The effect of previous Giardia infection on the presentation of new Giardia infections among children in Bangladesh.

Kirkby D. Tickell

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Committee:

Judd Walson

Ali Rowhani-Rahbar

Patricia Pavlinac

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Abstract

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Kirkby D. Tickell

Chair of the Supervisory Committee:

Dr. Judd Walson

Department of Global Health

Background: *Giardia Lamblia* (Giardia) is considered to be pathogenic in high-resource settings, but observational studies among children in resource-limited settings suggest an inverse relationship between Giardia and diarrhea. To test the hypothesis that an adaptive immune response prevents diarrhea in Giardia infections following a child's first exposure, we examined the association between first (compared to subsequent) Giardia infections and diarrhea among Bangladeshi children in their first two years of life.

Methods: Children were followed from birth to two years with bi-weekly home visits and stool was collected from children monthly and during episodes of diarrhea. Children with less than two unique Giardia infections were excluded from the primary analysis. The odds of diarrhea at a child's first Giardia detection was compared to the odds of diarrhea at presentation of subsequent Giardia infections using Generalized Estimated Equations with an independent correlation structure. Breast feeding, age, nutritional status,

maternal education and head of household education were considered potential confounding factors and maintained in the logistic regression model if their inclusion changed the odds ratio by more than 10%.

Results: Among 265 children enrolled in the parent study, the mean birth weight at enrollment was 2.8 kg (standard deviation [SD]=0.4kg) and exclusive breastfeeding was maintained for an average of 4.8 months (SD = 1.8 months). The incidence rate of Giardia infection was 30.6 per 1000 child months and 32.7% of all giardia infections were first detected in diarrheal stool. Forty children had at least two unique Giardia infections, and adjusting for head of household education and age of the child, the odds of diarrhea was not significantly different between first and subsequent Giardia infections (OR 1.22, 95% CI: 0.42 to 3.54, p-value: 0.71).

Conclusion: We did not find significant evidence to suggest that diarrhea was more common in children with a first episode of Giardia. However, the analysis may have been underpowered to detect smaller odds ratios than we observed. Future studies that include more frequent sampling of asymptomatic children, molecular diagnostics, and a larger sample size of multiply infected children are needed to explore this hypothesis further.

Introduction

Diarrheal disease remains a leading cause of child mortality in Asia and Sub-Saharan Africa, where children are frequently exposed to enteric pathogens. *Giardia Lamblia* (Giardia) is a pathogen traditionally associated with diarrheal disease and linear growth failure.[1] However, recent data suggest that Giardia may frequently be asymptomatically carried.[1-3] A host immune response to previous infection may moderate the pathogenicity associated with Giardia in infections following a child's first exposure.[3]

In high resource settings, Giardia is a common cause of traveller's diarrhea and Giardia has associated with diarrhea outbreaks related to contaminated water sources in both the United States and Europe.[4] Experimental human studies in the 1950's and 1980's also confirmed Giardia as a diarrheal pathogen among adults from the high-income countries.[5-7] However, in low-resource settings, the association of Giardia with diarrheal disease has come into question. [1, 8] The Global Enteric Multicenter Study (GEMS) sampled over 20,000 children with and without diarrhea in seven low and middle-income countries and found Giardia infection to be more common among asymptomatic controls than among diarrhea cases.[8] This inverse association between Giardia and diarrhea has also been observed in several cross-sectional studies conducted in low-resource settings. [1, 3, 8] In these endemic settings, prevalence estimates among children can reach 20%, and cumulative childhood exposure is thought to be nearly universal.[1, 3, 9] Given that multiple exposures to Giardia are common among children living in low-resource settings, it is plausible that an acquired immune response may alter the presenting symptoms of the infection.

Using previously collected data from a large prospective birth cohort in an urban slum in Bangladesh, we tested the hypothesis that diarrhea is more common when a child is first exposed to Giardia and that second or subsequent Giardia infections are

more likely to be asymptomatic. Specifically, we compared children presenting with diarrhea who had giardia isolated from their stool for the first time to children with isolation of asymptomatic Giardia infection during surveillance screening. We hypothesize that a child's first exposure to Giardia infection will result in diarrhea more frequently than subsequent infections, which will be more likely to be asymptomatic.

