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Immune response markers in sera of children infected with *Giardia duodenalis* AI and AII subassemblages

Flávia T.F. Pacheco[a], Silvia S. Carvalho[a], Luciana S. Cardoso[a], Lorena S. Andrade[a], Gisele M.T. das Chagas[a], Daisy C. Gomes[a], Cristiane F. Mercês[a], Felipe C. Rocha[a], Luciano K. Silva[b], Neci M. Soares[a], Márcia C.A. Teixeira[a,*]

[a] Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, Brazil
[b] Fundação Oswaldo Cruz, Centro de Pesquisa Gonçalo Moniz, Salvador, Brazil

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ABSTRACT

In this study, we evaluated serum markers of immune responses in children infected with *G. duodenalis* and compared them with the characterized parasite isolates. The reactivity indexes (RI) of IgG (1.503 ± 0.819) and IgA (2.308 ± 1.935) antibodies were significantly higher ($P < 0.001$) in infected children than in non-infected children. There were also statistically significantly higher serum levels ($P < 0.05$) of IFN-γ (393.10 ± 983.90 pg/mL) as well as serum (30.03 ± 10.92 µmol/L) and saliva nitric oxid derivatives (NO_x) (192.4 ± 151.2 µmol/L) in children infected with *G. duodenalis* compared to the group of non-parasitized children (127.4 ± 274.30 pg/mL; 25.82 ± 7.74 µmol/L and 122.5 ± 105.90 µmol/L, respectively). Regarding the characterized genetic variants of *G. duodenalis* and the immune response profiles, no differences were observed in terms of antibody reactivity or levels of serum cytokine and NO_x among children infected with AI or AII subassemblages. The elevated levels of IFN-γ and NO_x indicate that *G. duodenalis* intestinal infection in humans induces a cellular immune response detectable at the systemic level. Moreover, no significant differences in the antibody reactivity profile or the cytokine and NO_x production in the sera of children infected with AI or AII *G. duodenalis* variants were observed, suggesting that subtypes of the parasite do not influence the immune response profile.

1. Introduction

G. duodenalis (syn. *Giardia intestinalis* and *Giardia lamblia*) is one of the most common agents of diarrhea in the world, with more than 200 million people infected annually (Ankarklev et al., 2010; Feng and Xiao, 2011). The parasite colonizes the small intestine but does not penetrate the epithelial barrier. Infected individuals may be asymptomatic or may develop gastrointestinal symptoms, characterized by acute or chronic diarrhea, epigastric pain, nausea, vomiting, weight loss, abdominal distension and flatulence (Cama and Mathison, 2015; Cotton et al., 2011; Minetti, 2016). In children, especially in malnourished ones, infection can also lead to impairment in physical and cognitive development (Berkman et al., 2002; Yentur Doni et al., 2015). It is not fully understood why some individuals develop clinical giardiasis while others remain asymptomatic. The severity of the disease is probably determined by the interaction between the parasite genetic diversity, the host's immune response and nutritional status, the nature of the intestinal microflora and the presence or absence of other pathogens (Fahmy et al., 2015).

Several lines of evidence suggest that the immune response against *Giardia* involves innate and adaptive mechanisms (Lopez-Romero et al., 2015). Antibodies, mainly secretory IgA, contribute to protective immunity in both human and animal models (Eckmann, 2003; Langford et al., 2002; Stark et al., 2009). However, other studies suggest the presence of additional mechanisms, independent of antibodies, in the elimination of *G. duodenalis* (Davids et al., 2006; Singer, 2015). The cellular immune response plays an essential role in the elimination of *Giardia* infections. In humans and animals, the decrease in $CD4^+$ T cell levels contributes to the development of chronic giardiasis, although the mechanisms are poorly understood. It is possible that $CD4^+$ T cells induce B cells in the production of antibodies or that other mediators are involved (Singer, 2015). Studies using animal models have reported elevated serum levels of different cytokines, including TNF, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, IL-17, IL-22 and IL-23 (Abdul-Wahid and

* Corresponding author at: Av Barão de Jeremoabo, nº 147, Campus Universitário de Ondina, Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, Bahia, 40170-115, Brazil.
E-mail address: marciat@ufba.br (M.C.A. Teixeira).

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Faubert, 2007; Grit et al., 2014; Kamda and Singer, 2009; Larocque et al., 2003; Solaymani-Mohammadi and Singer, 2011; Zhou et al., 2007). Although the role of cytokines in parasitic infections has been widely investigated in animal models, there are limited studies in human giardiasis.

Nitric oxide (NO) is synthesized by immune cells and by the intestinal epithelium, presenting antimicrobial actions on a wide range of bacteria and parasites (Brunet, 2001; Eckmann, 2003; Fang, 1997). NO inhibits the proliferation of *G. duodenalis* trophozoites as well as the encystation and excystation processes (Eckmann et al., 2000) *in vitro*. Despite the evidence for the protective role of NO in giardiasis (Zarebavani et al., 2017), understanding of the effect of this mediator in the immune response against *G. duodenalis* in humans is still limited.

Although *G. duodenalis* is considered a unique species, advances in molecular biology techniques have revealed that this protozoan is a complex species with genetic diversity but with morphologically identical, exhibiting adaptations to different hosts (Cacciò et al., 2002; Thompson, 2004). Therefore, genetically related *G. duodenalis* isolates were grouped into 8 major assemblages: A, B, C, D, E, F, G and H as well as their respective subassemblages (Plutzer et al., 2010; Thompson, 2004; Thompson and Ash, 2016). Feng and Xiao (2011), in a review of molecular characterization of more than isolates of *G. duodenalis,* showed that, almost exclusively, assemblages A and B were associated with infection in humans. Some researchers suggest that genetically distinct parasite isolates differ in their immunopathological characteristics and in producing symptoms. While some studies show that assemblage A is more highly associated with cases of symptomatic giardiasis (Fouad et al., 2014; Haque et al., 2005; Sahagún et al., 2008), other studies have found that infections with assemblage B were significantly correlated with clinical symptoms (ElBakri et al., 2014; Ghoshal et al., 2016; Mohammed Mahdy et al., 2009; Molina et al., 2011). However, more recent reports found no association between genetic variants and clinical manifestations (Alyousefi et al., 2013; García-Cervantes et al., 2017).

Different mechanisms have been suggested to explain the variation in the clinical manifestations and the immune response elicited by *G. duodenalis*. Among these mechanisms, it is assumed that genetically different isolates of the parasite induce an assemblage-specific immune response (Babaei et al., 2016; Solaymani-Mohammadi and Singer, 2011). Therefore, the objective of this study was to evaluate the production of innate (NO), humoral (IgG, IgA, IL-5 and IL-6 cytokines), cellular (IFN-γ and TNF) and regulatory (IL-10) mediators in children infected with *G. duodenalis*, as well as to associate the levels of these immunological parameters with the *G. duodenalis* assemblages and subassemblages identified.

2. Materials and methods

2.1. Origin of samples

This cross-sectional study was conducted in children, with ages ranging yr, who were examined at the public Laboratory of Clinical Analysis of the Faculty of Pharmacy, Federal University of Bahia, Salvador, Bahia, Brazil. Samples of feces, saliva and serum were collected from 83 children, who were divided into three groups: Group G - 39 monoinfected with *G. duodenalis*; Group N – 26 without intestinal parasite infection; and Group P - 18 parasitized by other intestinal protozoa (7 children infected with *Endolimax nana*, 5 with *Entamoeba coli*, 4 with *Iodamoeba butschlii* and 2 with *Blastocystis hominis*). A questionnaire was applied for obtaining demographic, socioeconomic and hygienic-sanitary data of the children's families, as well as gastrointestinal symptom complaints, such as diarrhea, abdominal pain, nausea, vomiting, constipation, bloating and abdominal distension.

This study was approved by the Ethics Committee of the Nursing School of the Federal University of Bahia, protocol Nº 907.867. A signed informed consent form was obtained from the legal guardians of the children. Children with parasitic infections were treated by their pediatricians when necessary.

2.2. Parasitological analysis of fecal samples

Stool samples were subjected to the following parasitological techniques: zinc sulphate flotation (density of the solution 1.18 g/ml) (Faust et al., 1938); centrifugal sedimentation in water (Pacheco et al., 2013); and modified Ziehl-Neelsen (ZN) staining (Henriksen and Pohlenz, 1981). The fecal sediments obtained by the sedimentation technique were examined on slides with iodine for the detection of helminths and protozoa and were stained by ZN for the diagnosis of *Cryptosporidium*. In addition to the microscopic examination, the fecal samples were tested for *G. duodenalis* antigens by a commercial enzyme-linked immunosorbent assay (ELISA; RIDASCREEN® *Giardia*, R-Biopharm AG, Germany) according to the manufacturer's instructions. For samples up to twice the cut-off point provided by the manufacturer (optical density 0.15), with negative parasitological examination for *Giardia*, the ELISA was repeated twice for confirmation. Positive controls of the assay included *Giardia* antigen (supplied by the manufacturer) and two fecal samples positive for *Giardia* cysts. Wells containing diluent instead of feces served as the negative control for the assay.

