Pre-and Post-treatment Evaluation of Intestinal Inflammation in Giardia and Blastocystis Infected Children: A Community-based Study

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Abstract

Giardia intestinalis and *Blastocystis hominis* cause frequent infections in children in developing countries. However, the role of intestinal inflammation in their pathogenesis is still poorly understood**.** Faecal calprotectin (FC) level is used as an indicator of intestinal inflammation and neutrophil migration in the intestinal tract. The present study aimed to evaluate intestinal inflammation by measuring FC level among children infected with either *G. intestinalis* or *B. hominis* before and after treatment. Stool samples were collected from 282 children inhabiting a rural area in Egypt and examined microscopically for intestinal parasites. FC level was estimated in a group of children infected with *G. intestinalis* (n=12) or *B. hominis* (n=12) before and three weeks after receiving nitazoxanide (200 mg twice daily for three days) and compared to a control group (n=18) of parasite-free children. Cases of mixed infection were excluded. Nitazoxanide cure rate was 83% in both infections with a remarkable reduction of infection intensity in uncured children. Both parasites caused non-significant elevation in FC level compared to the uninfected children and the difference between pre and post-treatment estimations was not statistically significant. Elevated levels were observed before treatment in three children (two infected with *G. intestinalis* and one with *B. hominis*) who displayed normal post-treatment levels. Although *G. intestinalis* and *B. hominis* infections appear to cause no remarkable intestinal inflammation, they may induce abnormally elevated FC levels in a subset of children who may be suspected of having inflammatory bowel disease**.**

Introduction

Enteric protozoa constitute a diverse group of unicellular microparasites colonizing the intestinal tract of high vertebrate hosts including humans. They are transmitted through ingestion of cysts/ oocysts in contaminated food or water or through the person-to-person route. (Fletcher et al. 2012). Inadequate sanitary conditions facilitate the dissemination of infection in developing countries. Several species of intestinal protozoa are linked to gastrointestinal symptoms in humans worldwide. The flagellate *Giardia intestinalis* and the stramenopile *Blastocystis hominis* are the most frequent and affect mainly young children in endemic areas with high rates of reinfection (Cama and Mathison 2015). However, the pathogenesis and host-parasite interaction of these infections are still poorly understood (Podewils et al. 2004; Torgerson et al. 2014).

Intestinal colonization by *G. intestinalis* causes villous flattening or atrophy and microvillus shortening although it is frequently asymptomatic (Farthing et al. 2009). The role of intestinal inflammation in giardiasis is unclear. Experimental infection with *G. intestinalis* has demonstrated neutrophilic infiltration in the jejunum during colonization (Chen et al. 2013). However, the absence of significant mucosal inflammation was reported in *G. intestinalis* infected individuals (Campbell et al. 2004). *B. hominis* has been linked to irritable bowel syndrome (Yakoob et al. 2010). Histologic examination of the caecum and colon of a murine model of *B. hominis* showed marked inflammatory cell infiltration with oedema of the lamina propria and sloughing of the mucosal layer (Moe et al. 1997).

One of the commonly used markers of intestinal inflammation is faecal calprotectin (FC). It is a 36.5 kDa oligomer of one heavy and two light subunits. It constitutes 5% of the total protein and 60% of the cytosolic protein in neutrophils and it is also found in monocytes and macrophages (Pathirana et al. 2018; Haisma et al. 2019). Assessment of FC level plays a pivotal role in the workup evaluation of patients with chronic diarrhea (Carrasco-Labra et al. 2019). It is mainly used as a marker for primary screening and follow-up of patients with inflammatory bowel diseases with 95% sensitivity and 91% specificity (Henderson et al. 2014; van Rheenen et al. 2010). An elevated level of FC is also found in allergic colitis, drug-induced enteropathy, and colorectal cancer (Hanevik et al. 2007; Damms and Bischoff 2008; Schoepfer et al. 2008). Elevated levels are also induced by gastrointestinal pathogens (Lam et al. 2014). The contribution of intestinal parasitic infections to intestinal inflammation and changes in FC levels in endemic areas is unclear. The present study aimed to estimate FC level before and after treatment of *G. intestinalis* and *B. hominis* infection among children residing in an Egyptian rural area.

