Molecular analysis of *Giardia duodenalis* isolates from symptomatic and asymptomatic children from La Habana, Cuba

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ABSTRACT

Giardiasis is considered the most common intestinal parasitic disease in humans worldwide. In Cuba, this infection has particularly a strong clinical impact on the child population. *Giardia duodenalis* is a highly diverse protozoan, which comprises a complex of eight morphologically identical genetic assemblages, further divided into sub-assemblages. The present study used triose phosphate isomerase (*tpi*) and small-subunit ribosomal RNA (SSU rRNA) genes as genetic markers for the identification of *G. duodenalis* assemblages and sub-assemblages in correlation with clinical and epidemiological data in children attended at the Paediatric Hospital "William Soler" and at Pedro Kouri Institute, between 2015 and 2016. A prevalence of 8% of *G. duodenalis* infection was recorded in stool samples after concentration techniques from 68 children out of 847 analysed. A 100% detection of *Giardia* DNA was achieved by a SSU-rRNA PCR, whereas DNA from 63 of 68 (92.6%) was successfully amplified by *tpi*-PCR. By this assemblage-specific *tpi*-PCR 32 (50.8%) assemblage B, 17 (27.0%) assemblage A and 14 (22.2%) mixed infection (A + B) were identified. Assemblage B was significantly ($P < 0.02$) more frequently found in children with diarrhoea. Sequence analysis of the *tpi* gene of *Giardia* isolates from symptomatic children showed that assemblage A belonged to the sub-assemblage AII, and 4 sub assemblages BIV and 1 sub assemblage BIII were also recorded. Only 2 discordant genotyping results were observed by phylogenetic comparison of SSU-rRNA and *tpi* sequences. Further studies with novel molecular tools for a better discrimination at the sub-assemblage level are needed to identify the dynamics of spread of giardiasis and to verify possible correlations between *Giardia* genetic diversity and clinical manifestation.

1. Introduction

Giardiasis is a worldwide parasitic infection caused by *Giardia duodenalis* (syn. *G. intestinalis*, *G. lamblia*). This flagellate causes one of the most frequently diagnosed intestinal protozoal infections reported, especially in areas of developing countries (Feng and Xiao,

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2011). Annually, there are > 200 million cases of notified giardiasis according to the World Health Organization (WHO) and due to the negative impact on growth and cognitive development in childhood, giardiasis was included in the WHO's 'Neglected Diseases Initiative' (Savioli et al., 2006).

Giardiasis is a frequently intestinal parasitic infection which contributes to approximately 5% of gastroenteritis incidences in developed countries and as high as 15–55% in developing countries (Alum et al., 2012). In Cuba, infection by *Giardia* represents one of the most important intestinal parasitic infections in children (Núñez et al., 2003).

Although this parasite was not considered pathogenic until 1978, currently is accepted that the infection by *Giardia* ranges from asymptomatic cyst passage and acute diarrhoea to a syndrome of chronic diarrhoea, weight loss, and malabsorption. The pathogenic mechanisms of this disease are now considered multifactorial, including apoptosis of enterocytes, loss of epithelial-barrier function, hypersecretion of chloride ions, inhibition of brush-boarder enzymes, and malabsorption of glucose, water, and sodium ions (Ankarklev et al., 2010).

A considerable amount of data has shown that *G. duodenalis* should be considered as a species complex, whose members show little variation in their morphology, and they can be assigned to at least eight distinct genetic groups or assemblages (A to H) based on protein or DNA polymorphisms (Cacciò and Ryan, 2008; Ryan and Cacciò, 2013). Studies in the molecular characterization of *Giardia* carried out in humans and animals at several loci, including glutamate dehydrogenase (*gdh*), β-giardin, small subunit ribosomal RNA (SSU rRNA), and the triose phosphate isomerase (*tpi*) genes, have revealed that two major genetic groups or assemblages, designed as A and B are responsible for causing the majority of human infections (Feng and Xiao, 2011).