2.3. Molecular characterization of G. duodenalis

2.3.1. DNA extraction from feces and PCR

DNA from *G. duodenalis* cysts was purified using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with some modifications. For example, the time and temperature of the cell lysis step were increased to 10 min at 95 °C, and the DNA elution volume was reduced to 100 µL of buffer.

A 753-bp fragment of the *β-giardin* gene was amplified using forward primer G7 and reverse primer G759, as described by Cacciò et al. (2002). In the sequential nested PCR reaction, a 511 bp fragment was amplified using forward primer G99 and reverse primer G609, as described by Lalle et al. (2005). Further, the isolates of *G. duodenalis* identified as assemblage A, through the analysis of the β-giardin gene, were submitted to a semi-nested PCR (snPCR) for amplification of the 384-bp fragment using the direct primers G376 and reverse G759 (Cacciò et al., 2002).

A 432 bp fragment of the *gdh* gene was amplified using semi-nested PCR, as described by Read et al. (2004). In the primary PCR reaction, the DNA fragment was amplified using the forward primer GDHeF and the reverse primer GDHiR. In the sequential semi-nested PCR reaction, a 432 bp fragment was amplified using the forward primer GDHiF and the reverse primer GDHiR (Read et al., 2004).

All the PCR products were analyzed by electrophoresis on ethidium bromide-stained 1% agarose gels.

2.3.2. Amplicon analyses by RFLP and sequencing

For the characterization of the *Giardia* assemblages, 10 µL of the 511 bp *β*-giardin amplicon was digested overnight with 10 U of *Hae*III in a final reaction volume of 32 µL at 37 °C (Cacciò et al., 2002). For identification of the A subassemblages (AI, AII/AIII), the 384-bp fragment produced by snPCR was digested with the endonuclease *Hha*I, as described above (Lalle et al., 2005). The *gdh* gene was digested overnight at 37 °C using 10 µL of the 432-bp amplicon of the snPCR and 10 U of the enzyme *Nla*IV (BspLI) in a final volume of 32 µL. The samples indicating the presence of assemblage B had the amplicons also digested with a second endonuclease, *Rsa*I, under the same conditions to specify subassemblages BIII and BIV (Read et al., 2004). Restriction fragments were analyzed by 3% agarose gel electrophoresis using a 50 bp molecular weight standard. Electrophoresis was performed at for two hours.

The isolates suggestive of mixed infections or inconclusive results in RFLP were subjected to amplicon sequencing of the β-giardin gene. The PCR products were purified and sequenced in both directions by the

Macrogen Inc. (Macrogen Inc., Seoul, Korea) sequencing service. Nucleotide sequences and electropherograms were analyzed and edited using the program CLC Main Workbench, version 8.0 (CLC Bio, Qiagen). To determine the assemblages of each sample, the tree phylogenetic analysis was performed using the Neighbor-Joining method with the MEGA 6 software (Tamura et al., 2013). β-giardin gene references corresponding to the different assemblages of *G. duodenalis* were obtained from GenBank (AY072723, subassemblage AII; KR051224, subassemblage AI; GQ337974, assemblage B; AY072726, subassemblage BIII; AY072725, subassemblage BIV; and GQ337973, assemblage E). The sequences were deposited in GenBank under accession numbers MG845536 to MG845549.

2.4. Determination of serum IgG and IgA anti-Giardia antibodies

2.4.1. Production of G. duodenalis antigens

G. duodenalis trophozoites (strain WB) were axenically cultured in TYI-S-33 medium, as described previously (Keister, 1983). For antigen production, trophozoites were resuspended in sterile phosphate buffered saline (PBS) buffer, with pH 7.2, and were disrupted with 15 cycles of ultrasonication (Branson Sonifier Cell Disruptor, USA) for at 90 Hz, with equal period intervals in an ice bath. Subsequently, the following protease inhibitors were added to the antigenic lysate: 0.05 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.05 mM TPCK/TLCK, and 1 µg/ml leupeptin. After homogenization, the lysate was centrifuged at x g for at 4 °C. The protein content of the supernatant was measured by the method of Lowry et al (LOWRY, 1951), and the aliquots of the antigenic solution were frozen at −20 °C until use.

2.4.2. Enzyme-linked immunosorbent assay (ELISA)

Serum IgG and IgA anti-*G. duodenalis* antibodies were detected by an *in-house* ELISA. Briefly, microplate wells were incubated with 20 µg/ml *G. duodenalis* antigens, diluted in 0.06 M carbonate-bicarbonate buffer (pH 9.6) and incubated for at 4 °C. Plates were then washed 3 times with 0.15 M PBS containing 0.05% Tween 20 (PBS-T) and blocked with 100 µl of PBS-T containing 5% skim milk (PBS-T-SM) for at 37 °C. Next, wells were washed 5 times and 100 µl of serum diluted 1:100 (IgG) or 1:25 (IgA) in PBS-T-SM were applied in duplicate, followed by incubation at room temperature. After another washing cycle, 100 µl of the anti-IgG (Sigma-Aldrich Company, St. Louis, USA) or anti-IgA peroxidase conjugate (Thermo Fisher Scientific, USA), diluted 1: in PBS-T-SM, were added and incubated for at 37 °C. After 3 washes as before and twice with PBS, the reaction was developed with 100 µl of the substrate (0.051 M citrate-phosphate buffer, pH 5.0, containing 0.0037 M p-phenylenediamine and 0.04% hydrogen peroxide 30 v) for, and stopped with 20 µl of 8 N sulfuric acid. Reaction was read at filter. The antibody levels were expressed as reactivity indexes (RIs), calculated as the ratio between the absorbance of the optical density (OP) of each tested sample and the OP at the cut-off point. The samples with RIs above 1 were considered positive.

2.5. Determination of serum cytokine levels

Serum cytokine levels of tumor necrosis factor (TNF) (BD Biosciences, San Diego, USA), interferon gamma (IFN-γ),and interleukins 5, 6 and 10 (IL-5, IL-6 and IL-10) (R & D Systems, Inc., USA) were measured in duplicate by ELISA, according to the manufacturer's instructions. The results were expressed in picograms/ml (pg/ml), and the sensitivities of the ELISAs were 15.6 pg/mL for TNF and IFN-γ; 9.4 pg/mL for IL-6; 31.3 pg/mL for IL-10; and 23.4 pg/ml for IL-5.

2.6. Dosing of nitric oxide derivatives

Nitric oxide metabolites nitrate/nitrite (NO_x) were measured according to Tatsch et al (Tatsch et al., 2011) in serum and saliva using the Griess colorimetric method (Green et al., 1982) with a 1:1 (v:v) solution of 1% sulfanilamide and 0.1% naphthylethylenediamine (NED) in 60% acetic acid. Briefly, a standard solution of sodium nitrite in 0.9% saline solution at concentrations of 200, 100, 25, 12, 12, 6, 3, 125, 1.563, 0.781, 0.391, and 0.195 µmol/L were used for the calibration curve. Fifty microliters of samples and 50 µl of the Griess reagent were added to a polystyrene microplate. Nitrite reacts with sulfonylamide forming a diazo composite which, upon reacting with the NED, generates a pink-colored chromophore (Miranda et al., 2001). Optical densities of the reactions were measured at 560 nm using a spectrophotometer, and the results were expressed in µmol/L.

2.7. Statistical analysis

Data were analyzed using the GraphPad Prism 5.0 program (GraphPad, San Diego, CA, USA). The age and gender of the children, as well as the presence of gastrointestinal symptoms of the different groups, were presented as proportions and were compared using the chi-square test. Numerical variables were presented with mean, standard deviation, median, minimum and maximum values. The D'Agostino-Pearson normality test was used to evaluate the type of distribution of the data. Cytokine levels and reactivity indexes of serum anti-*Giardia* IgG and IgA antibodies were compared using the Mann-Whitney test, and Student's was used for NO_x levels. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of the groups of children

Table 1 shows the demographic characteristics and the frequency of gastrointestinal symptoms of the groups of children included in this study. There was no statistically significant difference between the groups in relation to gender or age, which ranged years, with the majority (> 60.0% in all groups) fitting within the age range of years old.

In relation to the presence of gastrointestinal symptoms, 9 (23.1%) children with *G. duodenalis* infection presented complaints of gastrointestinal symptoms, and of these, two reported diarrhea, although there was no statistically significant difference compared with the groups without *G. duodenalis* (Table 1).