Methods

Study design: We conducted a case-control analysis nested in the Bangladeshi cohort of the interactions of Malnutrition & Enteric Infections: Consequences for Child Health and Development (MAL-ED) study, a multi-site prospective cohort study enrolling children at birth and following them until two years of age. The MAL-ED study aims to more clearly document the interaction between enteric infections and malnutrition. Between February 2010 and February 2012, children were visited twice a week, and their caregivers answered a feeding and health questionnaire, with particular attention paid to stool frequency and consistency. Routine stool samples were collected monthly in addition to symptomatic samples during episodes of diarrhea. All symptomatic and routine samples were tested for Giardia using GIARDIA II [TechLab Inc, USA], a commercially available enzyme linked immunosorbent assay (ELISA). [10] ELISA testing has demonstrated 97% sensitivity and 92% specificity for the detection of giardia when compared to microscopy.[11, 12] The parent study was approved by the Ethical Review Boards of at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b). This analysis was not human subjects research as all data were de-identified. . All analyses were conducted in STATA 13.

Population and setting: The study area, the Mirpur area of Dhaka city, is a large urban slum (111, 811 inhabitants) with a population density of 38,760 per square

kilometer. This area has a high incidence of diarrheal disease and Giardia is known to be endemic. [10] All children born in the catchment area during the enrollment period were eligible for enrollment, other than those with congenital conditions that effect nutritional status, including cardiac or renal malformations, cleft lip or palate, suspected cerebral palsy, and chromosomal disorders. For the primary analysis of this study, only children who had more than one giardia infection were included (Figure 1).

Exposure: The exposure of interest was first Giardia infection, detected by ELISA testing on fecal samples, as compared to all subsequent Giardia infections. The "subsequent infections" were defined by the first positive sample following a period of clearance. As there is no validated definition of clearance for Giardia, we defined clearance as two Giardia negative samples recorded at least one month apart, with no intervening positive samples. A second analysis was then conducted using the primary definition of clearance, but included all children with Giardia infections, even if no second infection was observed. Two final sensitivity analyses re-examined the data using a more conservative (three consecutive negative samples over at least six weeks, with no intervening positives) and a more liberal definition of clearance (two consecutive negatives with no time constraint).

Outcome and case definition: Diarrhea was defined as the passage of three or more watery stools in 24 hours, except in breast fed infants where the mother reporting an abnormal stool was considered diarrhea. At each episode of diarrhea, a stool sample was taken in the household (symptomatic sample). In addition, routine monthly stool samples were collected.

Cases were defined as children with diarrhea in whom Giardia was isolated. Controls were defined as children in whom Giardia was identified in a routine sample (asymptomatic infection).

Statistical Analysis: Pairwise t-tests and chi-square tests were used to compare means and proportions of clinical and sociodemographic characteristics of children with multiple Giardia infections to those with none and between children with multiple Giardia infections to a single infection. To compare the odds of diarrhea at a child's first ever detection of Giardia, to the odds of diarrhea at all subsequent Giardia infections, general estimating equation (GEE) with a logit link an binomial family was used. All analyses allowed for children with multiple Giardia infections to contribute to both the case and control pools. Therefore, was used for all models. Potential confounders, including baseline maternal and head of household education, breast-feeding at first infection, nutritional status at first infection, and age, were included stepwise in the model and maintained in the model if the estimate of effect changed by more than 10%. Confounding was evaluated in the primary model and covariates included in this model were included in both subsequent models without re-evaluation.

Results

Two hundred and sixty-five children were enrolled in the parent study and followed for twenty-four months. The mean birth weight at enrollment was 2.8 kg (standard deviation [SD]=0.4kg) and exclusive breastfeeding was maintained for an average of 4.8 months (SD = 1.8 months). By one year of age, 26.8% children were stunted and 16.2% were underweight. Socio-economic status was low; only 1% of mothers and 5% of head of households had completed high school.

Giardia infection was relatively common in the cohort and the incidence of infection increased as the children aged (Figure 2). The incidence rate of new infections, irrespective of symptoms, was 30.6 per 1000 child month. Among enrolled children, 75 (28.3%) had only one Giardia infection and forty children (15.1%) experienced two or

more Giardia infections. Fifty-four (32.7%) of all Giardia infections were first detected in a diarrheal sample. The mean age at first infection among children who had only one giardia infection was 16.8 months (SD = 5.8) and among those with at least two infections, the mean age of second infection was 14.3 (SD=5.3).

The 40 children with multiple Giardia infections were similar to the 75 children with one and 150 children with no Giardia infections over the study period across birth weight, and markers of socio economic status (Table 1). However, mean age at first infection was lower among those with multiple infections (mean: 14.3 months, SD: 5.3) than those with a single infection (mean 16.8 months, SD: 5.8). Children with one or more Giardia infection identified appear to have exclusively breastfed longer and weaned later than children with no Giardia identified.

