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Research paper

Occurrence and molecular characterization of *Giardia duodenalis* in child population from Colombia

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ABSTRACT

Giardia duodenalis is one of the most prevalent human intestinal parasite, with children living in developing countries being particularly at risk of infection. The occurrence and molecular diversity of *G. duodenalis* was investigated in stools specimens from 307 individuals aged one to nineteen years in Colombia. Samples were collected in three educational establishments (n: 163) and two hospital laboratories (n: 144) from urban and rural areas. Feces were concentrated using a biphasic sedimentation method and wet mounts of the sediment were examined by light microscopy. *G. duodenalis* assemblages and sub-assemblages were determined on positive samples by PCR of the triose phosphate isomerase (*tpi*), β-giardin (*bg*) and small-subunit (*ssu*) *rRNA* genes. *G. duodenalis* infection was detected by microscopy in 23 individuals (7.5%). The protozoan was more prevalent among specimens collected in educational establishments (11.6%) than in those obtained from hospital laboratories (2.8%). Infection was most common in individuals from urban areas and children aged 1–5 years. No significant association between diarrhea and infection could be demonstrated. Twenty *Giardia*-positive samples were successfully allocated to assemblage B (n: 11), sub-assemblage AII (n: 7), and assemblage A (n: 2). Results indicate the potential for transmission of *G. duodenalis* infection in children attending educational establishments and individuals from urban areas, where transmission seems to be primarily anthroponotic.

1. Introduction

Giardia duodenalis (syn. *G. intestinalis, G. lamblia*) is an intestinal protozoan responsible for acute and chronic diarrhea, and malabsorption syndrome in mammals. It is considered one of the most prevalent human intestinal parasite, with an estimated 2.8×10^8 cases *per annum*, and a major agent of parasitic waterborne outbreaks (Lane and Lloyd, 2002; Efstratiou et al., 2017). *G. duodenalis* infection is a significant cause of morbidity in children, especially in developing countries where poor sanitation, housing and socioeconomic conditions are risk factors that contribute to transmission (Botero-Garcés et al., 2009). Diagnosis is mainly based on the detection of cysts in stool specimens using microscopy or immunology-based methods, but molecular tools are needed to investigate the intraspecific diversity and subsequent classification into assemblages and sub-assemblages (Adeyemo et al., 2018). Eight genetically distinct groups (assemblages), designated A to H, have been described so far. Two major potentially zoonotic assemblages (A and B) are found in both humans and animals, and further subdivided into at least eight sub-assemblages that show preferences for human or animal hosts (AI to AIV, BI to BIV). The remaining six assemblages (C to H) are host-specific (Monis et al., 2003; Ryan and Cacciò, 2013).

Giardiasis is a significant public health problem in Latin America. Prevalence values ranging from 5.1% to 36% have been reported by microscopy in Venezuela, Argentina, Perú or Brazil (Molina et al., 2011; Villegas et al., 2012; Campos Ponce et al., 2013; Rosado-García et al., 2017; Seguí et al., 2018). In Colombia, incidence rates of Giardiasis ranging from 13.35 to 183.69 cases/100,000 population and costs of over US$ 18.4 million have been estimated in the period 2009–2016 (Bedoya-Arias et al., 2018). Laws for the mandatory surveillance of the protozoan in water for human consumption were established in 2007 in this country, although regulations are still in the implementation period by service companies (Rosado-García et al., 2017). Nevertheless,

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studies on the molecular characterization of *Giardia* are limited in Colombia. The most recent studies have revealed a prevalence of *G. duodenalis* infection from 23.7% to 48.1% based on feces microscopy, with the presence of a range of sub-assemblages (AI, AII, BIII and BIV) within assemblages A and B (Sánchez et al., 2017; Villalba-Vizcaíno et al., 2018). In this current study, the occurrence, distribution and molecular diversity of *G. duodenalis* was investigated in different groups of the Colombian human population. Samples from children attending day-care centers and schools, and specimens submitted for diagnosis to hospital laboratories were investigated. Sampling was conducted in the country's capital city and two Departments where the molecular epidemiology of this protozoa had not been previously studied.