3.2. Genotyping of G. duodenalis

Of the 39 isolates of *G. duodenalis* subjected to DNA amplification, 25 (64.1%) amplified at least one of the genes analyzed. RFLP analysis revealed assemblage A as the most frequent (92.0%, n = 23; $P < 0.05$) (Fig. 1A). Considering *G. duodenalis* subassemblages, the type AI was in the highest proportion (60.0%, n = 15; $P < 0.05$) of the amplified isolates, followed by AII (32.0%, n = 8) and BIV (4%, n = 1) (Fig. 1B). A *G. duodenalis* isolate identified as assemblage B could not be subtyped. Five isolates suggestive of mixed infections by RFLP patterns were not confirmed by β-giardin gene sequencing, being classified as 3 AI, 1 AII

Table 1
Demographic characteristics of groups of children.

		Groups of children n (%)		
		G (n = 39)	N (n = 26)	P (n = 18)
Gender	Male	19 (48.7)	12 (46.2)	9 (50.0)
	Female	20 (51.3)	14 (53.8)	9 (50.0)
Age range (yr)	0-2	3 (7.7)	1 (3.8)	0 (0.0)
	3-6	24 (61.5)	17 (65.4)	12 (66.7)
	7-10	6 (15.4)	4 (15.4)	3 (16.7)
	11-14	6 (15.4)	4 (15.4)	3 (16.7)
Gastrointestinal symptoms		9 (23.1)	7 (26.9)	3 (16.7)

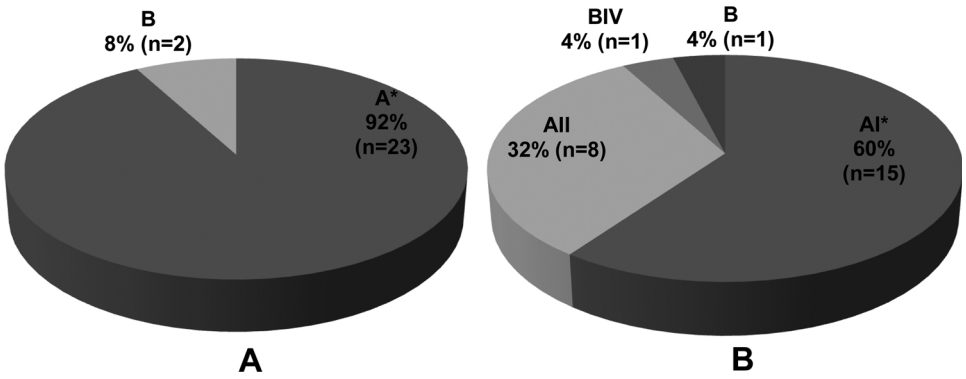

Fig. 1. Distribution of assemblages (A) and subassemblages (B) of *Giardia duodenalis* isolated from children. Significant frequency of the assemblage A (mostly subassemblage AI) (*P < 0.05, chi-square test).

and 1BIV subassemblages.

Among the nine cases of symptomatic giardiasis included in this study, seven had the protozoa assemblages identified. Of these children, two presented with diarrhea, and both were infected with the *G. duodenalis* AI assemblage. The remaining children with other gastrointestinal symptoms had molecular isolates AI (n = 2), AII (n = 2) and B (n = 1). There were no statistically significant differences between *G. duodenalis* assemblages and/or subassemblages and the presence of gastrointestinal symptoms.

3.3. Antibodies, cytokines and nitric oxide levels in sera of children

The reactivity indexes of IgG (1.503 ± 0.819) and IgA (2.308 ± 1.935) anti-*Giardia* antibodies in the sera of children infected with *G. duodenalis* were significantly higher when compared to the uninfected groups and the groups infected by other protozoa ($P < 0.001$) (Fig. 2A and B; Table 2).

Regarding the serum cytokine concentrations, the results showed that IFN-γ levels were higher in children with giardiasis (393.10 ± 983.90 pg/mL) than in the other groups ($P < 0.05$). Concentrations of TNF and IL-10 were similar among the groups of children. Levels of IL-5 and IL-6 were below the detection limits in most samples (96.4%; 80/83) (Fig. 2 and Table 2).

Serum and salivary NO_x levels were higher in the samples from subjects infected with *G. duodenalis* (30.03 ± 10.92 µmol/L and 192.4 ± 151.2 µmol/L, respectively; $P < 0.05$) than those observed in children without parasitic infection (25.82 ± 7.74 µmol/L and 122.5 ± 105.90 µmol/L, respectively) ($P < 0.05$) (Fig. 2 and Table 2). In addition, higher concentrations of serum and salivary NO_x (greater than 41 µmol/L and 301 µmol/L, respectively) were mostly observed in *G. duodenalis* infected children (Table 3).

3.4. Comparison of serum markers of immune responses with molecular isolates of G. duodenalis

Due to the low frequency of assemblage B (only two isolates), the systemic production of immune response mediators was evaluated by comparing the *G. duodenalis* subassemblages AI and AII, which were more frequent in the children in this study. There was no statistically significant difference in the immunological parameters analyzed among the children infected by these two molecular types (Fig. 3).

4. Discussion

Epidemiological studies suggest that previous infection with *Giardia* leads to a reduced risk of reinfection or to milder symptoms (Isaac-Renton et al., 1994; Istre et al., 1984; Kohli et al., 2008). In fact, the immune response is important for the eradication of the parasite in the gut and in the development of protective immunity (Lopez-Romero et al., 2015).

Most of the knowledge about immune response in giardiasis comes from *in vitro* studies by the stimulation of immune cells with *G. duodenalis* antigens or from animal experimental models infected with *G. duodenalis* or *G. muris* (Amorim et al., 2010; Dann et al., 2015; Eckmann, 2003; Faubert, 2000; Grit et al., 2014; Jiménez et al., 2014). However, the results obtained from animal models may not correctly represent what occurs in human giardiasis. Differences in the performed method, the race and immune status of the laboratory animal, the parasite strain, and the natural pathogens and host microbiota may affect the results and interpretation of the assays (Eckmann, 2003; Faubert, 2000). In this work, the parameters of the innate (nitric oxide), humoral (IgG, IgA, IL-6 and IL-5), cellular (IFN-γ, TNF-α) and regulatory (IL-10) immune responses in children with giardiasis were evaluated and compared to groups of children infected with other protozoa and those without infection by enteroparasites.

Evidence suggests that antibodies, especially those of the IgA class, contribute to protective immunity against giardiasis. Experimental studies in *Giardia*-infected animal models showed elevated IgG and IgA serum antibodies during infection, which remained elevated for months after resolution of the infection (Amorim et al., 2010; Daniels and Belosevic, 1994; Grit et al., 2014). However, the antibody response against *G. duodenalis* in humans is still poorly studied. In this work, there was significantly more reactivity of the IgG and IgA anti-*Giardia* serum antibodies observed in children infected with *G. duodenalis,* than in those infected with other protozoa or in those of the non-parasitized control group. Our results corroborate previous studies in patients with giardiasis (El-Gebaly et al., 2012; Rodríguez et al., 2004; Velazquez et al., 2005), showing that *G. duodenalis* infection activates the production of specific serum antibodies. The mechanisms by which the antibodies exert their anti-*Giardia* functions are not well understood but probably involve “immune exclusion”,i.e., immobilization of the trophozoites of the intestinal epithelium or mucus layer (Heyworth, 2014).

Other studies indicate that although antibodies are important in protection against giardiasis, there are cellular immune mechanisms independent of the antibodies involved (Hanevik et al., 2011; Lopez-Romero et al., 2015; Singer and Nash, 2000). The possible protective role of the Th1, Th2 or Th17 cellular immune response in *Giardia* infection is still unclear. IFN-γ, which is a crucial cytokine in the Th1 response, appears to play a role in giardiasis, as it is elevated in experimental infection models (Bienz et al., 2003; Jiménez et al., 2014; Solaymani-Mohammadi and Singer, 2011) and in humans infected with *G. duodenalis* (Matowicka-Karna et al., 2011, 2009), as also observed herein. Reports of the role of this cytokine in *Giardia* immunity have conflicting results, as observed in experimental models, that IFN-γ-deficient mice can eliminate this protozoan (Singer and Nash, 2000; Wakelin et al., 1993), suggesting that IFN-γ is not essential for the control of infection. In contrast, other studies have shown that IFN-γ, together with IL-5, is important in controlling giardiasis in

