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Concordance of *Giardia duodenalis* assemblages determined by different PCR methodologies in three observational studies in Cuba

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ABSTRACT

Giardia duodenalis is one of the most important intestinal parasites globally, especially in children, and in Cuba is the leading cause of chronic paediatric diarrhoea in this population. *G. duodenalis* is composed of eight genetic groups (or assemblages), two of which (A and B) are apparently zoonotic, occurring in both humans and other animals. However, consensus on the most appropriate genotyping scheme for optimal characterization of *G. duodenalis* isolates is lacking. In this article we present the results of three descriptive observational studies conducted in Havana, Cuba between 2010 and 2013, with the aim of comparing the results from molecular (PCR) approaches targeting different genes in order to assign with confidence 224 isolates of *G. duodenalis* to the correct assemblages. In each sub-study, following DNA isolation by the phenol/chloroform/isoamyl alcohol extraction method, PCR targeting the triose phosphate isomerase (*tpi*) gene was used for molecular characterization, as well as one additional PCR-method targeting another gene or pair of genes. DNA amplification was obtained in 87%, 83%, and 80% in the three sub-studies. Although excellent agreement (kappa index = 1) was recorded between results from some pairs of genes, for other combinations only moderate or substantial agreement was achieved. These results highlight the importance of interpretation of genotyping data, especially when single genetic markers are used. From the results of our studies, PCR targeting a combination of the *tpi* gene and the intergenic spacer region of rDNA may be a useful approach for the molecular characterization of *G. duodenalis* isolates.

1. Introduction

Giardia duodenalis (syn. *G. intestinalis*, *G. lamblia*) is one of the most prevalent intestinal human parasites worldwide. It is of particular importance in school-age children in low-income countries, where it is associated with persistent diarrhoea (Muhsen and Levine, 2012). In fact, the World Health Organization reports more than 200 million symptomatic infections annually caused by *G. duodenalis* in developing countries of Asia, Africa, and Latin America (Naguib et al., 2008). The emergence of this parasitic infection in these regions and the negative impact on growth and cognitive development in childhood, were the reason for the previous inclusion of giardiasis in the WHO's 'Neglected Diseases Initiative' (Savioli et al., 2006).

Some sub-types (genetic groups, or assemblages) of *G. duodenalis* also commonly infect other mammals, including pets and livestock (Thompson, 2004). Indeed, this enigmatic parasite is considered a species complex, divided at the genetic level into 'assemblages' (A to H) with different host specificities (Thompson and Monis, 2004). Molecular studies carried out at several loci of *G. duodenalis* isolates from humans and animals, including triosephosphate isomerase (*tpi*), glutamate dehydrogenase (*gdh*), β-giardin (*bg*), and the small subunit ribosomal RNA (SSU rRNA) genes, have shown that assemblages A and B are responsible for causing the majority of human infections (Feng and Xiao, 2011). As these assemblages are also found in other mammals,

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they are considered as zoonotic assemblages (Ryan and Cacciò, 2013).

Despite our understanding of these different assemblages, there is no consensus on which genotyping scheme should be adopted for the molecular characterization of *G. duodenalis* isolates, and different researchers often use different genetic loci. However, it is widely accepted that analysis and interpretation should be undertaken on the basis of at least two loci, because this provides the most robust information (Cacciò et al., 2005). Indeed, phylogenetic analysis of sequence data in GenBank has indicated that although three commonly used target genes (*tpi*, *gdh* and *bg* genes) gave relatively congruent tree topologies, the SSU rRNA gene did not resolve assemblages according to the current classification (Brynildsrud et al., 2018). Thus, the authors also conclude that multi-locus or (ideally) whole-genome approaches would be preferable, and use of the SSU rRNA gene as the sole marker should be avoided when possible.

Although sequencing can provide additional information, use of assemblage-specific markers has enabled differentiation between genetic variants to be other more rapid and cheaper, and also may allow better discrimination of mixed infections that are not detected by standard PCR (Cacciò et al., 2008).

