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Sub-assemblages distribution of *Giardia duodenalis* and its association with clinical symptoms in children from Sancti Spíritus, Cuba

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ABSTRACT

Objective: To determine the prevalence of sub-assemblages of *Giardia duodenalis* (*G. duodenalis*) causing infection in preschool children in a central region of Cuba.
Methods: A cross-sectional study was conducted on 417 children from Sancti Spiritus from January to June 2013. A PCR amplification of the glutamate dehydrogenase (*gdh*) gene was performed for all positive samples in direct microscopic examination to enable sub-assemblages identification.
Results: The prevalence of *G. duodenalis* in preschool children was 10.8%. DNA from 39 of 45 (86.7%) samples was successfully amplified by PCR-restriction fragment length polymorphism analysis of the *gdh* gene. Restriction fragment length polymorphism analysis classified the 39 *gdh* amplicons in sub-assemblages BIII (11, 28.2%), AII (8, 20.5%) and BIV (4, 10.3%). Mixed infections accounted for 41%, with the pattern AII/BIII (12, 30.7%) being the predominant. Abdominal pain was statistically associated with infection with sub-assemblage BIII.
Conclusions: Sub-assemblage BIII was the most commonly identified in the population of children studied, and significantly associated with abdominal pain in the symptomatic children. More defining tools to discriminate sub-assemblage and genotype levels to correlate with clinical data are needed in further studies. Large studies in endemic settings are required to elucidate the role that assemblage types play in *G. duodenalis* infections in vulnerable populations, such as children.

1. Introduction

Giardia duodenalis (synonyms: *Giardia lamblia*, *Giardia intestinalis*) (*G. duodenalis*) is a protozoan parasite that infects a wide array of vertebrates, including humans, pets, livestock, wildlife, and marine animals[1]. According to estimates, about 200 million people worldwide have symptoms of intestinal giardiasis, and 500 000 new cases occur annually. Due to its impact on health, especially among children in developing countries, *Giardia* has been included in the Neglected Diseases Initiative of the World Health Organization since 2004[2]. The symptoms of *Giardia* infection are highly variable and some individuals may shed infectious cysts in their faeces without showing any overt clinical signs. It is not fully understood why some individuals develop clinical giardiasis while others remain asymptomatic[3]. However, host factors and "strain" variation of the parasite are both likely to be involved. In children in developing countries, giardiasis is associated with stunting and malnutrition and documented to have adverse effects on success at school and cognitive function[4].

Molecular characterizations of *Giardia* from humans and animals have been carried out at several loci, including glutamate dehydrogenase (*gdh*) gene, β-giardin, small subunit ribosomal RNA, and the triosephosphate isomerase genes[5]. It is generally accepted that at least eight major assemblages exist in the *G. duodenalis* species complex[6], most of which have distinct associations with particular host species. Only assemblages A and B appear to be associated with human infections, but *G. duodenalis* isolates within these assemblages have also been identified from various animals, including cattles, dogs, and cats[6].

These assemblages are broad clusters of genetically related isolates and four human infective sub-assemblages (AI, AII, BIII and BIV) have been previously described. Genetic diversity within assemblage B, however, is higher than assemblage A; assemblage B subgroups are unresolved; and numerous assemblage B genotypes

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contribute to human and animal infection[1,7]. Identification of different genetic types that contribute to disease enables differences in host specificities, transmission cycles, and sources of infection to be more closely examined[6].

Molecular techniques such as PCR provide alternative methods for specific detection of pathogens in stool, and in combination with techniques, such as restriction fragment length polymorphism (RFLP) or nested PCR, they have been used to genotype organisms[8]. PCR-RFLP has been used previously to target the *gdh* gene locus. This gene has been shown to be a reliable, easy and cost-effective method to identify *G. duodenalis* isolates directly from stool samples[9,10].

Several epidemiological studies of giardiasis have been conducted in Cuba[11-13], but few have performed molecular analyses[14,15], to identify genetic subtypes contributing to high infection rates and to correlate these results with the symptomatology manifested in children. The previous studies made by Pelayo *et al*. and Puebla *et al*.[14,15], 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.

The purpose of the present study was to determine the genetic variability of *G. duodenalis* sub-assemblages by a PCR-RFLP technique, and to associate these results with clinical data collected.

2. Materials and methods

2.1. Sample collection and surveillance data

A descriptive cross-sectional study was conducted on preschool children from the municipality of Fomento, province of Sancti Spiritus. Children stool samples microscopically positive for *G. duodenalis* cysts ($n = 45$) by a wet smear stained with Lugol's iodine and followed by formalin ethyl acetate concentration technique were collected at the Provincial Center of Hygiene and Microbiology, from January to June 2013.