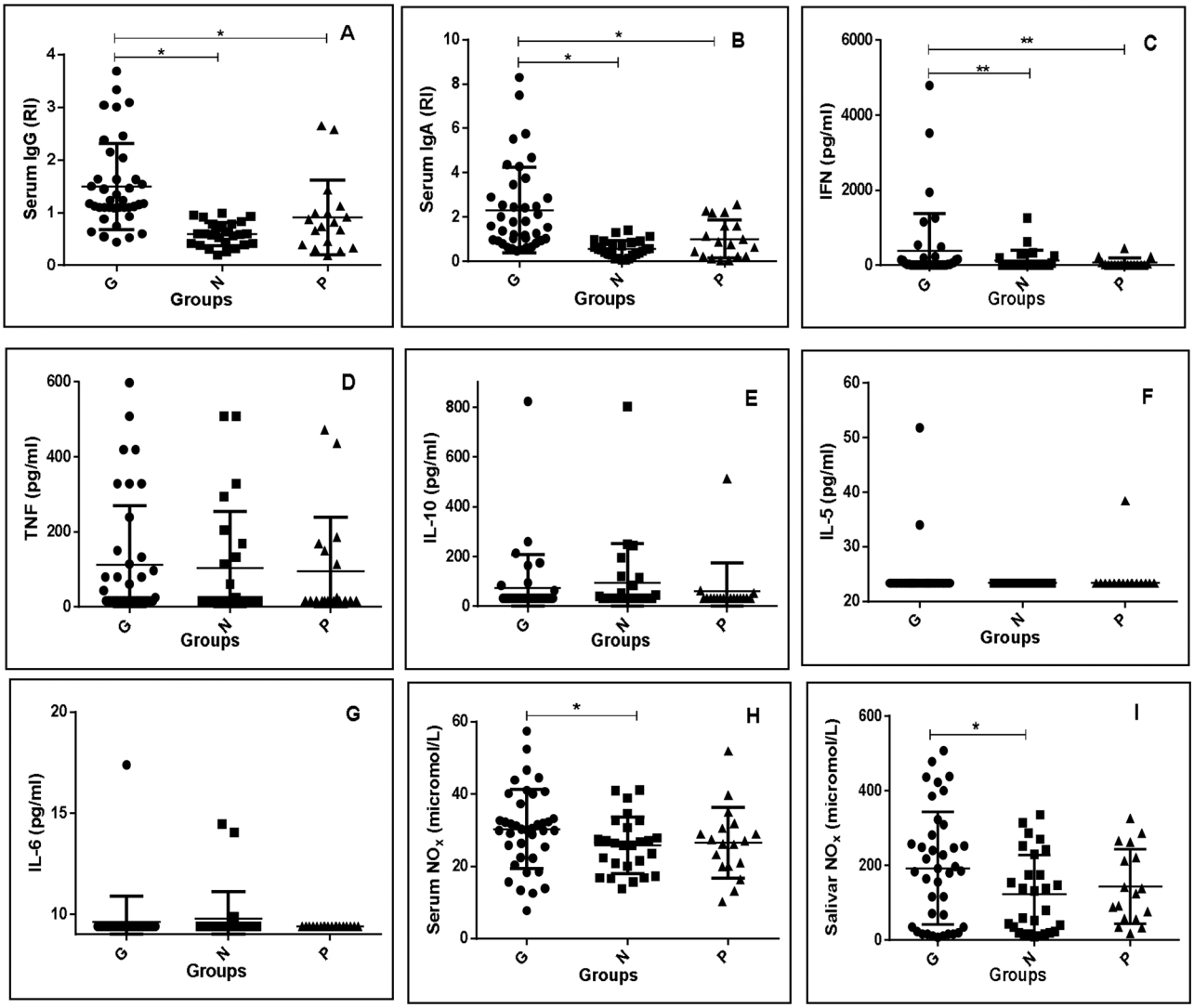

Fig. 2. IgG (A) and IgA (B) anti-*Giardia* reactivities and levels of cytokines IFN-γ (C), TNF (D), IL-10 (E), IL-5 (F), and IL-6 (G) and NOx (H) serum and salivary NOx (I) in 39 children infected with *G. duodenalis* (●), 26 without enteroparasite infection (■) and 18 infected with other intestinal protozoa (▼). *$P < 0.001$; **$P < 0.05$ (Mann Whitney test). Bars indicate mean ± standard deviation.

Table 2
Reactivities of anti-*G. duode*nalis IgG and IgA, concentrations of cytokines and NOx in sera, and levels of NOx in saliva of children.

	Group G (n = 39)			Group N (n = 26)			Group P (n = 18)			p
	Median	Mean ± SD	Minimum - Maximum	Median	Mean ± SD	Minimum - Maximum	Median	Mean ± SD	Minimum - Maximum	
IgG (RI)*	1.176[a]	1.503 ± 0.819	0.441 – 3.684	0.592[a]	0.592 ± 0.226	0.199 – 0.993	0.779	0.911 ± 0.704	0.184 – 2.654	< 0.001
IgA (RI)*	1.588[b]	2.308 ± 1.935	0.471 – 8.294	0.537[b]	0.562 ± 0.375	0.044 – 1.397	0.816[b]	1.005 ± 0.863	0,029 – 2.559	< 0.001
NO_x serum (µmol/L)**	30.20	30.03 ± 10.92[c]	7.75 – 57.45	26.13	25.82 ± 7.74[c]	13.76 – 41.07	26.63	26.53 ± 7.74	10.21 – 51.86	< 0.05
NO_x saliva (µmol/L)**	184.60	192.4 ± 151.2[d]	7.81 – 506.90	105.50	122.5 ± 105.90[d]	9.48 – 335.80	125.00	143.3 ± 99.90	17.64 – 326.5	< 0.05
IFN-γ (pg/mL)*	25.60[e]	393.10 ± 983.90	15.60 – .00	15.60[e]	127.4 ± 274.30	15.60 – .00	15.60[e]	70.51 ± 120.40	15.60 – 464.90	< 0.05
TNF (pg/mL)	15.60	111.8 ± 158.40	15.60 – 597.20	15.60	103.2 ± 151.80	15.60 – 507.90	15.60	95.91 ± 143.50	15.60 – 472.20	
IL-10 (pg/mL)	31.30	72.91 ± 134.80	31.30 – 823.50	31.30	93.88 ± 158.80	31.30 – 803.50	31.30	61.11 ± 113.30	31.30 – 513.50	
IL-5 (pg/mL)	23.40	24.43 ± 4.87	23.40 – 51.78	23.40	23.40 ± 0.00	–	23.40	24.28 ± 3.65	23.40 – 38.44	
IL-6 (pg/mL)	9.40	9.61 ± 1.29	9.40 – 17.38	9.40	9.79 ± 1.32	9.40 – 14.46	9.40	9.40 ±	–	

[a;b;c;d;e] equal letters indicate a statistically significant difference among groups. *Mann-Whitney test - significant differences in RI of IgG and IgA anti-*Giardia* and IFN-γ concentration in sera of *Giardia*-positive children (Group G); **Student's - significant differences in NOx concentrations in sera and saliva of individuals from Group G compared to non-parasitized children (Group N). RI - Reactivity Index; SD- Standard Deviation.

Table 3
NO_x serum and saliva concentration ranges according to the groups of children.

	Groups of children		
	G n (%)	N n (%)	P n (%)
NOx in sera (μmol/L)			
0 - 20	7 (17.9)	6 (23.1)	4 (22.2)
21 - 40	26 (66.7)	19 (73.1)	13 (72.2)
41 - 60	6 (15.4)*	1 (3.8)	1 (5.6)
TOTAL	39 (100.0)	26 (100.0)	18 (100.0)
NO_x in saliva (μmol/L)			
0 - 100	13 (33.3)	14 (53.8)	8 (44.4)
101 - 300	17 (43.6)	10 (41.7)	9 (50.0)
> 301	9 (23.1)*	2 (7.7)	2 (11.1)
TOTAL	39 (100)	26 (100.0)	18 (100.0)

* Differences in the frequencies of high concentrations of serum and saliva NO_x from children with *G. duodenalis* compared to the other groups; $P < 0.05$ (chi-square test).

experimentally infected mice (Jiménez et al., 2014) and that the intensity of *G. muris* infection was increased in mice treated with anti-IFN-γ neutralizing antibodies (Venkatesan et al., 1996). Interestingly, Long et al. (2010) showed that elevated levels of IFN-γ, IL-4 and IL-5 in fecal samples from infected individuals were associated with the increased duration of *G. duodenalis* infection, suggesting that these cytokines may favor persistence of the infection.