Although several clinical and epidemiological studies of giardiasis have been conducted in Cuba (Núñez et al., 2003; Escobedo et al., 2007; Cañete et al., 2012; Escobedo et al., 2016), there are few works which have been addressed to the molecular analyses of this intestinal parasite associated with the symptomatology manifested in children (Pelayo et al., 2008; Puebla et al., 2014; Jerez-Puebla et al., 2015). The previous studies made by Pelayo et al. (2008) and Puebla et al. (2014) in a group of children from La Habana, have found that children harbouring assemblage B of *Giardia* were more likely to have symptomatic infections than children with isolates from assemblage A.

In the present study, we performed a molecular characterization of *G. duodenalis* isolated from children, addressing the SSU-rRNA and the triose phosphate isomerase (*tpi*) genes, which are among the most commonly genetic markers used for genotyping studies. In particular, we determined the prevalence of different *G. duodenalis* assemblages among children, and the associations with clinical and epidemiological data collected. Furthermore we assessed the intra-assemblage level of genetic variation at the different loci for assemblages A and B.

2. Materials and methods

2.1. Study design

A descriptive cross-sectional study was conducted in 847 children remitted to the Paediatric Hospital "William Soler" with gastrointestinal disturbances and from kindergartens and primary schools located in the municipalities of Boyeros, Arroyo Naranjo and La Lisa from the province of La Habana. As part of an intestinal parasite surveillance addressed by the Ministry of Public Health, which takes place every year all over the country, the present investigation was performed in the Parasitology Department from the Academic Paediatric Hospital "William Soler" and the Institute of Tropical Medicine "Pedro Kouri" in the period between January 2015 and March 2016.

Epidemiological and clinical data from each *Giardia*-positive children were recorded in standard questionnaires that had been completed by the parents, or caregivers, of the patients, following informed consent of their agreement to participate in this investigation. These surveillance data included information about some epidemiological variables (sex, age, residing area, ethnic group) and general health status of the participants (i.e. symptoms related to intestinal parasitic infections such as diarrhoea, nausea, vomiting, flatulence, fatigue, loss of weight, abdominal pain and a history of receiving anthelmintic treatment).

All cases in which co-infection with other intestinal parasites of medical importance was diagnosed, were not included in the investigation correlating infecting assemblages and clinical picture developed in children.

2.2. Stool sample collection and DNA extraction

Fresh faecal samples were collected in 50-mL screw-caps clearly labelled containers. Three stool samples were examined from 847 children for intestinal parasites by a wet smear stained with Lugol's iodine and followed by formalin ethyl acetate concentration techniques (Garcia, 2001). In parallel, these samples were processed by the brine flotation technique for the detection of parasite eggs. The diarrhoeic stool samples were stained by modified acid-fast for the diagnosis of the following intestinal coccidians: *Cryptosporidium* spp., *Cyclospora cayetanensis* and *Cystoisospora belli* (Garcia, 2001).

All *Giardia*-positive cyst/trophozoites were preserved in 2.5% potassium dichromate and stored at − 20 °C for further molecular analysis at the Institute of Tropical Medicine "Pedro Kourí".

Giardia cysts were purified and concentrated from faecal samples in a sucrose gradient with a specific gravity of 0.85 M, following the protocol described elsewhere (Babaei et al., 2011). The cyst wall was disrupted by 5–8 freeze–thaw cycles in liquid nitrogen alternated with a 95 °C water bath.

Whole DNA was extracted directly from the specimens following the conventional DNA extraction method of phenol/chloroform/ isoamyl alcohol (PCI). Briefly, purified cysts were mixed with 300 μL of buffer lysis (50 mM Tris–HCl, pH 7.5; 25 mM EDTA, 25 mM

NaCl), and 1% of sodium dodecyl sulphate (SDS). After adding 100 μg/mL of proteinase K, the suspension was incubated at 56 °C for 2 h. The DNA lysate was then treated with phenol/chloroform/isoamyl alcohol (24:24:1), followed by chloroform/isoamyl alcohol (24:1) according to Sambrook and Russell (2001). The DNA was precipitated by the addition of 1 mL chilled ethanol and stored at − 20 °C until use. The dried DNA was re-suspended in 50 μL distilled water and used as a template for PCR.