A standard form was used to collect information on the age, gender and symptoms of each cyst-positive child, from that child's parents/guardians. This surveillance data included information about epidemiology in order to identify risk factors of *G. duodenalis* infection, standardized questionnaires, covering demographic data, sanitary behaviors including cooking and eating habits, sources and treatment methods of drinking water, pets or animal contact, and a history of present gastrointestinal symptoms, were used.

All *Giardia* positive samples were stored in potassium dichromate (2.5%) at 4 ℃ and transferred to Pedro Kourí Institute for molecular characterization.

The research protocol was approved by the Ethics Committee of the Pedro Kourí Institute. Written informed consent was obtained from parents/guardians of children to participate in the study.

Epidemiological data such as age, gender and clinical signs and symptoms of some cases were also collected by using a predesigned questionnaire.

2.2. Purification of G. duodenalis cysts and DNA extraction

Giardia cysts were purified and concentrated from stool samples in a sucrose gradient with a specific gravity of 0.85 mol/L and then washed with distilled water, following the protocol described by Hawash *et al*.[16]. The cyst wall was disrupted by 8–10 freeze-thaw cycles in liquid nitrogen alternated with a 95 ℃ water bath. After that, purified cysts were mixed with 300 μL of buffer lysis (50 mmol/L Tris-HCl, pH 7.5; 25 mmol/L ethylene diamine tetraacetic acid, 25 mmol/L NaCl, and 1% of sodium dodecyl sulphate) and vortexed. After adding 100 μg/mL of proteinase K, the suspension was incubated at 56 ℃ 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 protocol of Sambrook and Russell[17]. The DNA was precipitated by the addition of 1 mL chilled ethanol and stored at -20 ℃ until use. The dried DNA was re-suspended in 30 μL distilled water and used as a template for PCR.

2.3. Genotypic characterization

2.3.1. PCR amplication

PCR amplification of the *gdh* was performed for all positive samples in direct microscopic examination to enable sub-assemblages identification. A 432 base pairs (bp) fragment of the *gdh* gene was amplified following the semi-nested protocol and cycling conditions described by Read *et al*., by using primers GDHeF, GDHiF and GDHiR[8] (Figure 1).

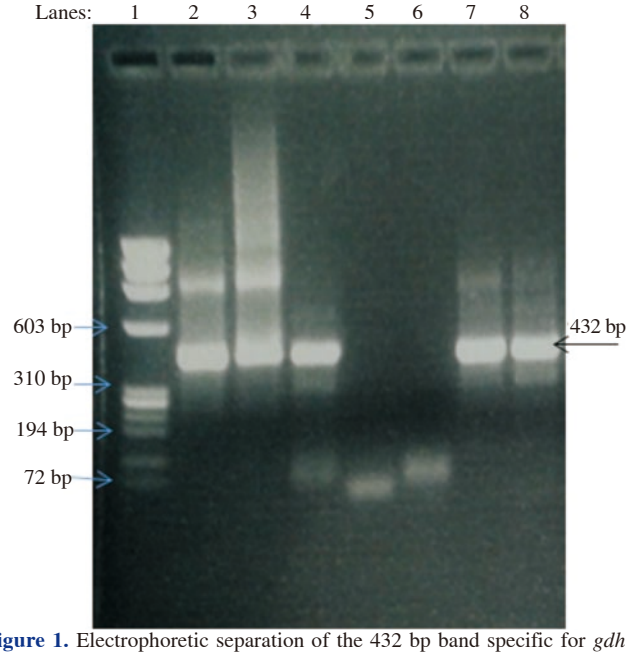

Figure 1. Electrophoretic separation of the 432 bp band specific for *gdh* nested PCR product.
Lane 1: Molecular weight ϕX 174 DNA/HaeIII; Lane 2: *G. duodenalis* strains WB-C6; Lanes 3, 4, 7, 8: Representative samples positive to PCR-*gdh*; Lanes 4 and 5: Negative control to the first and second round of this nested PCR-*gdh*, respectively.

The PCR reaction mixture was done by using a AmpliTaq® DNA polymerase with GeneAmp® 10× PCR buffer kit (Applied Biosystems, USA) in a total volume of 25 μL, and comprised 2.5 μL of 10× PCR buffer (Applied Biosystems, USA), 0.2 mmol/L of each deoxynucleoside triphosphate (Applied Biosystems, USA), 1

IU of Taq polymerase (Applied Biosystems, USA), 0.4 μmol/L of each primer, 5 μL of DNA template, with ultrapure water used as a negative control.

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

2.3.2. RFLP

To identify *G. duodenalis* sub-assemblages and examine the population structure of individual infections, PCR products were analysed by RFLP using the method previously described by Read *et al*.[8]. Restriction digests were carried out directly on PCR products in 20 μL reactions. Ten microliter of PCR product was added to 1× reaction buffer and 2 IU NlaIV (New England Biolabs). For those isolates which showed the genotyping profile of assemblage B, 2 IU RsaI (New England Biolabs) restriction enzyme was used to differentiate between BIII and BIV sub-assemblages. Digestion took place at 37 ℃ for 3 h. Profiles were visualized on 2% high resolution grade agarose stained with ethidium bromide.