The cytokine IFN-γ has been associated with an increase in NO production by macrophages in *Giardia* infection (Byrd et al., 1994; Holland et al., 2000). Studies show that NO synthases (NOS) are induced mainly in Th1 inflammatory conditions by the cytokines IFN-γ, TNF-α and IL-1β, whereas the Th2 immune response profile inhibits their production (Munder et al., 1999). In our study, IFN-γ as well as the mean levels of serum and salivary NO in patients infected with *G. duodenalis* were higher than those in the control group without parasitosis, corroborating previous studies (Bayraktar et al., 2005; Matowicka-Karna et al., 2011; Zarebavani et al., 2017). Matowicka-Karna et al. (2011) demonstrated that giardiasis stimulates NO production in humans, which did not decrease even after anti-parasitic treatment. Other reports have shown that the molecule inhibits the proliferation of *G. duodenalis* trophozoites but does not kill the parasite, thus having a cytostatic effect (Eckmann et al., 2000). Despite the effects of NO on *Giardia* trophozoites, the parasite has developed strategies to evade this host defense mechanism by downregulating the expression of NOS in intestinal cells (Maloney et al., 2015; Stadelmann et al., 2013) and by consuming arginine, which is the main source for the production of NO (Eckmann et al., 2000; Maloney et al., 2015; Ringqvist et al., 2008).

It is important to note that no significant differences were observed between the concentrations of serum and salivary NO in the group of children with *G. duodenalis* compared to children parasitized by other protozoa, although the mean values of the *Giardia* group were higher.

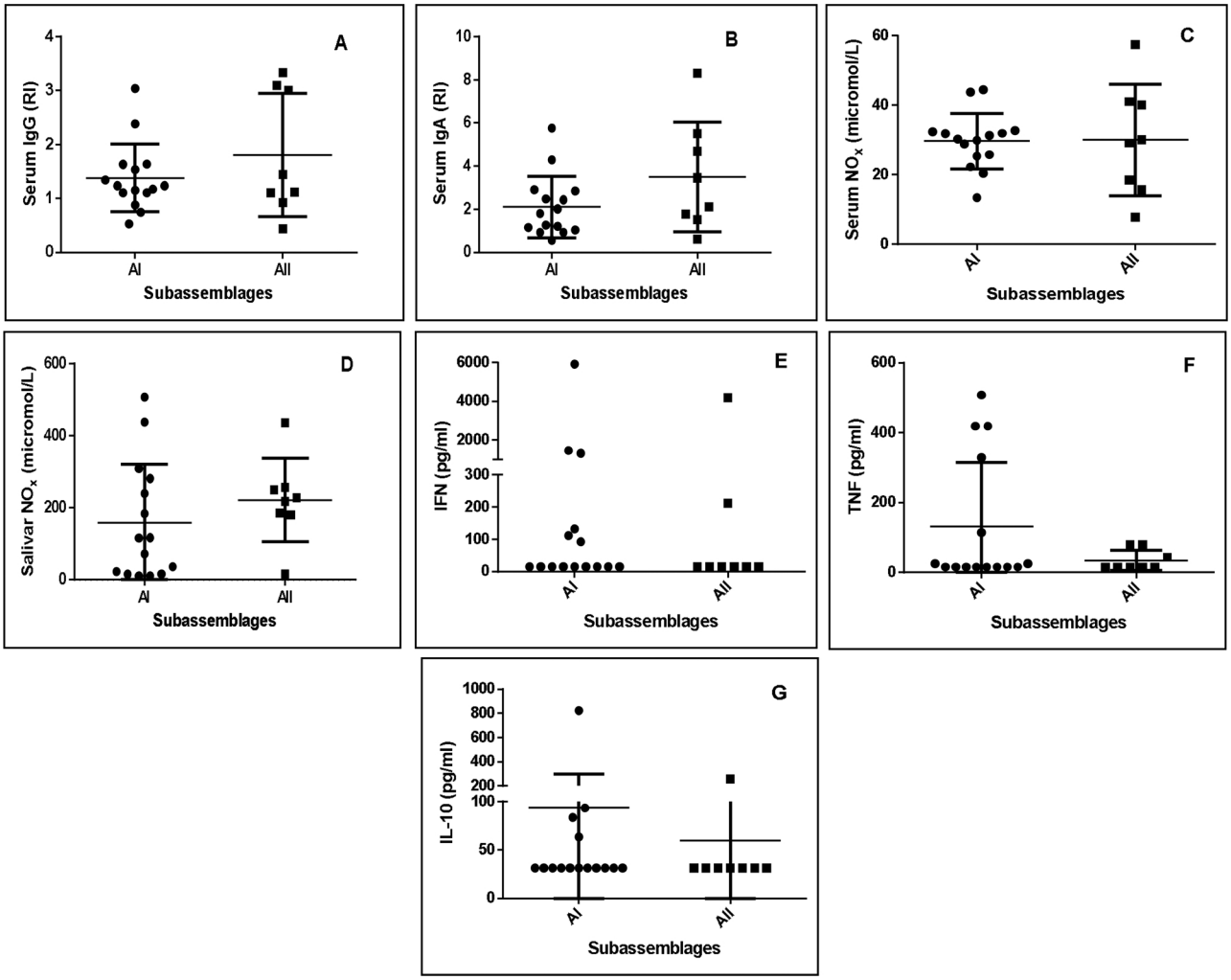

Fig. 3. Profile of systemic mediators in 15 children infected with *G. duodenalis* subassemblage AI (●) and 8 infected with AII (■). Anti-*Giardia* IgG (A) and IgA (B) reactivity indexes and levels of NOx (C) and cytokines IFN-γ (E), TNF (F) and IL-10 (G) in serum and salivary NOx (D). Bars indicate mean ± standard deviation.

These data may be explained by the stimulation of the local immune response at basal levels by protozoa considered to be non-pathogenic. Thus, the results of our study suggest that NO may have a role in immunity in human giardiasis and that asymptomatic infections do not affect NO production. More studies are needed to understand the multiple effects of NO in giardiasis.

Tumor necrosis factor (TNF) is a proinflammatory cytokine released during *Giardia* infection (Grit et al., 2014; Solaymani-Mohammadi and Singer, 2011). TNF-deficient mice exhibit delay in elimination of the protozoan (Zhou et al., 2007), and a human study showed that patients with giardiasis had elevated serum TNF levels (Bayraktar et al., 2005), suggesting that this cytokine participates in the immune response during infection. However, the production of cytokines such as IL-10 and TGF-β during giardiasis prevented the recruitment of inflammatory cells (Grit et al., 2014; Jiménez et al., 2014; Kamda and Singer, 2009). In our study, there was no significant difference in serum levels of TNF and IL-10 among individuals infected with *G. duodenalis* and control individuals, which may be due to sample size and/or low production of these cytokines in children with subclinical infections.

Similar to TNF, IL-6 is a proinflammatory cytokine that induces an innate cell response and mediates IgA production and the development of a Th17 cell response (Kimura et al., 2007; Scheller et al., 2011). Mice deficient in this cytokine fail to eliminate *Giardia* (Bienz et al., 2003; Zhou et al., 2003). IL-5 also contributes to the humoral immune response, being produced by Th2 lymphocytes and inducing the differentiation of B and T cells, in addition to stimulating the proliferation, differentiation and chemotaxis of eosinophils (Faccioli et al., 1997; Weltman, 2000). A study by Matowicka-Karna (Matowicka-Karna et al., 2011) found lower levels of IL-5 and IL-6 in *G. duodenalis*-infected individuals when compared to healthy controls, and levels of these cytokines increased after treatment of the infection. In our study, the levels of these molecules were below the limit of detection in most patients. This result may be due to their low concentrations in sera, requiring either the use of more sensitive detection methods or analysis in the culture supernatant of cells stimulated with *Giardia* antigens.

Infection with *G. duodenalis* presents a broad clinical spectrum, ranging from asymptomatic cases to acute or chronic gastrointestinal symptoms. The severity of the disease is probably determined by the interaction between parasites with different degrees of virulence and the immune status of human hosts (Bartelt and Sartor, 2015; Certad et al., 2017). The genetic diversity of the parasite has been implicated in the pathogenesis of the disease (Puebla et al., 2014). The correlation between *G. duodenalis* molecular types and clinical presentation has long been a controversial issue. While some studies have shown that assemblage A is associated with cases of symptomatic giardiasis (Fouad et al., 2014; Haque et al., 2005), others have reported that infections with assemblage B were significantly correlated with clinical symptoms (ElBakri et al., 2014; Hussein et al., 2017). In the study described herein, the *G. duodenalis* assemblage A (n = 23; 92.0%) was the most frequent, with predominance of subassemblage AI compared to AII. Only two children were infected with assemblage B.

We found that 76.9% (n = 30) of the *G. duodenalis*-infected children did not present diarrhea or relevant complaints of gastrointestinal symptoms at the time of collection. Among the cases of giardiasis included in this study, 9 were symptomatic and 7 had the assemblages identified. Two children with diarrhea had assemblage AI, and the others with different gastrointestinal symptoms presented with 2 assemblages of AII, 2 AI and 1 B. Thus, it was not possible to evaluate associations among the assemblages, antibody reactivity, cytokines and NO_x levels with clinical symptoms, due to the limited number of symptomatic individuals in our study.