2.3. Amplification of tpi and SSU-rRNA genes

Amplification of the *tpi* gene was performed as a single PCR according to Bertrand et al. (2005). The PCR reaction included 5 μL of DNA template and was done with 1 unit of GoTaq® DNA Polymerase (Applied Biosystems, USA) in a total volume of 25 μL comprising 5.0 μL of 5 × PCR buffer (Applied Biosystems, USA), 0.2 mM of each deoxynucleoside triphosphate (dNTPs) (Applied Biosystems, USA), and 0.4 μM of A-for and B-for forward primers and 0.4 μM of A-rev and B-rev; reverse primers. Cycling parameters were 15 min at 95 °C (initial heat activation step), followed by 50 cycles of 30 s at 94 °C, 30 s at 62 °C, and 30 s at 72 °C, with a final extension of 7 min at 72 °C. A 148-bp fragment of the assemblage A gene and 81-bp fragment of assemblage B gene were obtained with these primers.

DNA from axenic cultures of *G. duodenalis* strains WB-C6 (assemblage A) and GS (Assemblage B), were used as positive controls, while ultrapure H_2O instead of DNA was included in negative control PCRs.

The DNA amplification products from the SSU rRNA and the *tpi* PCR were visualized on 2% agarose gels stained with 0.5 μg/mL of ethidium bromide (Sambrook and Russell, 2001).

For sequence analysis of the *tpi* gene, a nested PCR approach was done by amplification of 530-bp segment of the *tpi* gene according to Sulaiman et al. (2003). For the primary PCR, a PCR product of 605 bp was amplified by using primers AL3543 and AL3546. The PCR reaction included 5 μL of DNA template and was done with 1 unit of GoTaq® DNA Polymerase (Applied Biosystems, USA) in a total volume of 25 μL comprising 5.0 μL of 5 × PCR buffer (Applied Biosystems, USA), 0.2 mM of each deoxynucleoside triphosphate (dNTPs) (Applied Biosystems, USA), and 0.5 μM of primers. The reactions were performed for 35 cycles (94 °C for 45 s, 50 °C for 45 s, and 72 °C for 60 s) in a BIO RAD T100™ thermocycler, with an initial hot start (94 °C for 5 min) and a final extension (72 °C for 10 min).

For the secondary PCR, a fragment of 530 bp was amplified by using primers AL3544 and AL3545 and 2.5 μL of primary PCR reaction. The conditions for the secondary PCR were identical to the primary PCR. The PCR products were analysed by 2.0% agarose gel electrophoresis and visualized after ethidium bromide staining (Sulaiman et al., 2003).

Giardia DNA was detected in a nested SSU-rRNA PCR by using specific primers RH11 and RH4 to amplify a 130 bp product from the SSU-rRNA gene (Hopkins et al., 1997) and the primers GiarF and GiarR for the secondary PCR (Read et al., 2002) as previously described.

PCR reactions were performed in a total volume of 25 μL, with the primary PCR reaction mixture containing 1unit GoTaq® DNA Polymerase (Applied Biosystems, USA) with 5.0 μL of 5 × PCR buffer GeneAmp® PCR buffer kit (Applied Biosystems, USA), 0.5 μM of RH11 and RH4 primers, 0.2 mM of deoxynucleotide triphosphates (dNTPs) and 1.25 μL of dimethyl sulfoxide (DMSO). The thermocycler (BIO RAD T100™, Singapore) conditions consisted of 96 °C for 5 min for 1 cycle, 96 °C for 45 s, a50°C for 30 s and 72 °C for 45 s for 35 cycles followed by 72 °C for 7 min.

Two microlitres from the first PCR were used as template in the secondary PCR with forward GiarF, and reverse GiarR primers under the same amplification conditions with the exception that 55 °C instead of 50 °C was applied as annealing temperature (Read et al., 2002).

2.4. Giardia phylogenetic analysis

In order to assess the genetic variability of *Giardia* isolates from symptomatic children, PCR-sequencing-based genotyping using marker genes *tpi* and SSU-rRNA was performed.

PCR products were purified using the QIAquick® PCR Purification kit (QIAGEN Ltd.), and were sequenced in both directions with the respective forward and reverse primers using the Beckman Coulter Genomics sequencing system (Essex, United Kingdom).

The sequences obtained were aligned using the BioEdit v7.0.1 package, then compared with gene sequences of *Giardia* available from the NCBI server, using the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the *Giardia* assemblage, sub-assemblage and sub-type.