2.4. Statistical analysis

All data were analysed by using EPi Info 6.04 and EPiData 3.1 statistical programmes. *Chi* square test and proportion tests were employed to assess the significance of the associations. The Fisher's exact test was used when required by data scarcity. The $P < 0.05$ was considered as statistically significant for all tests. The odds ratio (OR) with 95% confidence interval (*CI*) were performed as measures of association. ORs with 95% *CI* and *P* values were calculated for *G. duodenalis* sub-assemblages infection.

3. Results

3.1. Characteristics of the study population

Of the 417 specimens examined by microscopy, 45 were positive for *G. duodenalis*, including 20 girls and 25 boys. From these *Giardia*-infected children, 21 of them presented symptomatology whereas the others were asymptomatic. No co-infection with other parasitic and bacterial intestinal pathogens was recorded from them.

3.2. Genotyping results

Nested PCR amplification of the *gdh* gene was successful in 39 samples (86.67%) (Figure 2). These negative samples showed very low excretion cysts in the stool samples (less than 2 per slide). After using RFLP analysis, different types of genetic profiles were present in the samples. Within assemblage A, sub-assemblage AII was detected in 8/39 (20.5%) samples. Sub-assemblage AI, as sole infection, was not identified. Within assemblage B, both sub-assemblages were detected separately in samples: BIII (11/39 samples, 28.2%); BIV (4/39 samples, 10.3%). Mixed infections were recorded in the patterns AII/BIII (12/39 samples, 30.7%), AII/BIV (3/39, 7.7%) and 1 child with AI/BIV (2.6%).

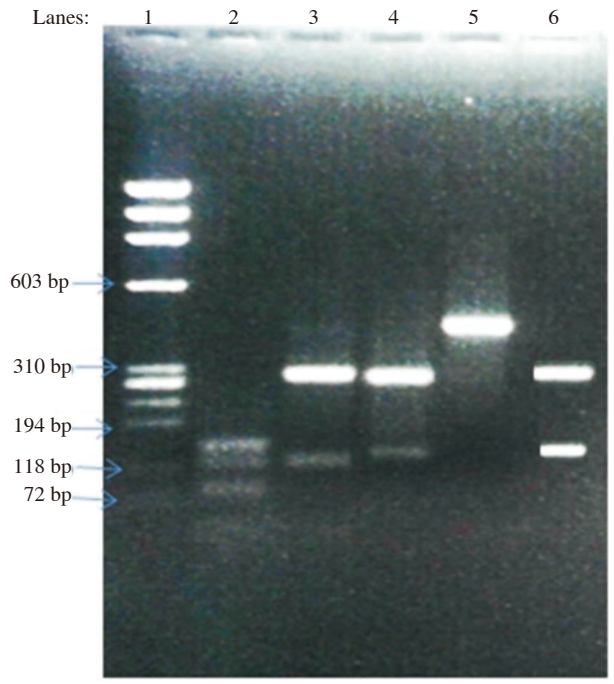

Figure 2. Ethidium bromide stained 2% showing DNA amplified from the control strains digested with NlaIV and RsaI.
Lane 1: Molecular weight ϕX 174 DNA/HaeIII; Lane 2: *G. duodenalis* strains WB-C6 (assemblage A) digested with NlaIV; Lanes 3 and 4: *G. duodenalis* strain GS (assemblage B) digested with NlaIV; Lane 5: *G. duodenalis* assemblage B digested with RsaI ((pattern of sub-assemblage BIV); Lane 6: *G. duodenalis* assemblage B digested with RsaI (pattern of sub-assemblage BIII).

Children were aged 0–5 years and there were slightly more males (22/39, 56.4%) than females (17/39, 43.6%) included in the study (Table 1). There was no difference in the total number of positive males and females, or between those aged 0–5 years related to infection with a specific sub-assemblage of *Giardia* ($P > 0.05$).

Table 1
Characteristics of children infected with *G. duodenalis* and distribution of assemblages A and B subtypes identified by *gdh* PCR-RFLP amplification. [*n* (%)].