Material And Methods

Study subjects

A cross-sectional community-based survey was carried out to detect *G. intestinalis* and *B. hominis* infection among 282 children aged 6-12 years residing in a rural area in Alexandria, Egypt. The study was approved by the Research Ethics Committee of the Medical Research Institute, Alexandria University, Egypt. Oral informed consent for participation in the study was obtained from parents /guardians of children.

Collection and examination of stool samples

Fresh stool samples were collected and examined using the formalin ethyl acetate concentration (FEAC) technique to detect intestinal parasitic infection (Garcia 2016). The intensity of *G. intestinalis* and *B. hominis* infection were estimated by the number of organisms/10 HPF as follows: few ≤ 2; moderate 3-9 and heavy ≥ 10.

Stool samples of *G. intestinalis* and *B. hominis* positive children showing no other detectable parasites by FEAC were further examined using Kato-Katz and modified Ziehl-Neelsen stain to exclude associated helminthic and coccidial infections respectively. Fresh stool samples microscopically negative for *B. hominis* were cultured in Jones' medium and examined 48-72 hours later to confirm the absence of *B. hominis* infection (Zhang et al. 2012).

FC level

Children infected with *G. intestinalis*, a corresponding group of children with *B. hominis* infection, and a control group of parasite-free children were subjected to history taking, clinical examination, and estimation of faecal calprotectin level. Children with mixed parasitic infection, including combined *G.*

intestinalis and *B. hominis* infection, children with known chronic diseases such as diabetes mellitus and chronic kidney diseases, and those who have received antibiotics or anti-inflammatory drugs within two weeks before the study were excluded. Infected children received nitazoxanide in a dose of 200 mg twice daily for three days under medical supervision. Treated children were revaluated three weeks post-treatment for improvement of symptoms, parasitological cure and FC level.

FC level was estimated using sandwich enzyme-linked immune- sorbent (ELISA) Calprest R kit (Eurospital, Trieste, Italy; REF:9031). Fresh stool samples were mixed with the extraction solution provided by the kit and stored at -20 ^{o} C for later measurement. Levels above 150 mg/kg stool were considered abnormally elevated (D'Angelo et al. 2017)

Statistical analysis

Data analysis was performed using the statistical package for social sciences (SPSS, version 17.0, 2006; SPSS Inc., Chicago, Illinois, USA). Chi-square test was used to study age and gender distribution of infection. Improvement of symptoms after treatment was analyzed by the Marginal Homogeneity Test. FC level analyzed using Mann Whitney test for comparison between two groups and Kruskal Wallis for more than two groups and Wilcoxon signed ranks to compare measurements before and after treatment.

Results

Out of 282 examined children, 108 children (38.2%) were infected with one or more intestinal parasites. *B. hominis* was the most frequent (91 children, 32.2 %) followed by *G. intestinalis* (21 children, 7.4%). Among children infected with *G. intestinalis* (n=21), nine children (42.9%) had mixed infection with *B. hominis*. Parasites detected in the study population and the children eligible for estimation of FC level are shown in figure 1. There was no significant age or gender differences in both *G. intestinalis* and *B. hominis* infections (Table 1).

Cure rates according to intensity of infection were assessed in 12 *G. intestinalis* and 12 *B. hominis* infected children three weeks after nitazoxanide treatment. The overall cure rate for each parasite was 83.3%. Treatment failure was recorded in two (out of 5) children with heavy *Giardia* infection and two (out of 8) children with heavy *B. hominis* infection (figure 2). All uncured children showed a reduction in infection intensity.

All treated children were complaining of abdominal pain before receiving nitazoxanide. Significant improvement occurred after receiving nitazoxanide where nine *G. intestinalis* infections (75%) and 11 *B. hominis* infections (91.7%) became asymptomatic ($p< 0.05$) (Table 2). Abdominal pain persisted in children with uncured *G. intestinalis* infection and in one of the two children with uncured *B. hominis*. However, abdominal pain was also recorded in one child after elimination of *G. intestinalis.*

Among *G. intestinalis* infected children, FC levels ranged from 6- 460 mg/kg. The median value was slightly higher than that of the control (50 versus 40 mg/kg, $p>0.05$). Only two *G. intestinalis*-infected

children displayed abnormally elevated levels. After nitazoxanide treatment, the level ranged from 2.2-820 mg/kg and the median value was non-significantly elevated compared to the pretreatment value (70 versus 50 mg/kg). Elevated post-treatment levels were recorded in two children, one of them continued to excrete few *G. intestinalis* cysts and the other had a slightly elevated level (160mg/kg).