A phylogenetic tree for *tpi* and SSU-rRNA was constructed based on the neighbour-joining algorithm. Distance-based analyses were conducted using Kimura 2-parameter distance estimates including alignments obtained from ClustalW using the Molecular Evolutionary Genetics Analysis version 6.06 (MEGA 6) (Tamura et al., 2013). Bootstrap proportions were calculated by the analysis of 1000 replicates of the phylogenetic tree.

SSU-RNA reference sequences used for *G. duodenalis* were sub assemblage AI: M54878, AII: AF199446, AIII: DQ100287, sub assemblage BIII: AF199447 and BIV: AF113898. *Trichomonas tenax* U37711 was used as outgroup. Reference sequences for *tpi* gene from *G. duodenalis* were sub assemblage AI: L02120, AII: U57897, AIII: DQ650647, sub assemblage BIII: AF069561, BIV: AF069560; assemblage C: AY228641, assemblage D: DQ246216, assemblage E: KF891311 and assemblage G: EU781013. *Giardia ardeae* AF069564, was used as outgroup.

2.5. Statistical analysis

The Epi-Info 6.04 (Centers for Disease Control and Prevention; Atlanta, GA, USA) and EPIDAT 3.1 statistical programmes were used for the selection of the sampled population and the analyses of data derived from questionnaires and parasitological examinations, respectively. Chi-square test and proportion tests were employed to compare categorical variables. The Fisher's exact test was used when required by data scarcity.

The medians of age in years, was compared using the Kruskal-Wallis non parametric test. The P values < 0.05 were considered as statistically significant for all tests. The odds ratio (OR) with 95% confidence interval (CI) were performed as measures of association.

2.6. Ethics statement

The study was approved by the Ethics Committee of the "Pedro Kourí" Institute, under the following name: Genetic characterization of *G. duodenalis* isolates from symptomatic and asymptomatic children and its clinical and epidemiological correlation (reference CEI-IPK-15-13).

All parents and/or tutors of participating children were informed about the study objectives and voluntary written consent was sought and obtained before inclusion.

3. Results

3.1. Prevalence and identification of G. duodenalis assemblages and mixed assemblages

From 847 children examined, a total of 68 (8.0%) scored positive for *Giardia* by microscopy. Co-infection with other parasites was found in 11 children (4 cases with *Entamoeba histolytica/E. dispar*, 2 *Enterobius vermicularis*, 2 *Trichuris trichiura*, 1 *Ascaris lumbricoides*, 1 *Cyclospora cayetanensis*, 1 *Cryptosporidium* spp.).

PCR products of the expected size were successfully amplified by the SSU-rRNA in 100% of microscopically positive cases, and in 63/68 (92.6%) by the assemblage specific tpi PCR.

The PCR-*tpi* classified 32 (50.8%) isolates as assemblage B and 17 (27.0%) as assemblage A, whereas in 14 (22.2%) cases mixed infections (A + B) were recorded.

3.2. Associated epidemiological and clinical factors for Giardia duodenalis assemblage infections

The association of *G. duodenalis* assemblages causing infections and sociodemographic characteristics are shown in Table 1. No statistically difference was found between assemblage of *Giardia* and the variables sex, educational institution, residence (urban or rural) and age ($P > 0.05$).

The clinical data and the association with infections by *G. duodenalis* assemblages are shown in Table 2. Assemblage B of *Giardia* was significantly ($P < 0.02$) more associated with diarrhoea in children. No correlation was found between other symptoms and signs with specific assemblage infections compared with the analysed groups ($P > 0.05$).

Table 1
Association between *Giardia duodenalis* assemblages and epidemiological data related to giardiasis in studied children.