2. Material and methods

2.1. Sampling procedure and microscopic detection of Giardia

A convenience sampling study was conducted during 2014. Stool specimens from 307 individuals aged one to nineteen years were collected in three educational establishments (daycare centers/schools) (n: 163) and two hospital laboratories (n: 144) from three different locations in central and southwest Colombia, including urban areas [Bogotá D.C. (n: 159) and Department of Valle del Cauca (n: 40)] and rural areas [Department of Nariño (n: 108)]. Participants were divided into four age groups: babies (younger than 1 year), toddlers and pre-schoolers (1–5 years), grade schoolers (6–12 years) and teenagers (> 12 years) (Table 1). The age was unknown for 14 individuals. Only one fecal sample per person could be collected. The presence/absence of diarrhea was recorded for all participants but no information about other clinical symptoms or epidemiologic data (*i.e.* sanitary or socio-economic conditions) was available. Diarrhea was considered according to the current WHO definition as the passage of three or more loose or liquid stools per day. Fecal samples were concentrated using a biphasic (water/ethyl acetate) sedimentation method. Wet mounts and iodine-stained preparations of the sediment were examined by light microscopy for *Giardia* cysts and other gastrointestinal parasites (Ramo et al., 2017). Infection intensities by *Giardia* spp. were evaluated semi-quantitatively according to the average number of cysts per field at ×200 magnifications as follows: few (0–1 cysts), moderate (1–5 cysts) and many (> 5 cysts). Smears of the sediment were stained by the modified Ziehl-Neelsen technique (Henriksen and Pohlenz, 1981) and examined for the presence of *Cryptosporidium* oocysts.

2.2. Molecular characterization of Giardia

The molecular characterization of *G. duodenalis* was conducted on microscopy-positive samples. For this purpose, total DNA was extracted from 200 µl. of each sediment using the QIAamp DNA minikit (Qiagen) according to the manufacturer's instructions. An initial step involving three cycles of freezing with liquid nitrogen (1 min) and thawing at 100 °C (5 min), followed by incubation at 56 °C for 30 min in lysis buffer containing proteinase K and purification over a spin column was incorporated into the protocol. DNA extracts were stored at −20 °C. *Giardia* species and assemblages were determined by nested PCRs of the small-subunit rRNA (*ssu-rRNA*), β-giardin (*bg*) and triose phosphate isomerase (*tpi*) genes, using primers and protocols previously described (Appelbee et al., 2003; Sulaiman et al., 2003; Lalle et al., 2005). PCR products were first separated on 1.5% agarose gels, and visualized by staining with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA) to confirm DNA amplification.

Positive PCR products were purified and sequenced in both directions on a 3500xL Genetic Analyser (Applied Biosystems®, Life Technologies) according to the manufacturer's instructions. Nucleotide sequences were edited and aligned with reference sequences from GenBank using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and Bioedit version 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), respectively. The consensus sequences of both the sense and the anti-sense strands were analyzed using a BLAST search on the NCBI databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Representative sequences generated in the current study were deposited in the GenBank database under accession numbers MG924430, MG924431, MG924451 to MG924454, and MG924457 to MG924462.

2.3. Statistical analysis

Chi-square and Fisher's exact test were used to compare infection rates between different groups according to the origin of specimens and the presence of diarrhea. A $P < 0.05$ value was required for significance.

3. Results

Cysts of *G. duodenalis* were identified by microscopy in stool specimens from 23 individuals (7.5%). Semiquantitative counting of *Giardia* positive preparations showed that most patients excreted few (43.5%) or moderate (47.8%) number of cysts, and only 8.7% excreted many cysts. Infection was not detected in children younger than 1 year nor teenagers older than 16 years. The protozoan was most prevalent in children aged 1–5 years (11.6%), although differences were significant only with children younger than 1 year ($P < 0.05$). *Giardia*-positive cases were more common among specimens collected in educational centers (11.6%) than in those obtained from hospital laboratories (2.8%) ($P < 0.005$). Moreover, *G. duodenalis* was more frequent in specimens from urban areas (Bogotá D.C.: 8.2%; Valle del Cauca: 17.5%) than in those from rural areas (Nariño: 2.8%) ($P < 0.05$) (Table 1). Diarrhea was recorded in 109 patients (35.5%) but no statistical association with *G. duodenalis* infection was seen. In fact, the protozoan was more common in individuals without diarrhea (8.6%) than in those with diarrhea (5.5%) (Table 2). The presence of other parasites was identified in 74 stool specimens (24.1%), including a range of enteric protozoa in either mono-infections or mixed infections. In addition to *G. duodenalis*, the most prevalent parasites were *Cryptosporidium* spp. (9.1%) and *Blastocystis* spp. (6.5%) (Table 3).