Babaei et al. (2016) reported that individuals infected with the AI subassemblage showed higher levels of serum IFN-γ and IL-10 than those infected with the AII subassemblage, suggesting that distinct molecular types may differ considerably in their ability to induce immunopathogenicity and tissue damage. In our study, IFN-γ levels were higher than pg/mL in 3 AI and in 1 AII-infected individual. In addition, TNF levels were higher than 100 pg/mL in 4 cases of AI carriers. Despite the high levels of inflammatory cytokines in some children infected by the AI species, considering all the results together, there was no difference in cytokine levels, nitric oxide, and IgG and IgA antibody response among groups of children infected with these two subassemblages. This result may indicate that the immune response profile against *G. duodenalis* is not influenced by molecular subtypes of the parasite, corroborating the study by Hanevik et al. (2011). Studies with a larger number of *G. duodenalis* isolates, including cases of symptomatic giardiasis, are necessary to better understand the diversity in immune responses, pathogenesis and the development of symptoms elicited by different assemblages/subassemblages of *G. duodenalis*.

Declarations of interest

None.

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References

Abdul-Wahid, A., Faubert, G., 2007. Mucosal delivery of a transmission-blocking DNA vaccine encoding *Giardia lamblia* CWP2 by *Salmonella typhimurium* bactofection vehicle. Vaccine 25 (50), 8372–8383. https://doi.org/10.1016/j.vaccine.2007.10.012.

Alyousefi, N.A., Mahdy, M.A.K., Xiao, L., Mahmud, R., Lim, Y.A.L., 2013. Molecular characterization of *Giardia duodenalis* in Yemen. Exp. Parasitol. 134 (2), 141–147. https://doi.org/10.1016/j.exppara.2013.03.001.

Amorim, R.M.R., Silva, Da.O., Taketomi, E.A., Morato, M.G.V.A., Mundim, M.J.S., Ribeiro, D.P., Oliveira, T.C., Viana, J.C., Gomes, M.A., Cury, M.C., 2010. *Giardia duodenalis*: kinetics of cyst elimination and the systemic humoral and intestinal secretory immune responses in gerbils (*Meriones unguiculatus*) experimentally infected. Exp. Parasitol. 125 (3), 297–303. https://doi.org/10.1016/j.exppara.2010.02.007.

Ankarklev, J., Jerlström-Hultqvist, J., Ringqvist, E., Troell, K., Svärd, S.G., 2010. Behind the smile: cell biology and disease mechanisms of *Giardia* species. Nat. Rev. Microbiol. 8 (6), 413–422. https://doi.org/10.1038/nrmicro2317.

Babaei, Z., Malihi, N., Zia-Ali, N., Sharifi, I., Mohammadi, M.A., Kagnoff, M.F., Eckmann, L., Singer, S.M., Solaymani-Mohammadi, S., 2016. Adaptive immune response in symptomatic and asymptomatic enteric protozoal infection: evidence for a determining role of parasite genetic heterogeneity in host immunity to human giardiasis. Microbes Infect. 18 (11), 687–695. https://doi.org/10.1016/j.micinf.2016.06.009.

Bartelt, L.A., Sartor, R.B., 2015. Advances in understanding *Giardia*: determinants and mechanisms of chronic sequelae. F1000Prime Rep. 7, 62. https://doi.org/10.12703/P7-62.

Bayraktar, M.R., Mehmet, N., Durmaz, R., 2005. Serum cytokine changes in Turkish children infected with *Giardia lamblia* with and without allergy: effect of metronidazole treatment. Acta Trop. 95 (2), 116–122. https://doi.org/10.1016/j.actatropica.2005.05.006.

Berkman, D.S., Lescano, A.G., Gilman, R.H., Lopez, S.L., Black, M.M., 2002. Effects of stunting, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: a follow-up study. Lancet Lond. Engl. 359 (9306), 564–571. https://doi.org/10.1016/S0140-6736(02)07744-9.

Bienz, M., Dai, W.J., Welle, M., Gottstein, B., Müller, N., 2003. Interleukin-6-deficient mice are highly susceptible to *Giardia lamblia* Infection but exhibit normal intestinal immunoglobulin a responses against the parasite. Infect. Immun. 71 (3), 1569–1573. https://doi.org/10.1128/IAI.71.3.1569-1573.2003.

Brunet, L.R., 2001. Nitric oxide in parasitic infections. Int. Immunopharmacol. 1 (8), 1457–1467. https://doi.org/10.1016/S1567-5769(01)00090-X.

Byrd, L.G., Conrad, J.T., Nash, T.E., 1994. *Giardia lamblia* infections in adult mice. Infect. Immun. 62 (8), 3583–3585.

Cacciò, S.M., De Giacomo, M., Pozio, E., 2002. Sequence analysis of the beta-giardin gene and development of a polymerase chain reaction-restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. Int. J. Parasitol. 32 (8), 1023–1030. https://doi.org/10.1016/S0020-7519(02)00068-1.

Cama, V.A., Mathison, B.A., 2015. Infections by intestinal Coccidia and Giardia duodenalis. Clin. Lab. Med. 35 (2), 423–444. https://doi.org/10.1016/j.cll.2015.02.010.

Certad, G., Viscogliosi, E., Chabé, M., Cacciò, S.M., 2017. Pathogenic mechanisms of *Cryptosporidium* and *Giardia*. Trends Parasitol. 33 (7), 561–576. https://doi.org/10.1016/j.pt.2017.02.006.

Cotton, J.A., Beatty, J.K., Buret, A.G., 2011. Host parasite interactions and pathophysiology in *Giardia* infections. Int. J. Parasitol. 41 (9), 925–933. https://doi.org/10.