Characteristics	Assemblage B (n = 32)		Assemblage A (n = 17)		Mixed assemblages A + B (n = 14)		P values
	n	%	n	%	n	%	
Sex							
Male	18	56.3	13	76.5	7	50.0	P = 0,26
Female	14	43.7	4	23.5	7	50.0	P = 0,26
Institution							
Kindergarten	17	53.1	7	41.2	5	35.7	P = 0,49
School	10	31.3	7	41.2	5	35.7	P = 0,79
House	5	15.6	3	17.6	4	28.6	P = 0,58
Residing area							
Urban	28	87.5	16	94.1	12	85.7	P = 0,71
Rural	4	12.5	1	5.9	2	14.3	P = 0,68
Age							
Mean value (± SD)	4.6 (± 3.7)		5.4 (± 4.3)		4.4 (± 2.9)		P = 0,71[a]
Median	3.0		4.0		4.0		P = 0,6759
Interval	1–16		1–13		1–13		

[a] Kruskal-Wallis test.

Table 2
Association between clinical data and infecting *G. duodenalis* assemblages in children only infected with *Giardia* (no mixed intestinal infection).

Clinical data	Assemblage B (n = 28)		Assemblage A (n = 13)		Mixed assemblages A + B (n = 11)		P values
	n	%	n	%	n	%	
Symptomatic children	24	85.7	10	76.9	7	63.6	P = 0,31
Diarrhoea	13	46.4	2	15.4	1	9.1	P = 0,02[a]
Abdominal pain	13	46.4	6	46.1	3	27.3	P = 0,52
Flatulence	8	28.6	1	7.7	5	45.5	P = 0,11
Nausea	4	14.3	1	7.7	2	18.2	P = 0,74
Vomiting	2	7.1	4	30.8	3	27.3	P = 0,11
Headache	1	3.6	0	0	0	0	P = 0,65
Anorexia	12	42.9	4	30.8	1	9.1	P = 0,09
Fever	4	14.3	4	30.8	1	9.1	P = 0,33
Loss of weight	12	42.9	4	30.8	3	27.3	P = 0,58
Fatigue	6	21.4	2	15.4	1	9.1	P = 0,64
Duration of diarrhoea (days)							
Mean (± SD)	4.5 (± 3.5)		3.2 (± 2.0)		3.3 (± 0.58)		P = 0,76[b]
Median	3.0		3.0		3.0		
Interval	1–12		1–7		3–4		

[a] Significant difference by Chi squared test.
[b] Kruskal-Wallis test.

3.3. DNA sequence analyses of tpi and SSU-rRNA amplicons and identification of Giardia sub-assemblages

From 22 strong PCR products obtained in the nested PCR-*tpi*, PCR amplicons from 15 symptomatic children (10 assemblages A and 5 assemblages B) were successfully sequenced. Based on phylogenetic analysis of the *tpi* gene (Fig. 1), all 10 of the assemblage A isolates were classified as subtype A2, 4 revealed a subtype BIV and 1 BIII (Table 3). A complete coincidence between PCR results and sequence analysis was obtained for the *tpi* marker gene.

In the case of the SSU-rRNA gene, sequences from 12 out of the same 15 symptomatic children were obtained. The SSU-rRNA-based phylogenetic tree was not able to differentiate between *G. duodenalis* sub-assemblages (Fig. 2). An agreement of 83.3% (10 out of 12) between sequences results of RNA and *tpi* genes was recorded (Table 3).

Sequences obtained at the *tpi* and SSU rRNA loci were deposited in GenBank under the following accession numbers: KY271712-KYP271726, and KY271727-KYP271738, respectively.

4. Discussion

The present study provides information on the status of *Giardia* infection among children of Havana, Cuba. The overall prevalence rate of *Giardia* infection was 8.0%. This is in agreement with previous studies made in the last few years where the prevalence rates among children have been reported from 9 to 22% (Núñez et al., 2003; Escobedo et al., 2008; Cañete et al., 2012; Puebla et al., 2014).

The symptomatology developed in giardiasis is probably determined by several factors, among which the virulence of the parasite, nutritional, and immunological status of the host, the nature of intestinal microbiota, and the presence or absence of other co-pathogens are considered the most important ones (Cotton et al., 2011). In the last years, an approach about how the genetic diversity of the parasite can influence the outcome, as a possible factor involving in differences observed between symptomatic and asymptomatic children, has been ascertained (Kohli et al., 2008).