Among *B. hominis* infected children, FC level showed a wide range (2-900 mg/kg) and the median value was lower than that of the control (18.6 and 40 mg/ kg respectively) with a non-significant difference. Abnormally elevated levels were found in three children. After nitazoxanide treatment, lower median FC level was observed (9.4 versus 18.6 mg/ kg, P = 0.06) and all children displayed normal FC level ranging from 60-100 mg/kg. Of note, *G. intestinalis* infected children displayed significantly higher post-treatment FC levels than *B. hominis* infected children (p 0.01) (figure 3).

Based on our objective to estimate the changes in fecal calprotectin level (mg/kg) in *B. hominis* infected children three weeks after nitazoxanide treatment, we calculated post-hoc power analysis and concluded 90% power of our study to determine the median difference of fecal calprotectin before treatment (18.6, Range: 2-900) and after treatment (median 9.4, range 0.60 – 100.0). Power analysis was calculated after non-parametric correction of the Independent sample t-test at 0.05 significance level using R software.

Discussion

Giardiaintestinalis and *B. hominis* infections are common worldwide. Rates of infection in the current study are relatively higher than those reported in developed countries (Harhay et al. 2010) but concur with a previous study in Egypt indicating a constant unchanging pattern of infection (Omaran et al. 2013). Neither age nor gender-related differences were detected suggesting equal exposure and/or susceptibility to infection. Generally, dissemination of intestinal protozoa reflects the sanitary, hygienic, and socio-economic standards of the population as they are transmitted primarily through the faeco-oral route (Fletcher et al. 2012).

The parasitological and clinical cure rates of nitazoxanide observed in the present study agree with previous reports which reported that nitazoxanide rapidly and efficiently reduced the duration of illness in *G. intestinalis* infected patients even before the parasitological cure was achieved (Rossignol et al. 2005; Escobedo and Cimerman 2007; Ordóñez-Mena et al. 2018). Treatment failure that was observed in four children may be attributed to high infection intensity and/or variable drug sensitivity among genetically different isolates. An in vitro study illustrated that nitazoxanide had highest efficacy against *Blastocystis* subtype 1 isolate at 0.1 mg/ml. However, at an increased concentration, subtype 5 was more sensitive to the treatment with an efficacy of 95.1 % (Rossignol et al. 2012; Girish et al. 2015).

Despite significant improvement of symptoms in nitazoxanide-treated children, abdominal pain persisted after clearance of *G. intestinalis* infection in one child. A similar observation was previously reported after treatment of *G. intestinalis* infection acquired in a waterborne outbreak. Possible explanations include bacterial overgrowth, prolonged lactose intolerance, or post-infectious functional disorder (Hanevik et al. 2007).

High calprotectin level in feces is an indicator of neutrophil migration towards the intestinal tract and it correlates with the degree of intestinal mucosal inflammation (D'Angelo et al. 2017). Data of the present study indicate that *G. intestinalis* and *B. hominis* infection had no significant impact on calprotectin levels in children. The present result is consistent with a recent study in rural Bangladesh demonstrating a non-significant association between the presence of *G. intestinalis* in stool and high FC among 203 children (George et al. 2018). Among Guatemalan children, neither the presence nor the intensity of *G. intestinalis* infection had an association with FC (Soto-Méndez et al. 2016). Fecal myeloperoxidase, another marker of neutrophil inflammation, was found to be unexpectedly lower in children with *giardias*is (McCormick et al. 2017). Other authors suggested that *G. intestinalis* infection is classically characterized by little or no inflammatory intestinal response (Roxström-Lindquist et al. 2006).