Twenty of the twenty-three *Giardia*-positive samples by microscopy were successfully amplified at one or more of the three loci (Table 4).

Table 1
Microscopy positive cases for *Giardia* [infected/studies (%)] in individuals from three locations in Colombia (Bogotá DC., Departments of Valle del Cauca and Nariño) according to the place of sampling and geographical area.

Age category (years)	Sampling		Geographical area		Total
	Educational establishments	Hospital laboratories	Urban	Rural	
< 1	0/1	0/45	0/46	0/0	0/46
1–5	13/38 (34.2)	0/74	13/112 (11.6)	0/0	13/112 (11.6)
6–12	3/66 (4.5)	3/13 (23.1)	4/20 (20)	2/59 (3.4)	6/79 (7.6)
13–19	3/54 (5.5)	1/2 (50)	3/7 (42.9)	1/49 (2)	4/56 (7.1)
Unknown	0/4	0/10	0/14	0/0	0/14
Total	19/163 (11.6)	4/144 (2.8)	20/199 (10.1)	3/108 (2.8)	23/307 (7.5)

Table 2
Microscopy positive cases for *Giardia* [infected/studies (%)] by clinical status (presence/absence of diarrhea) in individuals from three locations in Colombia (Bogotá, DC., Departments of Valle del Cauca and Nariño).

Age category (years)	Diarrhea	No diarrhea	Total (%)
< 1	0/17	0/29	0/46
1–5	3/52 (5.8)	10/60 (16.7)	13/112 (11.6)
6–12	1/11 (9.1)	1/23 (4.3)	6/79 (7.6)
13–19	2/25 (8)	6/69 (8.7)	4/56 (7.1)
Unknown	0/2	0/12	0/14
Total	6/109 (5.5)	17/198 (8.6)	23/307 (7.5)

Sequence analysis of the sixteen PCR positive specimens at the *tpi* locus revealed *G. duodenalis* assemblage B (n: 9) and AII (n: 7). All these isolates were identical to sequences previously reported in humans or animals, except for two assemblage B isolates that exhibited a single nucleotide polymorphism with reference sequence AY228628. Eight of the fourteen isolates that amplified at the *bg* locus were typed as *G. duodenalis* assemblage B and had 100% identity with reference sequence AY072727, except for one isolate that showed a single nucleotide polymorphism. The remaining six isolates were typed as sub-assemblage AII. Seven isolates testing negative at *tpi* and/or *bg* loci were further amplified at the *ssu rRNA* gene and allocated to assemblage B (n: 4) and assemblage A (n: 3). The combined use of the three loci allowed the allocation of *G. duodenalis* isolates to assemblage B (n: 11) and sub-assemblage AII (n: 7). Two additional specimens that only amplified at the *ssu-rRNA* gene were identified as assemblage A. No mixed assemblage infections or discordant genotyping results among the three loci were detected.

4. Discussion

G. duodenalis is among the most frequent parasites infecting humans and still represents a problem of public health around the globe. In the present survey, 7.5% of stool specimens from humans in different areas of Colombia were found positive by microscopy for *G. duodenalis* cysts. This percentage is similar to that reported in children 5–12 years old from Bogotá (6.3%) (Boeke et al., 2010) or children 1–5 years old from daycare centers in Ibagué (Tolima) (11.2%) (Rodríguez et al., 2014), but lower than seen in other locations where *G. duodenalis* was reported as the most common intestinal parasite, such as the Department of Antioquia (27.6%) or the Colombian Caribbean coast (48.1%) (Botero-Garcés et al., 2009; Villalba-Vizcaíno et al., 2018). An even higher point-prevalence was seen in Armenia (Department of Quindío) after the 1999 earthquake, where *G. duodenalis* was identified in 60.4% of children aged between 3 and 13 years as a consequence of poor hygienic and health conditions and the lack of potable water (Lora-Suarez et al., 2002).

It is significant to mention that infection rates detected in this study may be an underestimation since molecular methods have been recognized to be much more sensitive than microscopic techniques. In the Department of Cundinamarca, the prevalence of *G. duodenalis* infection among children was 13% by microscopy and 76–80% by PCR, depending on the molecular marker (Ramírez et al., 2015). A similar discrepancy was reported in indigenous children from the Colombian Amazon basin (23.7% *versus* 64.8%) (Sánchez et al., 2017). The low sensitivity of laboratory diagnosis might also be due to the intermittent excretion of *G. duodenalis* cysts in stool, as demonstrated by an increase in the number of positive samples after repeated sampling examinations (Uchôa et al., 2017).