Among children with multiple Giardia infections, the odds of diarrhea at first Giardia infection were similar to the odds of diarrhea at subsequent Giardia infection (OR: 0.93, 95%CI: 0.39 to 2.26, p-value: 0.88) among children with multiple infections. Although the odds ratio changed slightly after adjustment for age and head of household education (the two identified potential confounding factors), OR 1.22 (95% CI: 0.42 to 3.54, p-value: 0.71), the association remained not statistically significant. Subsequent sensitivity analyses did not alter the magnitude of association substantially (Table 3). Allowing all children with a single Giardia infection to contribute to the number of first infections identified resulted in an adjusted odds ratio of 1.18 (95% CI: 0.59-2.36, p-value: 0.65). After adjustment, the magnitude of effect increased but was not statistically significant (OR: 1.30, 95%CI: 0.62 to 2.72, p=0.48). Using a conservative clearance definition (three consecutive negatives with six weeks without Giardia identified) did not affect the result significantly (OR: 0.93, 95% CI: 0.14 to 6.33, p-value: 0.94). In addition, adjusting the definition of clearance to include two consecutive negative stools with no time constraint between them did not change the result (OR: 1.12 95%, CI: 0.41 to 3.09,

p-value: 0.82).

Discussion

Recent data from multiple observational studies in low-resource settings suggest Giardia is not always pathogenic in children. We sought to explore whether diarrhea was less common among children with repeated exposure to Giardia than in children exposed to Giardia for the first time. We did not observe an association between number of infections and odds of diarrhea and changing the definition of clearance in an attempt to more accurately differentiate first infection from subsequent infections did not alter this finding. However, design limitations such as timing of sampling and a limited number of children with multiple Giardia infections may have precluded our ability to exclude the possibility that children develop immunity to Giardia after the first infection. Interestingly, a high percentage (32.7%) of Giardia infections were first detected in diarrhea stool. This observation suggest that either there is association between Giardia and diarrhea among younger children, or a symptomatic response is more common in the acute phase of Giardiasis which maybe missed by cross-sectional studies that will oversample chronic cases relative to their incidence.

Although we did not find significant evidence to conclude a difference in disease likelihood with multiple infections, there is some evidence suggestive of an adaptive immune response. For example, a longitudinal study of Guatemalan children reported that the association between diarrhea and Giardia infection decreased with age, suggesting that the development of acquired immunity may protect against disease.[3] In addition, observations from two outbreaks five years apart in British Columbia suggested that adult residents known to have been previously infected with Giardia appeared to be largely protected against reinfection.[13] Experimental evidence has demonstrated a long-term, but variable, cellular response to Giardia infection. Under experimental conditions,

100% of adults exposed to repeated Giardia infection demonstrate antibody responses to infections.[5] In addition, when Belgian adults with a single known Giardia infection in the previous five years and adults with no known giardia exposure were experimentally exposed to Giardia antigens, the previously exposed group had a significantly larger CD4 T-cell mediated immune response. The same subjects were exposed to an array of other bacterial and yeast antigens with no significant difference in response between the groups.[14] This CD4 response may be important in modifying Giardiasis, as CD4 T-cell depleted mice appears unable to clear giardiasis while CD8 depleted mice are not similarly affected.[15, 16]

Although we did not evaluate immune response to Giardia infection, we presumed that an observed reduced diarrhea likelihood after more than one Giardia infections would be evidence of a functional immune response. Our study did not find significant evidence that supported this hypothesis, but was underpowered to detect small differences. There are several plausible biologic and study design reasons that we were unable to detect an association between repeated infection with Giardia and risk of diarrhea. It is possible that acquired immunity reduces the risk of infection rather than moderating symptoms after exposure. In addition, existing evidence for a functional immune response to Giardia largely comes from adults and older children. Very young children may not respond with a protective immune response and multiple exposures occurring at an older age may be required to develop a protective immune response. Given that children with multiple infections in our cohort were younger at their first infection than children with a single Giardia infection, it is plausible that multiply infected children tended to be too young to develop an adaptive immune response. Malnutrition was common in this cohort, and malnourished children also may not mount a protective immune response after Giardia infections. Finally, there is evidence that genotypic variation in Giardia assemblage is associated with pathogenicity and therefore it could

be Giardia assemblage, as opposed to host immune response, that explains the variable presentations of Giardia infections.[1]