Regarding *B. hominis,* a recent study in Italy found no association between *B. hominis* colonization and FC level in both symptomatic and asymptomatic patients (Sulekova et al. 2018). Nieves-Ramírez et al. (2018) detected even a significantly lower calprotectin concentration among *B. hominis* colonized compared to non – colonized persons. In contrast to these studies, Tibble et al. (2002) examined eight children with infectious diarrhea, with seven having confirmed giardiasis and observed excess FC level.

The extent of gut inflammation may be related to the clinical presentation of the studied patients. All children included in the present study were complaining of abdominal pain and none had diarrhea. Sulekova et al. (2018) found that FC level in *B. hominis* infection was significantly higher in patients with diarrhea than in the asymptomatic group or those with other gastrointestinal symptoms. They suggested the use of calprotectin as a marker for *B. hominis* pathogenicity. Furthermore, the present study was carried out in an endemic area with possibly high rates of reinfection. Reduction in inflammatory response and protection against disease severity probably occur with recurrent *G. intestinalis* infection (Kohli et al. 2008).

Abnormally elevated FC levels were observed in the present study in few children (two infected with *G. intestinalis* and three with *B. hominis*. Similarly, A study in Iraq reported FC positive test results in 10 out of 47 *G. intestinalis* cases and in one out of 31 *B. hominis* positive samples (Salman et al. 2017). In an Australian study, mild inflammation of the duodenal mucosa was observed in only 3.7% of 567 *G. intestinalis* patients (Oberhuber et al. 1997).

The parasite genotype may determine the degree of intestinal inflammation. *G. intestinalis* assemblage B has been linked to extensive mucosal damage and infiltration by inflammatory cells while *G. intestinalis* assemblage A does not induce overt intestinal pro-inflammatory responses and may attenuate intestinal neutrophil recruitment (Campbell et al. 2004; Cotton et al. 2014). Differential induction of the anti-inflammatory cytokine, IL-10 by *Blastocystis* subtypes was also reported (Yakoob et al. 2014).

Post-treatment FC level was higher in *G. intestinalis* infected than in *B. hominis* infected children. Moreover, a slightly elevated FC level was observed in a *G. intestinalis* infected child despite clearance of

infection. Slow recovery of mucosal inflammation was previously reported following treatment of *G. intestinalis* infection acquired in an outbreak (Asseman et al. 1999). A histopathological study using murine models demonstrated increased neutrophils infiltration and myeloperoxidase activity throughout the gut, 35 days after *Giardia* elimination (Campbell et al. 2004). On the other hand, the normal post-treatment FC level in *B. hominis* infected children denotes recovery of all affected children.

A limitation in the study was the difficulty in recruiting children with single *Giardia* infection; associated *Blastocystis* infection was detected in 9 out of 21 *Giardia* infected children (42.9 %) using the direct microscopic method and culture in Jones's medium. Due to the small number of children with a single infection who were initially examined for FC level, the pre and post-treatment comparison was performed to confirm the study findings.

In conclusion, both *G. intestinalis* and *B. hominis* infections appear to cause no remarkable intestinal inflammation. However, they may elicit abnormally elevated levels in a subset of children who may be erroneously diagnosed as having inflammatory bowel disease.

Declarations

Funding

No fund was obtained for the execution of the study.

Conflict of interest

No conflict of interest to declare.

Ethical approval

The study was approved by the Research Ethics Committee of the Medical Research Institute (MRI), Alexandria University (IORG 0008812). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments.

Consent to participate

Verbal consent was obtained from all participants (parents /guardians of children).

Consent for publication

All authors agree for publication.

Availability of data and materials

The data supporting the findings of this study are contained within the manuscript. The raw data are available by the corresponding author when requested.

Code availability

Not applicable

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Tables

Table 1 *Giardia* and *Blastocystis* infection among the examined children according to age and gender.

	No. examined	*G. intestinalis*		*B. hominis*	
		No.	%	No.	%
Age (years)					
6 – 8	156	10	6.4	46	29.5
9 – 10	74	6	8.1	26	35.1
11 – 12	52	5	9.6	19	36.5
P		0.748		0.559	
Gender					
Male	140	13	9.3	44	31.4
Female	142	8	5.6	47	33.1
P		0.243		0.764	

p: p value for comparing between the different categories. Diagnosis was based on microscopic examination of stool samples using FEAC technique.