1016/j.ijpara.2011.05.002.
Daniels, C.W., Belosevic, M., 1994. Serum antibody responses by male and female C57Bl/6 mice infected with Giardia muris. Clin. Exp. Immunol. 97 (3), 424–429.
Dann, S.M., Manthey, C.F., Le, C., Miyamoto, Y., Gima, L., Abrahim, A., Cao, A.T., Hanson, E.M., Kolls, J.K., Raz, E., Cong, Y., Eckmann, L., 2015. IL-17A promotes protective IgA responses and expression of other potential effectors against the lumen-dwelling enteric parasite Giardia. Exp. Parasitol. 156, 68–78. https://doi.org/10.1016/j.exppara.2015.06.003.
Davids, B.J., Palm, J.E.D., Housley, M.P., Smith, J.R., Andersen, Y.S., Martin, M.G., Hendrickson, B.A., Johansen, F.-E., Svärd, S.G., Gillin, F.D., Eckmann, L., 2006. Polymeric immunoglobulin receptor in intestinal immune defense against the lumen-dwelling protozoan parasite Giardia. J. Immunol. Baltim. Md. 1950 177 (9), 6281–6290. https://doi.org/10.4049/jimmunol.177.9.6281.
Eckmann, L., 2003. Mucosal defences against Giardia. Parasite Immunol. 25 (5), 259–270. https://doi.org/10.1046/j.1365-3024.2003.00634.x.
Eckmann, L., Laurent, F., Langford, T.D., Hetsko, M.L., Smith, J.R., Kagnoff, M.F., Gillin, F.D., 2000. Nitric oxide production by human intestinal epithelial cells and competition for arginine as potential determinants of host defense against the lumen-dwelling pathogen Giardia lamblia. J. Immunol. Baltim. Md 1950 164 (3), 1478–1487. https://doi.org/10.4049/jimmunol.164.3.1478.
ElBakri, A., Samie, A., Bessong, P., Potgieter, N., Odeh, R.A., 2014. Detection and molecular characterisation of *Giardia lamblia* genotypes in Sharjah, United Arab Emirates. Trans. R. Soc. Trop. Med. Hyg. 108 (8), 466–473. https://doi.org/10.1093/trstmh/tru083.
El-Gebaly, N.S.M., Halawa, E.F., Moussa, H.M.E., Rabia, I., Abu-Zekry, M., 2012. Saliva and sera IgA and IgG in egyptian *giardia*-infected children. Parasitol. Res. 111 (2), 571–575. https://doi.org/10.1007/s00436-012-2869-y.
Faccioli, L.H., Vargaftig, B.B., Medeiros, A.I., Malheiros, A., 1997. Cytokines in the modulation of eosinophilia. Mem. Inst. Oswaldo Cruz 92 (Suppl. 2), 109–114. https://doi.org/10.1590/S0074-02761997000800015.
Fahmy, H.M., El-Serougi, A.O., El Deeb, H.K., Hussein, H.M., Abou-Seri, H.M., Klotz, C., Aebischer, T., El Sayed Khalifa Mohamed, K., 2015. *Giardia duodenalis* assemblages in Egyptian children with diarrhea. Eur. J. Clin. Microbiol. Infect. Dis. 34 (8), 1573–1581. https://doi.org/10.1007/s10096-015-2389-7.
Fang, F.C., 1997. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. J. Clin. Invest. 99 (12), 2818–2825. https://doi.org/10.1172/JCI119473.
Faubert, G., 2000. Immune response to Giardia duodenalis. Clin. Microbiol. Rev. 13 (1), 35–54. https://doi.org/10.1128/CMR.13.1.35.
Faust, E.C., Sawitz, W., Tobie, J., Odom, V., Peres, C., Lincicome, D.R., 1938. Comparative efficiency of various technics for the diagnosis of protozoa and helminths in feces. J. Parasitol. 25 (3), 241–262.
Feng, Y., Xiao, L., 2011. Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. Clin. Microbiol. Rev. 24 (1), 110–140. https://doi.org/10.1128/CMR.00033-10.
Fouad, S.A., Esmat, S., Basyoni, M.M.A., Farhan, M.S., Kobaisi, M.H., 2014. Molecular identification of *Giardia intestinalis* in patients with dyspepsia. Digestion 90 (1), 63–71. https://doi.org/10.1159/000362644.
García-Cervantes, P.C., Báez-Flores, M.E., Delgado-Vargas, F., Ponce-Macotela, M., Nawa, Y., De-la-Cruz-Otero, M.D.-C., Martínez-Gordillo, M.N., Díaz-Camacho, S.P., 2017. *Giardia duodenalis* genotypes among schoolchildren and their families and pets in urban and rural areas of Sinaloa, Mexico. J. Infect. Dev. 11 (2), 180–187. https://doi.org/10.3855/jidc.8223.
Ghoshal, U., Shukla, R., Pant, P., Ghoshal, U.C., 2016. Frequency, diagnostic performance of coproantigen detection and genotyping of the *Giardia* among patients referred to a multi-level teaching hospital in northern India. Pathog. Glob. Health 110 (7-8), 316–320. https://doi.org/10.1080/20477724.2016.1254141.
Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. 126 (1), 131–138. https://doi.org/10.1016/0003-2697(82)90118-X.
Grit, G.H., Devriendt, B., Van Coppernolle, S., Geurden, T., Hope, J., Vercruysse, J., Cox, E., Geldhof, P., Claerebout, E., 2014. *Giardia duodenalis* stimulates partial maturation of bovine dendritic cells associated with altered cytokine secretion and induction of T-cell proliferation. Parasite Immunol. 36 (4), 157–169. https://doi.org/10.1111/pim.12095.
Hanevik, K., Kristoffersen, E., Svard, S., Bruserud, O., Ringqvist, E., Sørnes, S., Langeland, N., 2011. Human cellular immune response against *Giardia lamblia* 5 years after acute giardiasis. J. Infect. Dis. 204 (11), 1779–1786. https://doi.org/10.1093/infdis/jir639.
Haque, R., Roy, S., Kabir, M., Stroup, S.E., Mondal, D., Houpt, E.R., 2005. *Giardia* assemblage A infection and diarrhea in Bangladesh. J. Infect. Dis. 192 (12), 2171–2173. https://doi.org/10.1086/498169.
Henriksen, S.A., Pohlenz, J.F., 1981. Staining of cryptosporidia by a modified Ziehl-Neelsen technique. Acta Vet. Scand. 22, 594–596.
Heyworth, M.F., 2014. Diagnostic testing for Giardia infections. Trans. R. Soc. Trop. Med. Hyg. 108 (3), 123–125. https://doi.org/10.1093/trstmh/tru005.
Holland, M.J., Harcus, Y.M., Riches, P.L., Maizels, R.M., 2000. Proteins secreted by the parasitic nematode *Nippostrongylus brasiliensis* act as adjuvants for Th2 responses. Eur. J. Immunol. 30 (7), 1977–1987. https://doi.org/10.1002/1521-4141(200007)30:7<1977:AID-IMMU1977>3.0.CO;2-3.
Hussein, A.H., Rashed, S.M., El-Hayawan, I.A., Aly, N.S.M., Abou Ouf, E.A., Ali, A.T., 2017. Intestinal parasite infections and accuracy of direct thin and thick smear, formol-ether sedimentation, centrifugal flotation, and mini-FLOTAC techniques among patients with gastrointestinal tract disorders from the greater Cairo Region, Egypt. Am. J. Trop. Med. Hyg. 96 (3), 589–594. https://doi.org/10.4269/ajtmh.16-0436.
Isaac-Renton, J.L., Lewis, L.F., Ong, C.S., Nulsen, M.F., 1994. A second community outbreak of waterborne giardiasis in Canada and serological investigation of patients. Trans. R. Soc. Trop. Med. Hyg. 88 (4), 395–399.
Istre, G.R., Dunlop, T.S., Gaspard, G.B., Hopkins, R.S., 1984. Waterborne giardiasis at a mountain resort: evidence for acquired immunity. Am. J. Public Health 74 (6), 602–604.
Jiménez, J.C., Fontaine, J., Creusy, C., Fleurisse, L., Grzych, J.-M., Capron, M., Dei-Cas, E., 2014. Antibody and cytokine responses to *Giardia* excretory/secretory proteins in *Giardia intestinalis*-infected BALB/c mice. Parasitol. Res. 113 (7), 2709–2718. https://doi.org/10.1007/s00436-014-3927-4.
Kamda, J.D., Singer, S.M., 2009. Phosphoinositide 3-Kinase-Dependent inhibition of dendritic cell interleukin-12 production by Giardia lamblia. Infect. Immun. 77 (2), 685–693. https://doi.org/10.1128/IAI.00718-08.
Keister, D.B., 1983. Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. Trans. R. Soc. Trop. Med. Hyg. 77 (4), 487–488. https://doi.org/10.1016/0035-9203(83)90120-7.
Kimura, A., Naka, T., Kishimoto, T., 2007. IL-6- dependent and -independent pathways in the development of interleukin 17-producing T helper cells. Proc Natl Acad Sci U A.
Kohli, A., Bushen, O.Y., Pinkerton, R.C., Houpt, E., Newman, R.D., Sears, C.L., Lima, A.A.M., Guerrant, R.L., 2008. *Giardia duodenalis* assemblage, clinical presentation and markers of intestinal inflammation in Brazilian children. Trans. R. Soc. Trop. Med. Hyg. 102 (7), 718–725. https://doi.org/10.1016/j.trstmh.2008.03.002.
Lalle, M., Pozio, E., Capelli, G., Bruschi, F., Crotti, D., Cacciò, S.M., 2005. Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. Int. J. Parasitol. 35 (2), 207–213. https://doi.org/10.1016/j.ijpara.2004.10.022.
Langford, T.D., Housley, M.P., Boes, M., Chen, J., Kagnoff, M.F., Gillin, F.D., Eckmann, L., 2002. Central importance of immunoglobulin A in host defense against *Giardia* spp. Infect. Immun. 70 (1), 11–18. https://doi.org/10.1128/IAI.70.1.11-18.2002.
Larocque, R., Nakagaki, K., Lee, P., Abdul-Wahid, A., Faubert, G.M., 2003. Oral Immunization of BALB/c mice with *Giardia duodenalis* recombinant cyst wall protein inhibits shedding of cysts. Infect. Immun. 71 (10), 5662–5669. https://doi.org/10.1128/IAI.71.10.5662-5669.2003.
Long, K.Z., Rosado, J.L., Santos, J.I., Haas, M., Al Mamun, A., DuPont, H.L., Nanthakumar, N.N., Estrada-Garcia, T., 2010. Associations between mucosal innate and adaptive immune responses and resolution of diarrheal pathogen infections. Infect. Immun. 78 (3), 1221–1228. https://doi.org/10.1128/IAI.00767-09.
Lopez-Romero, G., Quintero, J., Astiazarán-García, H., Velazquez, C., 2015. Host defences against Giardia lamblia. Parasite Immunol. 37 (8), 394–406. https://doi.org/10.1111/pim.12210.
Lowry, O.H., 1951. Protein measurement with the Folin -Phenol reagent. J. Biol. Chemestry 193, 265–276.
Maloney, J., Keselman, A., Li, E., Singer, S.M., 2015. Macrophages expressing arginase 1 and nitric oxide synthase 2 accumulate in the small intestine during *Giardia lamblia* infection. Microbes Infect. Inst. Pasteur 17 (6), 462–467. https://doi.org/10.1016/j.micinf.2015.03.006.
Matowicka-Karna, J., Dymicka-Piekarska, V., Kemona, H., 2009. IFN-gamma, IL-5, IL-6 and IgE in patients infected with *Giardia intestinalis*. Folia Histochem. Cytobiol. 47 (1), 93–97. https://doi.org/10.2478/v10042-009-0013-3.
Matowicka-Karna, J., Kralisz, M., Kemona, H., 2011. Assessment of the levels of nitric oxide (NO) and cytokines (IL-5, IL-6, IL-13, TNF, IFN-gamma) in giardiasis. Folia Histochem. Cytobiol. 49, 280–284. https://doi.org/10.5603/FHC.2011.0039.
Minetti, C., Chalmers, R.M., Beeching, N.J., Probert, C., Giardiasis, Lamden K., 2016. BMJ 27, 355:i5369. https://doi.org/10.1136/bmj.i5369.
Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide Biol. Chem. 5 (1), 62–71. https://doi.org/10.1006/niox.2000.0319.
Mohammed Mahdy, A.K., Surin, J., Wan, K.L., Mohd-Adnan, A., Al-Mekhlafi, M.S.H., Lim, Ya.L., 2009. *Giardia intestinalis* genotypes: risk factors and correlation with clinical symptoms. Acta Trop. 112 (1), 67–70. https://doi.org/10.1016/j.actatropica.2009.06.012.
Molina, N., Pezzani, B., Ciarmela, M., Orden, A., Rosa, D., Apezteguía, M., Basualdo, J., Minvielle, M., 2011. Intestinal parasites and genotypes of *Giardia intestinalis* in school children from Berisso, Argentina. J. Infect. Dev. 5 (7), 527–534.
Munder, M., Eichmann, K., Morán, J.M., Centeno, F., Soler, G., Modolell, M., 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. J. Immunol. Baltim. Md 1950 163 (7), 3771–3777.
Pacheco, F.T.F., Silva, R.K.N.R., Martins, A.S., Oliveira, R.R., Alcântara-Neves, N.M., Silva, M.P., Soares, N.M., Teixeira, M.C.A., 2013. Differences in the detection of *Cryptosporidium* and *Isospora* (*Cystoisospora*) oocysts according to the fecal concentration or staining method used in a clinical laboratory. J. Parasitol. 99 (6), 1002–1008. https://doi.org/10.1645/12-33.1.
Plutzer, J., Ongerth, J., Karanis, P., 2010. *Giardia* taxonomy, phylogeny and epidemiology: facts and open questions. Int. J. Hyg. Environ. Health 213 (5), 321–333. https://doi.org/10.1016/j.ijheh.2010.06.005.
Puebla, L.J., Núñez, F.A., Fernández, Y.A., Fraga, J., Rivero, L.R., Millán, I.A., Valdés, L.A., Silva, I.M., 2014. Correlation of *Giardia duodenalis* assemblages with clinical and epidemiological data in Cuban children. Infect. Genet. Evol. 23, 7–12. https://doi.org/10.1016/j.meegid.2014.01.007.
Read, C.M., Monis, P.T., Thompson, R.C.A., 2004. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. Infect. Genet. Evol. 4 (2), 125–130. https://doi.org/10.1016/j.meegid.2004.02.001.
Ringqvist, E., Palm, J.E.D., Skarin, H., Hehl, A.B., Weiland, M., Davids, B.J., Reiner, D.S., Griffiths, W.J., Eckmann, L., Gillin, F.D., Svärd, S.G., 2008. Release of metabolic enzymes by *Giardia* in response to interaction with intestinal epithelial cells. Mol. Biochem. Parasitol. 159 (2), 85–91. https://doi.org/10.1016/j.molbiopara.2008.02.