This study had several notable strengths. The use of data from a large prospective birth cohort enabled careful examination of exposure over time. In addition, the high incidence of diarrhea, and the relatively high frequency of Giardia were notable. However, the study also had several limitations. Despite the size of the cohort and the number of events, the study may have been underpowered to detect meaningful differences in diarrhea associated with Giardia detection. In addition, the frequency and indications for stool sampling may have limited this analysis. If a child had diarrhea, their stool was sampled at that time, whereas children without diarrhea were only sampled monthly. Because the monthly sampling schedule was considerably shorter than the Giardia incubation period, some asymptomatic Giardia infections may have been missed, leading to misclassification of the exposure groups. Additionally, we cannot exclude the possibility that those children with asymptomatic Giardia infections on the day of monthly sampling go on to develop diarrhea. The sensitivity of the diagnostic test used to detect Giardia may have been another limitation. While the ELISA based test is a more sensitive detection method than traditional microscopy methods, molecular methods are a more sensitive method and could distinguish between repeated and new infections. Molecular techniques may also have enabled the detection of Giardia infections that were undetectable by ELISA. The use of ELISA may also have resulted in missing some first infections, leading to misclassification of the exposure groups, particularly in asymptomatic controls. Given the frequency of co-infection with other causes of diarrhea was common in diarrhea samples, we were unable to tease whether Giardia was the true cause of the diarrhea. Finally, while we have attempted to define new infections with various definitions of clearance, we cannot exclude the possibility that giardia persists in a chronic and undetectable state after the first infection, which

would limit our ability to distinguish between new and persistent infections.

In conclusion, we did not find significant evidence that children with repeated Giardia infection are at lower risk of developing diarrhea than children with a first Giardia infection. Future studies that include more frequent sampling, molecular diagnostics, and a cohort design are needed to exclude the possibility that children develop immunity to Giardia after first exposure to the pathogen.

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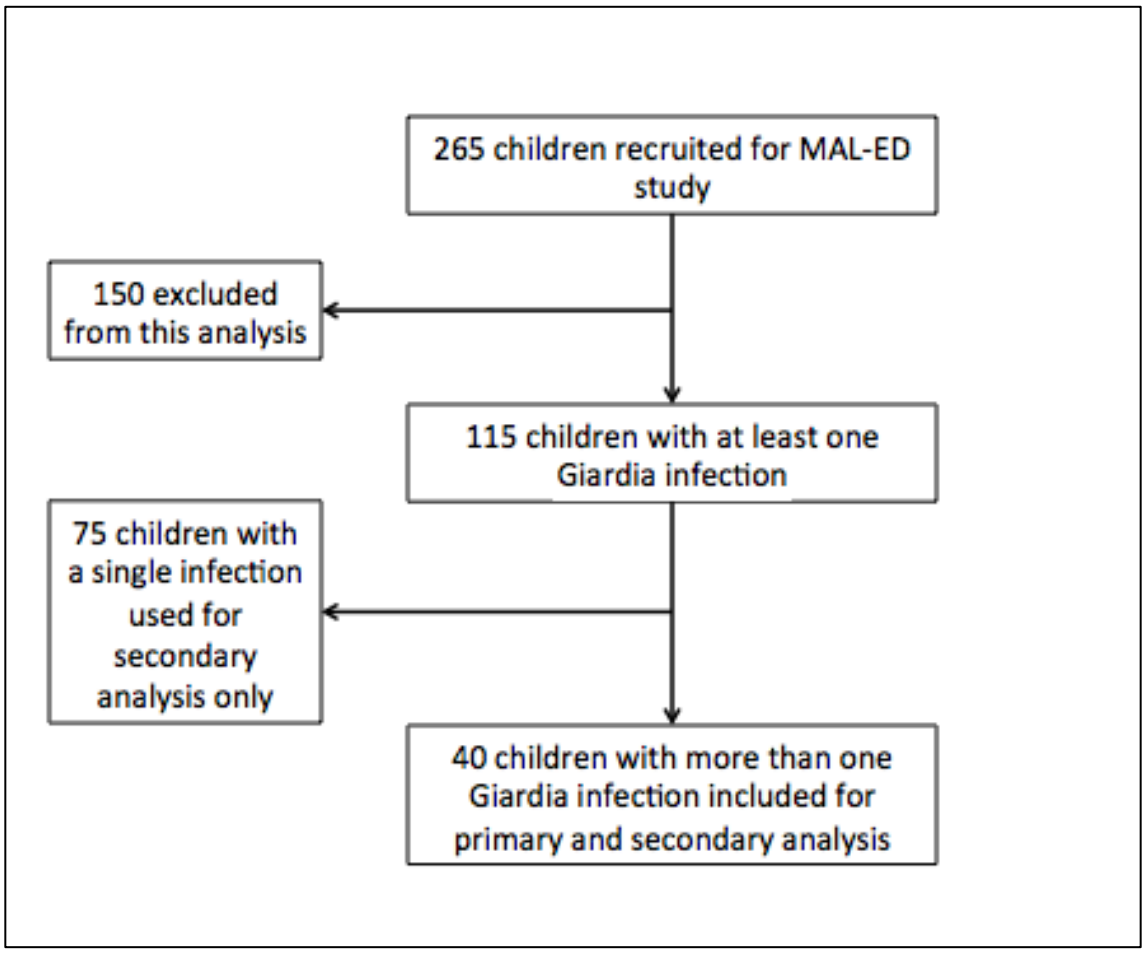