Table 3 Improvement of symptoms in *Giardia* and *Blastocystis* infected children three weeks after nitazoxanide treatment

Symptoms	*G. intestinalis* (n = 12)		*B. hominis* (n = 12)	
	No.	%	No.	%
Before treatment				
Asymptomatic	0	0.0	0	0.0
Symptomatic	12	100.0	12	100.0
After treatment				
Asymptomatic	9	75.0	11	91.7
Symptomatic	3	25.0	1	8.3
^{MH}p	0.003*		0.001*	

^{MH}p: p value of Marginal Homogeneity Test

*: Statistically significant at $p \leq 0.05$

Figures

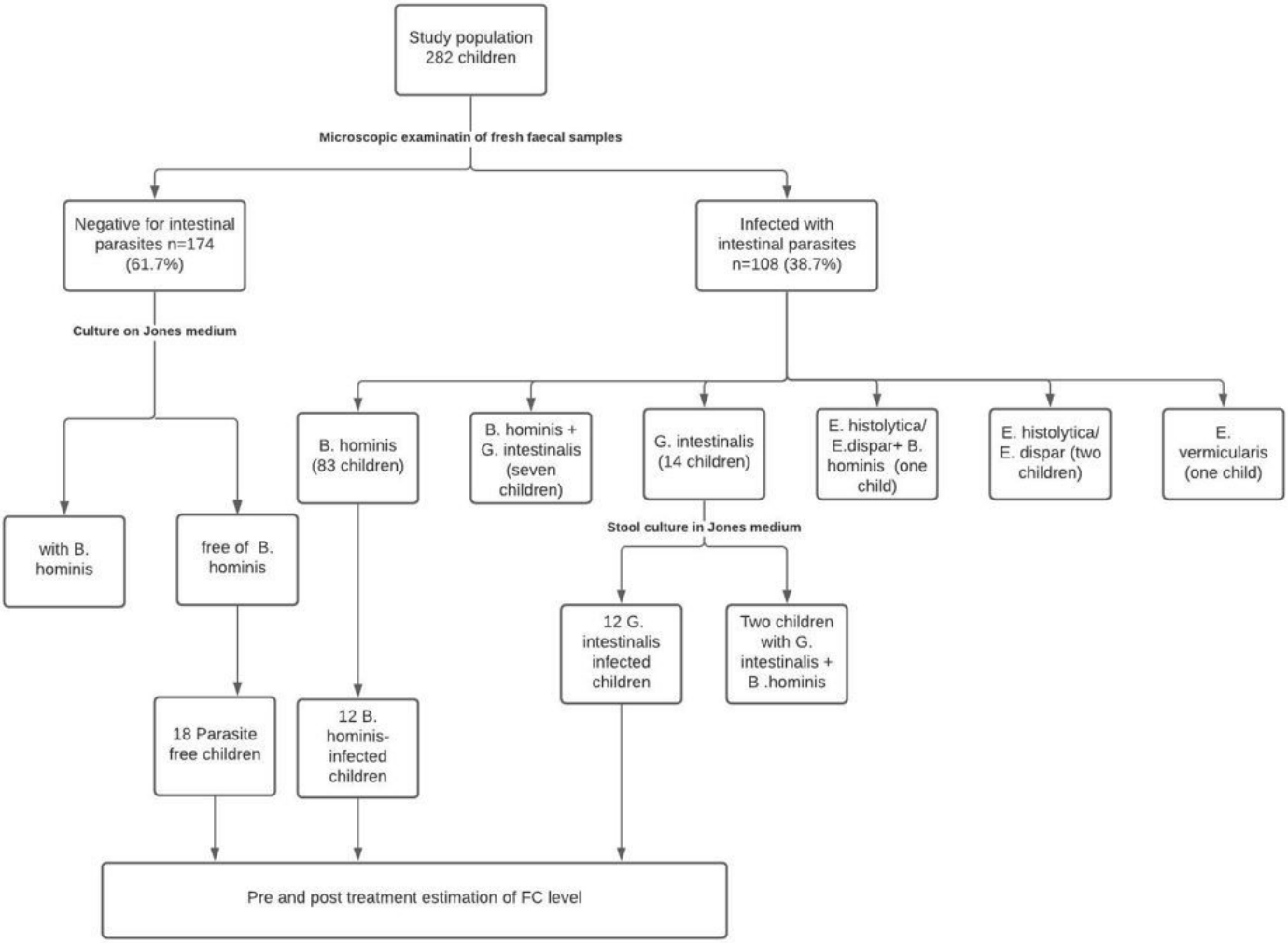

Figure 1

Flow chart showing parasites detected in the study population and the groups evaluated for FC level