The purpose of this study was to determine the concordance of PCR results using pairwise combinations of different assemblage-specific molecular markers in three observational sub-studies, with the aim of identifying the optimum combination of molecular markers that could be readily applied in a healthcare setting like ours in Cuba when investigating which assemblages of *G. duodenalis* are causing infection; this tool would be very useful for epidemiological investigations, particularly for determining potential sources and transmission routes during outbreaks.

2. Materials and methods

2.1. Study population, sample collection, and parasitological investigation

This study was conducted among paediatric populations in Cuba between 2010 and 2013. Included in the study were a total of 1500 children either attending the Pediatric Hospital “William Soler” in Havana with gastrointestinal symptoms, or asymptomatic children attending kindergartens in Havana and primary schools in Havana, or pre-school children in the municipality of Fomento in central Cuba. The division of the children into the sub-studies is described in section 2.2. Further details regarding the individual studies, including symptoms etc. maybe found in the relevant references listed in Table 1.

Stool samples were obtained from each child into a sterile container and were immediately examined for intestinal parasites by a wet smear stained with Lugol's iodine and followed by formalin ethyl acetate concentration technique. All diarrheic stool samples were stained by modified acid-fast trichrome for identification of infection with *Cryptosporidium* spp., *Cyclospora*, and *Cystoisospora* (Garcia, 2001).

2.2. Study set-up

Samples from the 1500 children were divided into three sub-studies for genetic analysis using different primer pairs targeting different genes and molecular methods. In each sub-study positive samples were characterised using the *tpi* molecular marker and one other, as shown in Table 1.

2.3. Cyst purification and DNA extraction

The 224 cases of microscopically-confirmed *Giardia* infection were taken to the National Laboratory of Intestinal Parasitic Infection in the Institute of Tropical Medicine “Pedro Kouri”, Havana for further analysis.

Cysts were purified and concentrated using a sucrose gradient and then washed with distilled water, following the protocol described by Babaei et al. (2011).

Following cyst purification, DNA was extracted using the conventional phenol/chloroform/isoamyl alcohol (PCI) protocol (Sambrook and Russell, 2001). Briefly, a suspension of purified cysts was 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)) and vortexed. After adding 100 μg/mL of proteinase K, the suspension was incubated at 56 °C for 2 h. The lysate was first treated with phenol/chloroform/isoamyl alcohol (24:24:1), and then by chloroform/isoamyl alcohol (24:1). DNA was precipitated by the addition of 1 mL chilled ethanol. The dried DNA was suspended in 50 μL distilled water and used as a template for PCR. All extracted DNA was stored at −20 °C until use.

2.4. Assemblage-specific PCR and RFLP

As described in section 2.2, three sets of sequential PCR were conducted on DNA purified from cysts in each sub-study using assemblage-specific primers for *G. duodenalis* of Assemblage A and Assemblage B from previously developed and published protocols (Bertrand et al., 2005; Lee et al., 2006; Vanni et al., 2012; Read et al., 2004). All PCR products from the protocols outlined below were analysed by 2% agarose gel electrophoresis, stained with 0.5 μg/mL of ethidium bromide and then visualized on a UV transilluminator (Syngene, U:Genius, Belgium).

DNA isolates from axenic cultures of G. *duodenalis* strains WB-C6 (genotype A) and Ad28 (genotype B), kindly donated by the Institute of Parasitology, Vetsuisse Faculty, University of Bern, Switzerland, were used as positive controls, while ultrapure water was included in negative controls for all PCR.

For cyst DNA in all sub-studies, PCR targeting the *tpi* gene was used according to the protocol of Bertrand et al. (2005), in which the primers amplify a 148-base pair (bp) fragment of the Assemblage A and 81-bp fragment of Assemblage B (Bertrand et al., 2005).

For the DNA isolated from cysts in sub-study 1, the *IGS*-targeted PCR was also conducted according to the protocol of Lee et al. (2006). Here,

Table 1
Sub-study set up by origin, number of samples, and PCR used for investigations.