Figure 1: Population selection flow chart. The primary analysis includes only children with multiple Giardia infections. A secondary analysis also includes those with only a single infection.

	Giardia status by two years of age		
	Never detected	**Single infection***	**Multiple infections$^{\zeta}$**
N	150	75	40
Birth weight (kg)	2.8 (0.4)	2.9 (0.4)	2.7 (0.5)
Age at 1st giardia detection (months)	--	16.8 (5.8)	14.2 (5.3) $^{\zeta}$
Exclusive breast feeding			
at 4 months	103 (68.7%)	54 (72.0%)	31 (77.5%)
at 6 months	48 (32.0%)	27 (36.0%)	14 (35.0%)
Weaning age (months)	14.2 (12.5)	16.0 (11.4)	17.9 (12.2)
Nutrition at 12 months			
Stunted (LAZ <-2)	34 (22.6%)	21 (28.0%)	16 (40.0%)
LAZ	- 1.66 (0.89)	- 1.56 (1.02)	-1.87 (0.89)
Underweight (WAZ <-2)	25 (16.7%)	12 (16.0%)	7(17.5)
WAZ	-1.20 (1.00)	-1.17 (1.05)	- 1.31 (1.04)
Wasted (WHZ <-2)	6 (4.0%)	2 (2.7)	1 (2.5%)
WHZ	-0.78 (0.89)	-0.80 (0.88)	-0.39 (1.03)
Nutrition at 24 months			
LAZ	- 2.07 (0.97)	- 1.80 (0.90)	-2.12 (0.93)
WAZ	-1.65 (0.93)	-1.53 (0.93)	- 1.41 (1.09)
WHZ	-0.49 (0.98)	-0.50 (0.99)	-0.49 (1.04)
Maternal Education			
None	28 (19 %)	16 (21 %)	7 (18 %)
Primary	63 (42 %)	38 (51 %)	18 (45 %)
Some high school	58 (38 %)	28 (21 %)	14 (35 %)
High school graduate	1 (1 %)	0 (0 %)	1 (2.5 %)
Head of household			
Father	115 (76%)	54 (72.0%)	28 (70.0%)
Mother	0 (0.0%)	0 (0.0%)	0 (0.0%)
Grandmother	11 (7.3%)	3 (4%)	2 (5%)
Grandfather	19(12.7%)	17 (22.7%)	9(22.5%)
Sibling	1 (0.7%)	0 (0.0%)	0 (0.0%)
Other	1 (0.7%)	0 (0.0%)	1 (2.5%)
Head of household education			
None	52 (35 %)	26 (35 %)	11 (28 %)
Primary	46 (31 %)	30 (41 %)	20 (50 %)
Some high school	41 (28 %)	16 (22 %)	6 (15 %)
High school graduate	8 (5 %)	2 (2.7 %)	3 (7.5 %)

Table 1: MALED cohort characteristics classified by cumulative observed giardia infections before age two, under our primary definition of giardia clearance: 1 month and two consecutive samples without Giardia detection. All continuous variable are mean (SD), and categorical variable are N (%). * Indicates a p-value < 0.05 for the comparison between single infection and no infection and $^{\zeta}$ indicates a p-value of less than 0.05 for the comparison between multiple infections to a single infection.