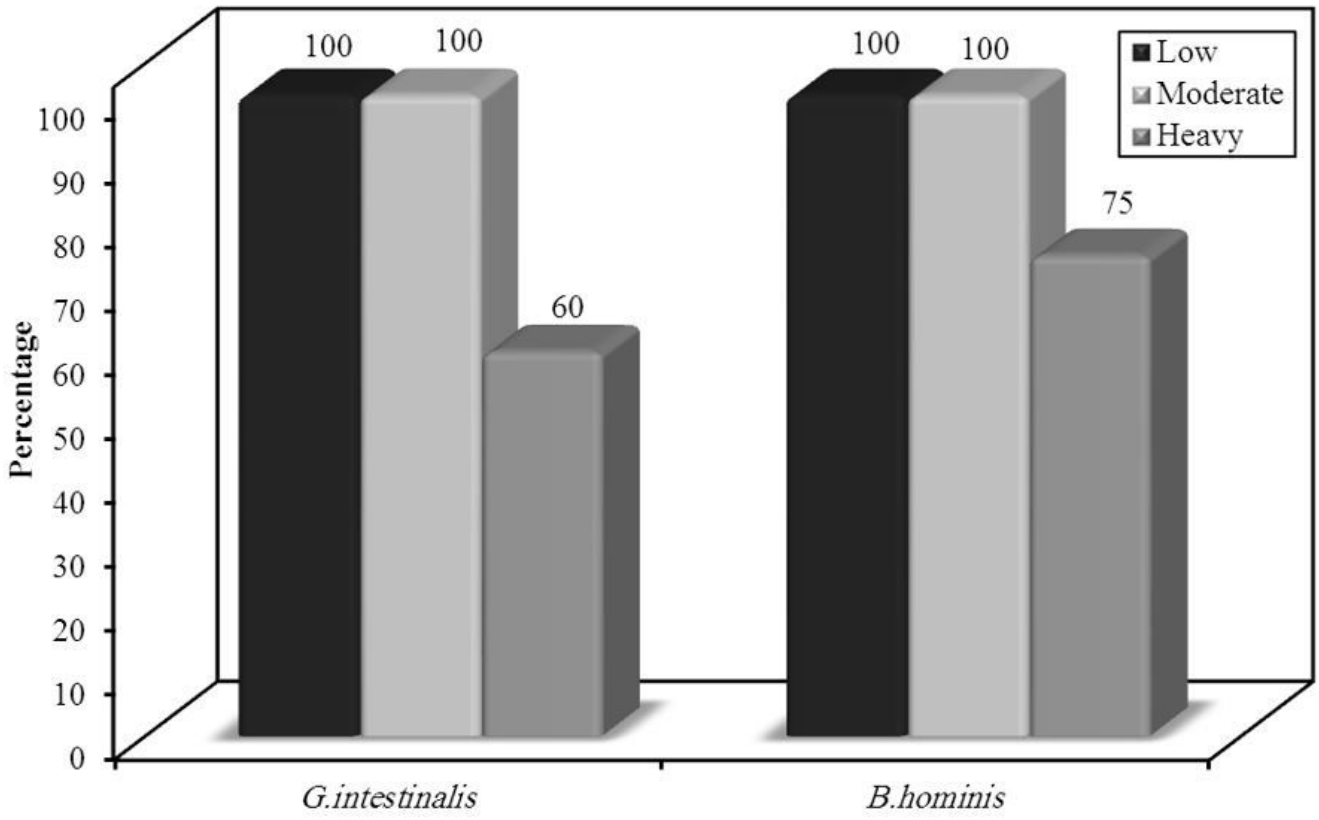

Figure 2

Nitazoxanide cure rate in G. intestinalis and B. hominis infected children according to intensity of infection