	Sub-study 1	Sub-study 2	Sub-study 3
Sample origins	Havana: Samples from 289 children at the hospital and 163 children from kindergartens	Havana: Samples from 639 schoolchildren from 4 primary schools	Fomento: Samples from 417 pre-school children
Number samples *Giardia*-positive by microscopy	103 *Giardia*-positive samples (23% prevalence)	76 *Giardia*-positive samples (12% prevalence)	45 *Giardia*-positive samples (11% prevalence)
Genes and molecular method used for genetic characterization	1) triose phosphate isomerase (*tpi*)- end-point PCR 2) intergenic spacer (*IGS*)- nested PCR	1) triose phosphate isomerase (*tpi*)-end-point PCR 2) 4E1-HP marker-end-point PCR	1) triose phosphate isomerase (*tpi*)-end-point PCR 2) glutamate dehydrogenase (*gdh*)-PCR-RFLP
References	Puebla et al. (2014)	Jerez Puebla et al., 2015	Puebla et al. (2015); Puebla et al. (2017)

the primers in the nested PCR amplify a 176-bp fragment (Sub-assemblage AI), a 261-bp fragment (Sub-assemblage AII), and a 319-bp fragment (Assemblage B) (Lee et al., 2006). For the DNA isolated from cysts in sub-study 2, the 4E1-HP single-copy marker on chromosome 4 was targeted, using the primers and protocol described by Vanni et al. (2012) in which a 165-bp amplicon results for Assemblage A and 272-bp amplicon for Assemblage B in an end-point PCR. Finally, for sub-study 3, in addition to the *tpi* PCR, the gdh gene was amplified using the primers and semi-nested protocol described by Read et al. (2004). This results in a 432 bp amplicon that can be further analysed by RFLP using the method previously described by Read et al. (2004). In brief, restriction digests were carried out directly on PCR products of the *gdh* PCR and showed digestions profile for Assemblage A of 90 bp, 120 bp, and 150 bp, and, for Assemblage B, of 120 bp and 290 bp. Profiles were visualized on 3% high resolution grade agarose stained with 0.5 μg/mL of ethidium bromide and then visualized on a UV transilluminator (Syngene, U:Genius, Belgium).

2.5. Statistical analysis

All data were entered into a spreadsheet and analysed using EPINFO 6.04 statistical programme. The Kappa index was used for measuring the concordance of PCR results by the molecular markers used in each sub-study. The scale of interpretation of Kappa index described by Landis and Koch (1997) was used.

3. Results

3.1. Genotyping results at tpi gene

Of the 224 isolates of DNA that were analysed by PCR targeting the *tpi* gene, results were obtained from 189 (84%); 90 from sub-study 1 (87%), 63 from sub-study 2 (83%), and 36 from sub-study 3 (80%). At this gene, Assemblage B was found to predominate in all sub-studies, ranging from 37 to 42% prevalence for single-assemblage infections. However, as mixed infections ranged from 15 to 34%, the prevalence of infections in which Assemblage B was found (including mixed and single infections) ranged from 52 to 74%. Results are summarized in Table 2.

3.2. Concordance between PCR results within each sub-study

For the other genes, the success rate ranged from 76% (4E1-HP in sub-study 2) to 87% (the other two PCR targets), with concordance between results obtained in by each PCR varying according both with primers/PCR target and with assemblage detected; see Table 3. In sub-study 1, there was complete concordance between the results obtained by PCR targeting the *tpi* gene and results obtained by PCR targeting IGS results (kappa index = 1). In sub-study 2, although there was concordance between results obtained by PCR targeting the *tpi* gene and results obtained by PCR targeting the 4E1-HP marker for 43 (4 Assemblages A, 28 Assemblages B, and 11 mixed infections), for 22 samples the results were not in agreement. In addition, 11 samples (14.5%) were negative by both PCR, 10 of which showed very few cysts in the coprological examination. The kappa index ranged between 0.39 (mixed infections) and 0.64 (Assemblage A); Table 3. In sub-study 3, concordant results were obtained between the 2 PCR for 32 of the isolates, with a lack of agreement for only 4 samples. In this sub-study, the kappa index ranged between 0.62 (Assemblage A) and 0.85 (mixed infections); Table 3.

Table 2
Results for PCR targeting *tpi* gene by study.