Molecular characterization of *Giardia* by several molecular markers is considered a key component in enhancing the understanding on the taxonomy, epidemiology and population genetics of this intestinal parasite (Feng and Xiao, 2011). Actually, several genetic loci are used to characterize *Giardia* at the genotype and subtype levels, among these, the most commonly used are the SSU rRNA, *tpi*, *gdh* and *bg* genes (Ryan and Cacciò, 2013).

It is known that *tpi* gene is a polymorphic gene, hence it is a profitable marker for genotyping and subtyping studies in *G. duodenalis* (Sprong et al., 2009); whereas the SSU rRNA, due to its multi-copy nature, results in greater sensitivity of PCR; however, the potential for discrimination is lower, and differentiation is possible only between assemblages, not between sub-assemblages or subtypes (Ryan and Cacciò, 2013). Because *Giardia* spp. have a clonal population structure, the use of a typing system based on sequence analysis of a single genetic locus with high sequence heterogeneity, such as *tpi*, can provide a resolution as high as multilocus sequence typing (Sulaiman et al., 2003). Similar reports were also reported in previous studies (Lalle et al., 2009; David et al., 2011; Mbae et al., 2016).

PCR for the SSU-rRNA gene proved to be the most sensitive method to detect *Giardia* DNA in all microscopically-*Giardia* positive stool samples. It has been reported that the *Giardia*-specific SSU rRNA PCR has a detection limit of 10 pg DNA/μL (Jaros et al., 2011).

Assemblage B of *Giardia* accounted 50% of infected children using assemblage-specific PCR-*tpi*, whereas assemblage A and mixed infection A + B had a similar distribution. Similar results in our country reveal a predominance of assemblage B (Puebla et al., 2014; Jerez-Puebla et al., 2015), which agrees with previous reports from other countries around the world (Gelanew et al., 2007; Lebbad
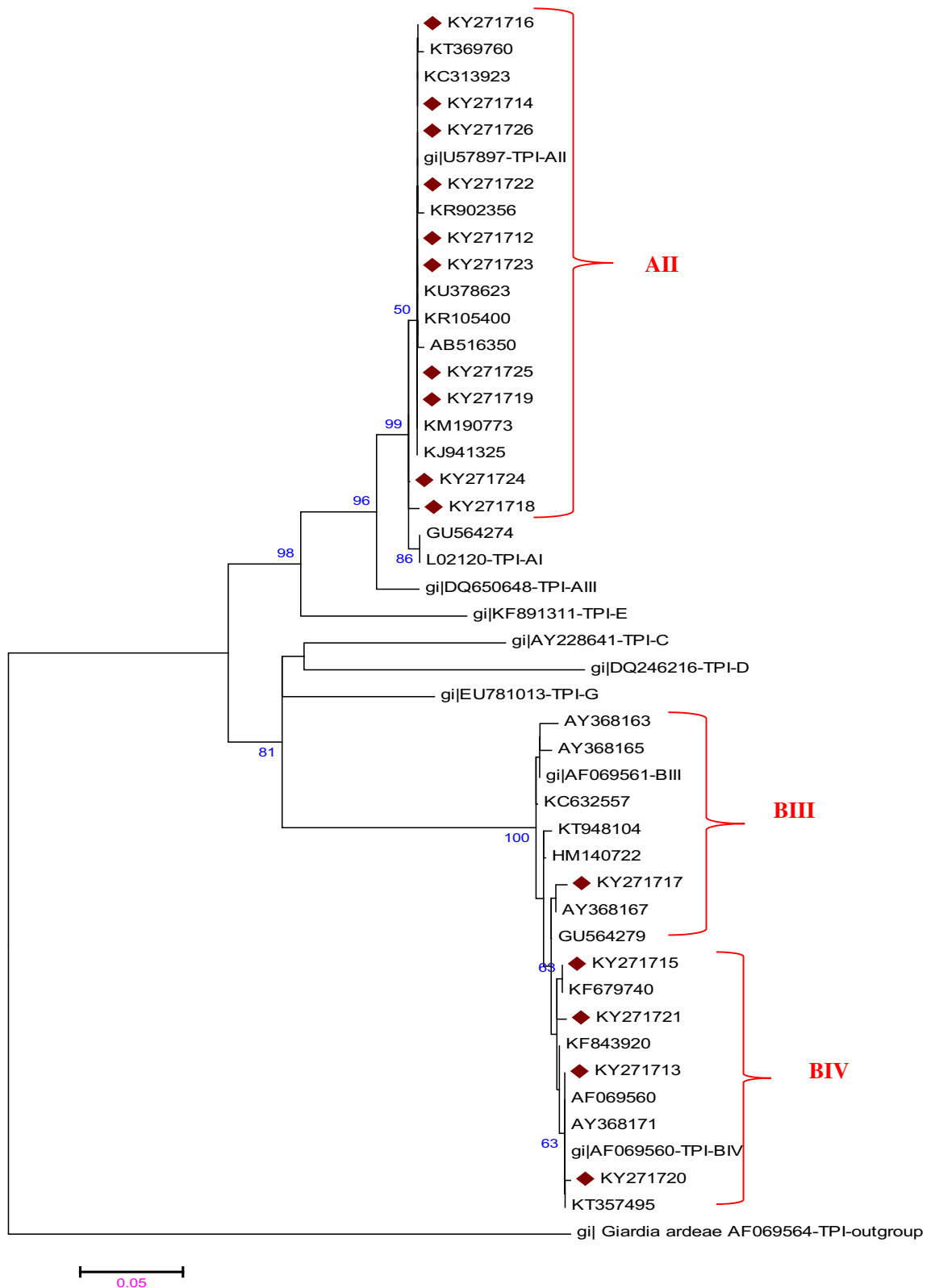
Fig. 1. Phylogenetic tree constructed from the *tpi* sequences of *Giardia duodenalis* isolates. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6 and further bootstrap analysis of 1000 replicas.