Attendance to daycare centers has been reported as an important risk factor for infection with *G. duodenalis*, which could explain the highest prevalence found in toddlers and pre-schoolers (Davies et al., 2009). Most *G. duodenalis* infections are self-limiting and symptoms may include chronic diarrhea, abdominal pain, severe malabsorption and weight loss (Feng and Xiao, 2011). However, asymptomatic giardiasis occurs frequently, especially in endemic areas from developing countries where reinfections are common (Squire and Ryan, 2017). Prevalence values of asymptomatic infections as high as 50% have been found in rural southern India and Bangladesh, or up to 80% in Argentina (Molina et al., 2011; Laishram et al., 2012; Taniuchi et al., 2013). Previous studies with Colombian school children showed that *G. duodenalis* was not significantly associated with gastrointestinal symptoms (Boeke et al., 2010). In the current study, diarrhea was reported for only 26% (6/23) of *Giardia*-infected individuals, with most of *Giardia*-positive cases seen among children without diarrhea from educational centers (16/23). This finding highlights the relevance of subclinical infections in the transmission of *G. duodenalis* among this group of the population.

The geographical distribution of *G. duodenalis* assemblages in humans does not have a uniform pattern worldwide. Multifactorial causes including the transmission pathways as well as socio-epidemiological and geo-climatic factors have been indicated to explain these differences (Ramírez et al., 2015; Azcona-Gutiérrez et al., 2017). Studies in Latin American countries have shown that humans are mostly infected by *G. duodenalis* assemblage A in Mexico (Eligio-García et al., 2008) or assemblage B in Nicaragua and Argentina (Lebbad et al., 2008; Minvielle et al., 2008). In contrast, no significant differences between assemblage distribution were seen in Cuba (Puebla et al., 2014). In Brazil, differences in the distribution of assemblages and sub-assemblages were found in three distinct biomes of the northeastern regions of the country (Nunes et al., 2018), while assemblage B predominated over assemblage A in children in the city of Fortaleza (Kohli et al., 2008), and both assemblages were proportionally distributed (52.7% *versus* 47.3%) in human clinical samples from Rio de Janerio (Faria et al., 2016). In Colombia, assemblage B was much more common than Assemblage A in children from the northern Department of Bolivar (91.1% *versus* 8.9%, respectively) or the center of the country (Cundinamarca) (90% *versus* 3%, respectively, and mixed infections in 7%). However, the distribution of assemblage B (52%) and assemblage A

Table 3
Microscopy positive cases for different enteric parasites [infected (%)] in individuals from three locations in Colombia (Bogotá DC., Departments of Valle del Cauca and Nariño).

Age category (years)	Enteric parasite[a]											Total (%)
	1	2	3	4	5	6	7	8	9	10	11	
< 1	0	0	0	0	0	0	0	0	0	0	0	0/46
1–5	10 (8.9)	13 (11.6)	3 (2.7)	0	0	0	0	0	0	0	0	26/112 (23.2)
6–12	10 (12.7)	5 (6.3)	9 (11.4)	1 (1.3)	0	1 (1.3)			1 (1.3)	1 (1.3)	0	28/79 (35.4)
13–19	5 (8.9)	3 (5.4)	5 (8.9)	0	2 (3.6)	1 (1.8)	1 (1.8)	1 (1.8)	1 (1.8)	0	1 (1.8)	20/56 (35.7)
Unknown	0	0	0	0	0	0	0	0	0	0	0	0/14
Total	25 (8.1)	21 (6.8)	17 (5.5)	1 (0.3)	2 (0.7)	2 (0.7)	1 (0.3)	1 (0.3)	2 (0.7)	1 (0.3)	1 (0.3)	74 (24.1)

[a] 1. *Cryptosporidium* spp. 2. *G. duodenalis*. 3. *Blastocystis* spp. 4. *Entamoeba coli*. 5. *Entamoeba histolytica/dispar* complex. 6. *Endolimax nana*. 7. *Cryptosporidium* spp. + *Blastocystis* spp. 8. *Cryptosporidium* spp. + *E. nana*. 9. *G. duodenalis* + *Blastocystis* spp. 10. *Cryptosporidium* spp. + *E. coli* + *E. histolytica/dispar* complex. 11. *E. coli* + *E. histolytica/dispar* complex.