005.
Rodríguez, O.L., Hagel, I., González, Y., Roque, M.E., Vásquez, N., López, E., Di Prisco, M.C., 2004. Secretory IgA antibody responses in Venezuelan children infected with Giardia duodenalis. J. Trop. Pediatr. 50 (2), 68–72. https://doi.org/10.1093/tropej/50.2.68.
Sahagún, J., Clavel, A., Goñi, P., Seral, C., Llorente, M.T., Castillo, F.J., Capilla, S., Arias, A., Gómez-Lus, R., 2008. Correlation between the presence of symptoms and the *Giardia duodenalis* genotype. Eur. J. Clin. Microbiol. Infect. Dis. 27 (1), 81–83. https://doi.org/10.1007/s10096-007-0404-3.
Scheller, J., Chalaris, A., Schmidt-Arras, D., Rose-John, S., 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim. Biophys. Acta 1813 (5), 878–888. https://doi.org/10.1016/j.bbamcr.2011.01.034.
Singer, S.M., 2015. Control of giardiasis by interleukin-17 in humans and mice–are the questions all answered? Clin. Vaccine Immunol. 23 (1), 2–5. https://doi.org/10.1128/CVI.00648-15.
Singer, S.M., Nash, T.E., 2000. T-cell-dependent control of acute *Giardia lamblia* infections in mice. Infect. Immun. 68 (1), 170–175.
Solaymani-Mohammadi, S., Singer, S.M., 2011. Host immunity and pathogen strain contribute to intestinal disaccharidase impairment following gut infection. J. Immunol. Baltim. Md 1950 187 (7), 3769–3775. https://doi.org/10.4049/jimmunol.1100606.
Stadelmann, B., Hanevik, K., Andersson, M.K., Bruserud, O., Svärd, S.G., 2013. The role of arginine and arginine-metabolizing enzymes during *Giardia* - host cell interactions in vitro. BMC Microbiol. 13, 256. https://doi.org/10.1186/1471-2180-13-256.
Stark, D., Barratt, J.L.N., van Hal, S., Marriott, D., Harkness, J., Ellis, J.T., 2009. Clinical significance of enteric protozoa in the immunosuppressed human population. Clin. Microbiol. Rev. 22 (4), 634–650. https://doi.org/10.1128/CMR.00017-09.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729. https://doi.org/10.1093/molbev/mst197.
Tatsch, E., Bochi, G.V., Pereira, R., da, S., Kober, H., Agertt, V.A., de Campos, M.M.A., Gomes, P., Duarte, M.M.M.F., Moresco, R.N., 2011. A simple and inexpensive automated technique for measurement of serum nitrite/nitrate. Clin. Biochem. 44 (4), 348–350. https://doi.org/10.1016/j.clinbiochem.2010.12.011.
Thompson, R.C.A., 2004. The zoonotic significance and molecular epidemiology of *Giardia* and giardiasis. Vet. Parasitol. 126 (1-2), 15–35. https://doi.org/10.1016/j.vetpar.2004.09.008.
Thompson, R.C.A., Ash, A., 2016. Molecular epidemiology of *Giardia* and *Cryptosporidium* infections. Infect. Genet. Evol. 40, 315–323. https://doi.org/10.1016/j.meegid.2015.09.028.
Velazquez, C., Beltran, M., Ontiveros, N., Rascon, L., Figueroa, D.C., Granados, A.J., Hernandez-Martinez, J., Hernandez, J., Astiazaran-Garcia, H., 2005. *Giardia lamblia* infection induces different secretory and systemic antibody responses in mice. Parasite Immunol. 27 (9), 351–356. https://doi.org/10.1111/j.1365-3024.2005.00793.x.
Venkatesan, P., Finch, R.G., Wakelin, D., 1996. Comparison of antibody and cytokine responses to primary *Giardia muris* infection in H-2 congenic strains of mice. Infect. Immun. 64 (11), 4525–4533.
Wakelin, D., Rose, M.E., Hesketh, P., Else, K.J., Grencis, R.K., 1993. Immunity to coccidiosis: genetic influences on lymphocyte and cytokine responses to infection with *Eimeria vermiformis* in inbred mice. Parasite Immunol. 15 (1), 11–19. https://doi.org/10.1111/j.1365-3024.1993.tb00567.x.
Weltman, J.K., 2000. Cytokines: regulators of eosinophilic inflammation. Allergy Asthma Proc. 21 (4), 203–207.
Yentur Doni, N., Yildiz Zeyrek, F., Simsek, Z., Gurses, G., Sahin, İ., 2015. Risk factors and relationship between intestinal parasites and the growth retardation and psychomotor development delays of children in Şanlıurfa, Turkey. Turk. Parazitolojii Derg. 39 (4), 270–276. https://doi.org/10.5152/tpd.2015.3620.
Zarebavani, M., Dargahi, D., Einollahi, N., Dashti, N., Safari, F., Rezaeian, M., 2017. Significance of nitric oxide level in giardiasis. Clin. Lab. 63 (1), 47–52. https://doi.org/10.7754/Clin.Lab.2016.160504.
Zhou, P., Li, E., Zhu, N., Robertson, J., Nash, T., Singer, S.M., 2003. Role of interleukin-6 in the control of acute and chronic *Giardia lamblia* infections in mice. Infect. Immun. 71 (3), 1566–1568. https://doi.org/10.1128/IAI.71.3.1566-1568.2003.
Zhou, P., Li, E., Shea-Donohue, T., Singer, S.M., 2007. Tumor necrosis factor α contributes to protection against *Giardia lamblia* infection in mice. Parasite Immunol. 29 (7), 367–374. https://doi.org/10.1111/j.1365-3024.2007.00953.x.