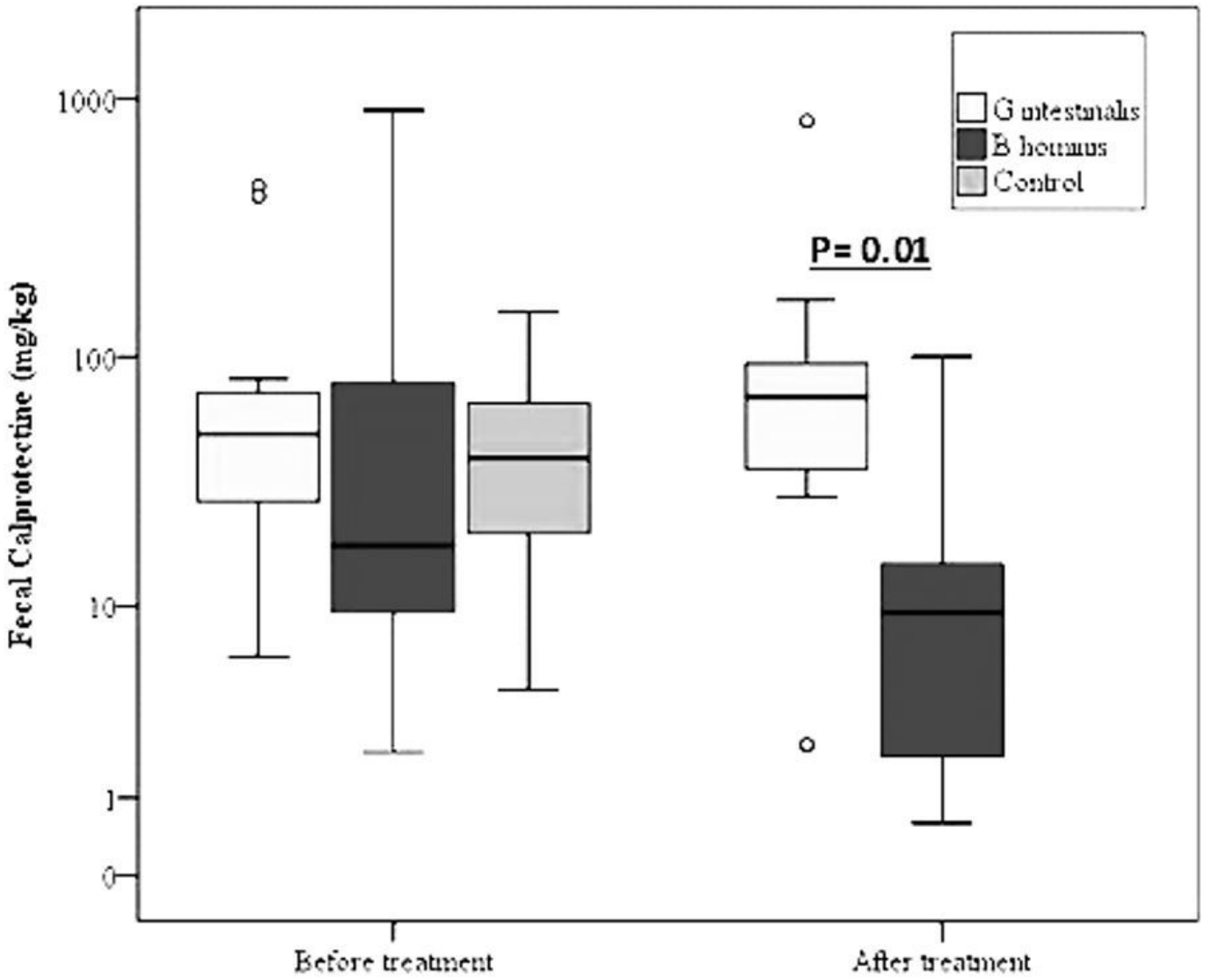

Figure 3

FC levels (mg/kg) in children infected with Giardia (n=12) and Blastocystis (n= 12) compared to healthy controls (n=17) and the corresponding post-treatment levels in infected children