Table 4
Assemblages and sub-assemblages of *Giardia duodenalis* identified in humans in Colombia at triosephosphate isomerase (*tpi*), β-giardin (*bg*) and small-subunit rRNA (*ssu rRNA*) loci. Similarity (%) with reference sequences from GenBank is indicated in brackets.

Sample ID	*tpi*	*bg*	*ssu rRNA*	Assemblage/Sub-assemb.
91H	n.a	n.a	B (100% KJ888984)	B
5JB	AII (100% U57897)	AII (100% AY072724)	n.t	AII
8JB	AII (100% U57897)	AII (100% AY072723)	n.t	AII
11JB	AII (100% U57897)	AII (100% AY072723)	n.t	AII
14JB	B (100% AY228628)	n.a	B (100% KJ888984)	B
19JB	B (100% KJ888985)	B (100% AY072727)	n.t	B
23JB	B (100% AY228628)	B (100% AY072727)	n.t	B
27JB	B (100% AY228628)	B (100% AY072727)	n.t	B
30JB	B (99% AY228628)	B (100% AY072727)	n.t	B
33JB	B (100% AY228628)	B (100% AY072727)	n.t	B
34JB	B (99% AY228628)	n.a	B (100% KJ888984)	B
36JB	B (100% AY228628)	B (100% AY072727)	n.t	B
42JB	B (100% AY228628)	B (100% AY072727)	n.t	B
43JB	n.a	B (99% AY072727)	B (100% KJ888984)	B
5V	AII (100% U57897)	AII (100% AY072723)	n.t	AII
96N	n.a	n.a	A (100% KF843922)	A
105N	AII (100% U57897)	n.a	A (100% KF843922)	AII
25V	n.a	n.a	A (100% KF843922)	A
26V	AII (100% KJ888992)	AII (100% AY072724)	n.t	AII
46V	AII (100% U57897)	AII (100% AY072723)	n.t	AII

n.a.: not amplified.
n.t.: not tested.

(48%) was similar among children from daycare centers in the Department of Tolima (Rodríguez et al., 2014; Arroyo-Salgado et al., 2014; Ramírez et al., 2015) and only assemblage A was identified among 47 samples collected in the city of Santa Marta at the Colombian Caribbean coast (Villalba-Vizcaíno et al., 2018).

In this study, more than a half of the successfully typed specimens (11/20) were allocated to assemblage B, which is more associated to humans than zoonotic (Ryan and Cacciò, 2013). It is worth mentioning that all these assemblage B isolates were collected in Bogotá D.C. and all but one came from 1 to 5 year old children attending daycare centers or schools, suggesting the predominance of human-to-human transmission in this environment. The remaining isolates (n: 9) were allocated to assemblage A, and most of them to sub-assemblage AII, which is also predominantly detected in humans (Ryan and Cacciò, 2013). No human infections with sub-assemblages AI and AIII were found. Sub-assemblage AI has a wide host range and sub-assemblage AIII is more specific for wild ungulates (Monis et al., 2003). Some authors have found a potential correlation between the *G. duodenalis* assemblage and the clinical status or even the presence of defined symptom patterns (Sahagún et al., 2008; Puebla et al., 2014). This association was not seen in the current study, since all six *Giardia*-positive cases from patients with diarrhea were allocated to either assemblages B (n: 3) or AII (n: 3).

This study is a contribution to our knowledge of the molecular diversity of *G. duodenalis* in humans in South America. Infection rates were similar to those seen in other areas of Colombia, with positive cases being more common among children aged 1–5 years from urban areas and those attending daycare centers and schools as compared to specimens from individuals submitted for diagnosis to hospital laboratories. Most *G. duodenalis* infections were not linked to diarrheal disease, which highlights the risk of transmission of this protozoan in educational establishments. Results of the molecular study suggest that *G. duodenalis* infections are primarily anthroponotic in the groups of the population surveyed.

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Ethical approval

The procedures followed were in accordance with the ethical standards of the Helsinki Declaration (1964, amended most recently in 2008) of the World Medical Association. The study design and consent procedures involved in this survey have been approved by the Research Ethics Committee of the Universidad del Valle (Act No: 04-013) to guarantee the voluntary character of the participation in the study and the anonymity and confidentiality of participants. Written informed consent was obtained from study participants and assent was given by guardians for persons under consenting age.

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

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