Table 3

Distribution of *G. duodenalis* assemblages A and B and sub assemblages based on PCR-*tpi* result and sequences obtained by SSU-rRNA and *tpi* genes.

Isolate	Sequence by tpi	GenBank accession no.	Sequence by SSU-rRNA	GenBank accession no.
1	AII	KY271712	A	KY271727
2	BIV	KY271713	B	KY271728
3	AII	KY271714	A	KY271729
4	BIV	KY271715	B	KY271730
5	AII	KY271716	B	KY271731
6	BIII	KY271717	A	KY2717132
7	AII	KY271718	A	KY271733
8	AII	KY271719	A	KY271734
9	BIV	KY271720	B	KY271735
10	BIV	KY271721	B	KY271736
11	AII	KY271722	A	KY271737
12	AII	KY271723	A	KY271738
13	AII	KY271724		Not sequenced
14	AII	KY271725		Not sequenced
15	AII	KY271726		Not sequenced

et al., 2008; Breathnach et al., 2010). Some reports have suggested that the higher *Giardia* excretion in assemblage B-infections as detected by microscopy or real-time PCR could explain the predominance of this assemblage in certain areas (Kohli et al., 2008).

Mixed infections were identified in 22.2% of *Giardia*-infected children. Data collected in our country have shown that mixed infections of *Giardia* ranges from 17 to 25% (Puebla et al., 2014; Jerez-Puebla et al., 2015). Interestingly, in both studies, the *tpi* gene was used as one of the molecular markers. It is indeed known that the use of assemblage-specific *tpi* primers allows the detection of a much higher number of mixed assemblages A and B infections than approaches based on the use of more general primers for PCR (Sulaiman et al., 2003). Besides, the occurrence of mixed infections with several assemblages/subtypes of *G. duodenalis* reflects the complex circulation of the parasite in the environment and the exposure of humans to multiple sources (Gelanew et al., 2007).

Several studies have described correlations between assemblages and symptoms, but there has been a lack of concordance among the data worldwide. *G. duodenalis* infection is known to vary widely in clinical manifestation including acute, chronic, and asymptomatic courses (Feng and Xiao, 2011). Among symptomatic children investigated in the present study, a significant association between diarrhoea and infection with assemblage B of *Giardia* was found. This is in agreement with previous studies made in Havana, Cuba, where assemblage B has been associated with diarrhoea, flatulence and abdominal pain in *Giardia*-infected children (Puebla et al., 2014; Jerez-Puebla et al., 2015, 2016).