	Sub-study 1 N = 103	Sub-study 2 N = 76	Sub-study 3 N = 45
No amplification; n (%)	13 (13)	13 (17)	9 (20)
Successful amplification and interpretation; n (%)	90 (87%)	63 (83%)	36 (80%)
Assemblage A; n (%)	36 (35)	7 (9)	4 (9)
Assemblage B; n (%)	38 (37)	30 (40)	19 (42)
Mixed (A and B); n (%)	16 (15)	26 (34)	13 (29)

Table 3
Concordance in results by PCR assays in the three sub-studies.

	Assemblage A	Assemblage B	Mixed (A and B)
Sub-study 1: *tpi* and *IGS* PCR			
Kappa index (95% CI)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)
Standard error	0	0	0
Sub-study 2: *tpi* and 4E1-HP PCR			
Kappa index (95% CI)	0.64 (0.31–0.96)	0.56 (0.37–0.74)	0.39 (0.18–0.60)
Standard error	0.17	0.56	0.11
Sub-study 1: *tpi* and *gdh* PCR			
Kappa index (95% CI)	0.62 (0.29–0.95)	0.81 (0.64–0.98)	0.85 (0.68–1.0)
Standard error	0.10	0.09	0.08

4. Discussion

Human giardiasis is caused by infection with one or both of two genetically very distinct genetic assemblages (A and B) of *G. duodenalis*. Various studies have investigated whether there is any association between assemblage and different factors, such as epidemiology, symptom spectrum, or co-infections with other pathogens (Robertson et al., 2010; Faria et al., 2017). However, no definitive conclusion has been reached. Nevertheless, for investigations of outbreaks (e.g., waterborne outbreak in Norway; Robertson et al., 2006) or for tracing dissemination of infection (e.g., in a welfare institute in China; Wang et al., 2018), it is often of relevance and interest to know the assemblage in a particular infection. A number of molecular assays, mostly based on amplification of specific gene fragments, have been developed to determine the genotype (for an overview please refer to Cacciò et al. (2005), Ryan and Cacciò (2013), and Koehler et al. (2014)), and these have been applied to both stool and environmental samples (Cacciò and Ryan, 2008).

Among these assays, the genes most commonly used are the small-subunit (SSU) rRNA gene, and various house-keeping genes coding for glutamate dehydrogenase (*gdh*), β-giardin (*bg*), elongation factor 1 alpha (*ef1-α*), and triosephosphate isomerase (*tpi*) (Feng and Xiao, 2011). Although some researchers have noted that the use of a single marker with high genetic heterogeneity can provide a resolution as high as multi-locus sequence typing, it is generally widely accepted that when only one molecular marker is used, particularly if that marker is the SSU rRNA gene, then the resolution may be insufficiently discriminatory to reach a firm conclusion (Ryan and Cacciò, 2013). Thus, it has been recommended that at least two molecular markers should be used (Cacciò et al., 2005). Although sequencing of PCR amplicons provides most information, this is often expensive and not readily accessible in countries such as Cuba, where the emphasis must be on rapid, low-cost testing. By using assemblage-specific primers sets, the relevant fundamental data can be obtained without requiring sequencing. Previous studies on which assemblages of *Giardia* circulate in Cuba have also indicated predominance of Assemblage B (Pelayo et al., 2008) using molecular approaches targeting 2 genes (*bg* and *gdh*), but in this study sequencing was used rather than assemblage-specific PCR. Although genotypes were obtained for 20 isolates, sequencing was only successful at both genes for 5 isolates, and concordant results were achieved for each of these (Pelayo et al., 2008). However, sequencing-based genotyping is currently too time-consuming and expensive for routine use in Cuba.

One problem that arises when using multi-locus PCR is discordance between results at different genes and thus difficulty in interpretation.

This may reflect mixed infections, where one assemblage may be preferentially amplified over another using a particular primer set. This can result in inconsistent results that are difficult to interpret (Cacciò et al., 2008; Huey et al., 2013). However, systematic data on concordance between results obtained by different PCR are scant, and in the work presented here we were able to compare concordance from PCR targeting different gene pairs where the amplified DNA is not analysed by sequencing.

