EAEC, and *A. lumbricoides* were −1.49, −1.67, and −2.11, respectively. It is important to mention that, to our knowledge, this investigation was the first report of EAEC detection in Panama, confirming the presence of this pathogen in this region of the country. In addition, the presence of intestinal inflammation also appeared to play a role in decreasing these young subjects' linear growth. The study participants with *A. lumbricoides* and EAEC infections in the presence of fecal lactoferrin showed another decrease of 0.19 and 0.13, respectively, in their HAZ means. The groups of children with the most frequently detected gastrointestinal pathogens and fecal lactoferrin tended to have lower means of HAZ than those study participants without enteric infections and without intestinal inflammation. Of note, even the study participants with no enteric pathogens and no fecal lactoferrin possessed a HAZ mean ~1 standard deviation below the mean of the reference population. This observation could be related to the fact that the study population lived in remote rural communities with high rates of poverty, which probably contributed to exposure to water and/or food contaminated with gastrointestinal pathogens and poor access to good diets and proper healthcare.

Because moderate stunting or chronic malnutrition is defined as an HAZ between 2 and 3 standard deviations below the international reference mean value, the previous findings could be indicative of a mild degree of stunting or chronic malnutrition within the study population. Given the high rate of gastrointestinal infections in these children, it is probable that many of the participants, regardless of whether they presented enteric pathogens at the time of the study, had been infected in the past. Overall, these infections may have

contributed to the low HAZ means reported. A cross-sectional analysis of the participants' anthropometric measurements by age revealed a decline of more than 1 standard deviation below the international reference mean and might denote mild stunting or chronic malnutrition in these children. These growth shortfalls may have been caused by a combination of factors, including an insufficient diet, the weaning process, and the introduction of water and/or food contaminated with gastrointestinal pathogens. It is important to note that the nutritional stage during this early period of life has been considered crucial for the linear growth.[25] A closely related situation has been described in Tanzania.[26] To improve the healthy growth of children, these authors recommend the promotion of exclusive breastfeeding during the first months of life and appropriate feeding and hygiene practices.[26]

Regarding the investigation's limitations, the study population may have been too small to provide accurate representation of each subset of children according to a particular gastrointestinal pathogen and the presence or absence of fecal lactoferrin. Another limitation comprised the use of only a single stool sample for detecting enteric pathogens, which was probably less accurate than repeated stool examinations. Furthermore, the study's enteric panel did not include viral or bacterial targets, which have been isolated frequently from children in other low-income settings associated with diarrhea or stunting.[27] In addition, as a cross-sectional study, this investigation did not provide a longitudinal assessment to track the children's linear growth and detect temporal patterns of enteric infections with a wider spectrum of bacteria and parasites. To truly describe growth faltering in relation to pathogens and lactoferrin measurements, a longitudinal study design would be necessary. Future studies in this area could test for other biomarkers of intestinal dysfunction, such as fecal alpha-1 antitrypsin or lactulose absorption, and its consequences, which include stunted growth in children.[28] Anemia may also be used as an indicator of stunting.[29,30] Nevertheless, it is urgent to call on health authorities to reinforce programs for the diagnosis, management, and prevention of child malnutrition and intestinal infections in this region.

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