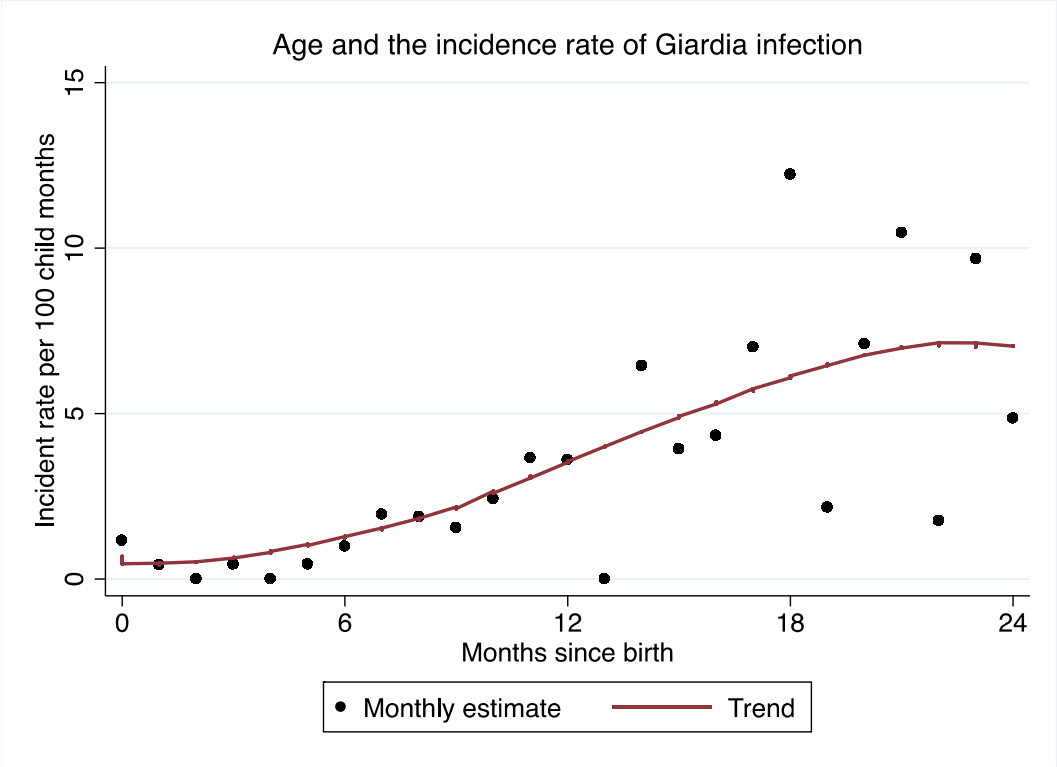

Figure 2: The incidence rate of Giardia infections per month among all children in the MALED cohort, Bangladesh. From the identification of second or higher infections the primary definition of clearance was used.

	Symptom status at Giardia detection among 40 children with ≥1 Giardia infection	
	Diarrhea	**Asymptomatic**
N	26	64
Age (months)	17.7 (4.3)	16.9 (5.5)
Breast feeding status		
EBF	0 (0%)	2 (3%)
Predominant	2 (8%)	0 (0%)
Partial	21 (81%)	55 (86%)
Weaned	3 (12%)	7 (11%)
Nutrition		
LAZ	-2.1 (0.9)	-1.9 (0.9)
WAZ	-1.4 (0.8)	-1.4 (1.0)
WHZ	-0.4 (0.7)	-0.6 (1.0)
Maternal Education		
None	4 (15%)	13 (20%)
Primary	12 (46%)	30 (47%)
Some high school	10 (31%)	21 (33%)
High school grad	2 (8%)	0 (0%)
Head of household education*		
None	12 (46%)	12 (19%)
Primary	9 (35%)	37 (58%)
Some high school	4 (15%)	10 (16%)
High school grad	1 (4%)	5 (8%)

Table 2: The host factors present in children at detection of giardia infection by diarrhea status at the time of sample collection. Numbers are mean (SD), unless the variable is categorical when N(%) is given. Length of infection is given for the primary definition of clearance. Variable marked with * are significantly different between the groups, test by a chi squared for categorical variables, and T-test for continuous measures.

Model	1st infections with diarrhea	Subsequent infections with diarrhea	Crude OR	Adjusted OR
Primary model	**13**	**17**	**0.93 (0.39 to 2.26)**	**1.22 (.42 to 3.54)**
Primary model including all 1st infections	43	17	1.18 (.59 to 2.36)	1.30 (.62 to 2.72)
Conservative clearance definition	5	8	0.55 (.12 to 2.47)	0.93 (.14 to 6.33)
Liberal clearance definition	15	18	1.02 (.43 to 2.39)	1.12 (.41 to 3.09)

Table 1: Logistic regression GEE models with adjustment for age and head of household education.