The *tpi* gene has been frequently used in molecular characterization of *G. duodenalis* due to its high level of polymorphism (Sulaiman et al., 2003), and PCR targeting this gene was used in all three sub-studies. The *IGS* gene was selected for our study as it is a multi-copy gene composed by variable regions that allows the differentiation of Assemblages A and B (Lee et al., 2006; Hussein et al., 2017). The *gdh* gene was chosen for the study as it has been used extensively in *Giardia* molecular assays and by using enzymatic digestion of PCR products the various possible assemblages can be identified. Finally, the assemblage-specific PCR described by Vanni et al. (2012) was used due to the rapid and simple differentiation of *G. duodenalis* assemblages by an end-point PCR.

We found 100% agreement (kappa index = 1) for results from PCR amplicons obtained by primers targeting the *tpi* and *IGS* genes, indicating that both molecular markers are suitable for *Giardia* characterization in our setting and produce concordant results that are therefore easy for interpretation. It would have been ideal to undertake further PCR with primers targeting the *IGS* gene in these sub-studies also, to see if the results could confirm those found in sub-study 1, but this was not possible due to logistical issues. As far as we are aware, only four previous studies have been published in which *IGS* gene was targeted for molecular characterization of *G. duodenalis*, either as a single marker (Lee et al., 2006; Al-Mohammed, 2011; Hussein et al., 2016) or in combination with another gene (Hussein et al., 2017).

For *tpi* and *gdh* genes, a good, but not perfect, agreement was found with the kappa index above 0.8 for Assemblage B and for mixed A and B infections, and 0.6 for Assemblage A. Huey et al. (2013) have reported a similar agreement using these genes in a study from Malaysia, where discrepancy between results was found in 9% of samples. We found a lower level of agreement between results obtained from the *tpi* gene and 4E1-HP target (kappa index from 0.39 to 0.64). As far as we are aware, this is the first report about concordance of these assemblage-specific PCRs for assemblages of *G. duodenalis*.

Inconsistencies in genotyping results have been reported in both human and animal isolates of *G. duodenalis* (particularly for isolates from dogs). According to Cacciò and Ryan (2008), these inconsistencies are often due to three distinct phenomena: (i) genetically differing cysts within the same faecal sample and with preferential amplification of a particular assemblage-specific marker gene; in other words, a true mixed infection followed by biased PCR amplification; or (ii) sexual recombination among two *Giardia* assemblages resulting in a mixed genotype regarding the two PCR targets or iii) introgression and retention of ancestral polymorphisms (Sprong et al., 2009; Ryan and Cacciò, 2013; Thompson and Ash, 2016).

Thus, interpretation of genotyping results when using a single marker may not provide a complete picture, and in genotyping more than 2,4000 samples discordant results were found between two markers (SSU rRNA, *bg, gdh*, and *tpi* genes) for 15%, with this inconsistency predominant in isolates from humans and dogs (Sprong et al., 2009; Huey et al., 2013).

The results of our study, which provides information on the concordance between different assemblage-specific PCR approaches for *G. duodenalis*, provides a basis for proposing an approach for assemblage identification of *Giardia* infections in children in Cuba, and similar countries where use of sequencing technologies, particularly whole genome sequencing, is currently not appropriate or feasible. We suggest that use of polymorphic genes, like the *tpi* and IGS, as targets is advantageous, and, in our study, these targets showed a high level of agreement. These results provide a foundation for future characterization work, and can also be a basis for evaluating other molecular markers and potentially resolving discrepancies when investigating new molecular markers.

Author statement

Luis Enrique Jerez Puebla: Conceptualization, Methodology, Investigation, Data curation, Writing – Original Draft, Writing – Review and Editing. Fidel A. Núñez Fernández: Conceptualization, Data curation, Supervision, Writing – Review and Editing, Jorge Fraga: Methodology, Data curation, Writing – Review and Editing, Lázara Rojas Rivero: Funding acquisition, Project administration, Iraís Atencio Millán: Investigation, Lucía Ayllón Valdés: Investigation, child treatment and follow-up, Isabel Martínez Silva: Investigation, Norbert Müller: Supervision, Resources, Writing – Review and Editing, Lucy J. Robertson: Supervision, Data curation, Writing – Original Draft, Writing – Review and Editing.

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