To date, there is still a lack of clear association between the assemblage of *Giardia* and the clinical outcome, with contradictory results worldwide. Some studies have found an association of diarrhoea with assemblage A infection (Haque et al., 2005; Ajjampur et al., 2009); other studies from various regions also suggest a correlation between the presence of symptoms and infection with assemblage B (Gelanew et al., 2007; Lebbad et al., 2008; Al-Mohammed, 2011). On the contrary, some reports have however found no relation between symptomatology and the infecting assemblage (Kohli et al., 2008).

Considering the level of genetic diversity within *G. duodenalis*, some authors point out that it will be important to analyse if intra-specific variation of this parasite would underlie differences in host infectivity/disease outcome among different isolates (Ryan and Cacciò, 2013). For that reason, a sequences analysis of *tpi* and SSU-rRNA genes was performed in some *Giardia* isolates from symptomatic children.

In the current study, sequences of *tpi* gene showed that all children infected with assemblage A had probably acquired their parasites through the anthroponotic route, since they harboured sub-assemblage AII, which is rarely found in animals (Sprong et al., 2009). This is similar with other studies where AII was the predominant sub-assemblage in human (Bonhomme et al., 2011; Lebbad et al., 2011). Distribution of subassemblage B showed that BIV accounted 4 out of 5 infected children. Although designation of isolates to subassemblages BIII or BIV is problematic, an accurate system to classify assemblage B isolates that enables comparison between studies is currently not available (Asher et al., 2014). The isolation of anthroponotic assemblages and sub-assemblages B, as well, implicates human as a potential source of the infection. Similar results of predominance of subassemblage AII have been found in our country in previous works (Puebla et al., 2014; Jerez-Puebla et al., 2015). In this study only one sub assemblage BIII was identified in the few samples that were sequenced and 4 sub assemblage BIV were classified according to the percentage of homology with references strains. In a previous research made in Sancti Spiritus, a high prevalence of subassemblage BIII was recorded by PCR-RFLP analysis (Jerez-Puebla et al., 2015).

Even though PCR for the SSU-rRNA gene proved to be the most sensitive method in identifying *Giardia* in faecal samples, phylogenetic analysis of this gene, as a subtyping tool proved to have a low discriminatory potential, and differentiation is possible only between assemblages, not between sub-assemblages or subtypes (Ryan and Cacciò, 2013).

In the present study, discordant genotyping results between *tpi* and SSU-rRNA genes were obtained in 2 cases out of 12 by sequences analysis. Although the underlying mechanisms remain uncertain, some factors that could contribute to the incongruent classification of assemblages by different makers include (i) the presence of a mixture of genetically different cysts in a faecal sample in combination with preferential amplification of a particular assemblage-specific marker gene compared to another marker gene indicative for a second assemblage (i.e., a true mixed infection followed by biased PCR amplification) (Wielinga and Thompson,

Fig. 2. Phylogenetic tree constructed from the SSU-rRNA sequences of *Giardia duodenalis* isolates using Kimura-2 parameter distance estimation method clustered by the NJ method in MEGA 6 and further bootstrap analysis of 1000 replicas.

2007); (ii) meiotic recombination, which suggests the potential of sexual reproduction in *Giardia*, and (iii) introgression that involves back crossing of hybrids with parental species and retention of ancestral polymorphism that could lead to presence of identical alleles in genetically distinct groups (Ryan and Cacciò, 2013).

In summary, the present study provides information on the distribution of *Giardia* assemblage's infection in paediatric population associated with some epidemiological and clinical parameters in Cuban children. The use of molecular epidemiological tools and particularly subtyping tools is important in understanding the epidemiology and dynamic transmission of *Giardia* infection thus contributing to an improved knowledge about the characteristics of this parasitic disease.

5. Conclusions

The high proportion of assemblage B of *Giardia* detected in this study among children confirms previous reports in our country. Mixed-assemblage infections had a high frequency. The only correlation observed between symptoms and assemblages was that diarrhoea was more commonly found in children infected with assemblage B. Subassemblage AII and BIV were most frequently identified in the population of children studied. Studies to be carried out with a higher number of samples from humans and animals and based on different sub-typing tools including high throughput next-generation sequencing will increase our understanding in the epidemiology and dynamic transmission of *Giardia*.

Acknowledgments

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