Characteristic	Gender		Age (years)				
	Male	Female	< 1	1–2	2–3	3–4	4–5
Sub-assemblage AII (*n* = 8)	4 (50.0)	4 (50.0)	0 (0.0)	0 (0.0)	2 (25.0)	3 (37.5)	3 (37.5)
Sub-assemblage BIII (*n* = 11)	6 (54.5)	5 (45.5)	0 (0.0)	2 (18.2)	1 (9.0)	5 (45.5)	3 (27.3)
Sub-assemblage BIV (*n* = 4)	2 (50.0)	2 (50.0)	0 (0.0)	0 (0.0)	1 (25.0)	1 (25.0)	2 (50.0)
Mixed AII/BIII (*n* = 13)	8 (61.5)	5 (38.5)	1 (7.7)	3 (23.1)	3 (23.1)	4 (30.7)	2 (15.4)
Mixed AII/BIV (*n* = 2)	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	0 (0.0)	1 (50.0)
Mixed AI/BIV (*n* = 1)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)
Total (*n* = 39)	22 (56.4)	17 (43.6)	1 (2.6)	5 (12.8)	8 (20.5)	14 (35.9)	11 (28.2)

3.3. Clinical data related to Giardia sub-assemblages

Table 2 presents clinical data from 36 of 39 *Giardia* infected children related to the infecting sub-assemblage. Due to the low number of cases of mixed AII/BIV and AI/BIV they were not included in the further analysis. The only correlation found between symptoms and infection with specific sub-assemblages was noted for abdominal pain, which was recorded more frequently, 5.8 times greater risk of developing this symptom, (95% *CI* = 0.898–37.700) from patients infected with sub-assemblage BIII ($P = 0.02$).

Table 2
Some clinical characteristics of children studied in relation to sub-assemblages of *G. duodenalis*. [*n* (%)].

Symptoms	Sub-assemblages			Mixed AII/BIII (*n* = 13)
	AII (*n* = 8)	BIII (*n* = 11)	BIV (*n* = 4)	
Diarrhoea	2 (25.0)	7 (63.6)	1 (25.0)	4 (30.8)
Abdominal pain	1 (12.5)	8 (72.3)	1 (25.0)	3 (23.1)
Flatulence	1 (12.5)	3 (27.3)	2 (50.0)	2 (15.4)
Nausea	3 (37.5)	3 (27.3)	1 (25.0)	5 (38.5)
Vomiting	2 (25.0)	2 (18.2)	1 (25.0)	2 (15.4)
Headache	0 (0.0)	2 (18.2)	1 (25.0)	1 (7.7)
Anorexia	4 (50.0)	4 (36.4)	2 (50.0)	3 (23.1)
Fever	1 (12.5)	1 (9.1)	1 (25.0)	1 (7.7)
Loss of weight	3 (37.5)	3 (37.5)	2 (50.0)	3 (23.1)
Fatigue	2 (25.0)	2 (18.2)	2 (50.0)	4 (30.8)

4. Discussion

In the present study we investigated the prevalence of *Giardia*, and genetic subtypes presented in preschool children living in a central region of Cuba. Screening by direct microscopy, *Giardia* had a prevalence of 10.8% in faecal samples collected over a 6-month period. *Giardia*sis in Cuba is one of the most important intestinal parasitic infections in children and the prevalence rates in the last few years have been reported from 10% to 55%[11–13].

In humans, transmission of *Giardia* is principally by faecal-oral contamination, which is reflected by higher levels of infection where levels of hygiene and sanitation are compromised, particularly in tropical and subtropical environments[2,9].

Realizing the differences in genotyping levels among different groups of *G. duodenalis* is of paramount interest in various areas of molecular medical research. These include, but are by no means limited to, comparisons of symptomatic and asymptomatic individuals to understand malfunctions in regulation and identification of genetical groups essential for diagnosis[18].

The *gdh* is one of the most popular and useful genetic markers for the genotypic analysis of *G. duodenalis*[6,8]. Analysis of the *gdh* locus has furthermore been shown to split assemblages A and B isolates into four subgroups, AI, AII, BIII and BIV[19]. The fixed genetic differences observed at the *gdh* locus support the divisions seen within *G. duodenalis* by using other genotyping methods and genetic loci[5].

Sub-assemblage BIII was seen to be the most predominant in our study (11 of 39, 28.2%). This is in agreement with previous studies reported in the international literature[4,19-21]. In this study, all clinical samples belonging to assemblage A had sub-assemblage AII except one case of mixed infection AI/BIV, which is predominantly associated with human infection, a result that is consistent with an anthroponotic origin of infection and the relation with person to person transmission pathway[22], but in other studies done in Brazil and Egypt, the sub-assemblage AI has been the predominant subtype identified[23,24], and this could be possible due to the contamination of public water with raw sewage from animal and human sources[24].

Detection of mixed genetic variants in 41% of cases (16 of 39) is higher when compared with the previous study done by Puebla *et al.* where they found a frequency of mixed assemblages infections of 17.8%[15]. It is of note that in the present study, *G. duodenalis* sub-assemblages BIII and AII together have been detected in thirteen samples. The larger proportion of mixed samples detected in this study might be indicative of environmental contamination and high frequency transmission of different *G. duodenalis* subtypes, and frequent host contact, which might contribute to the higher prevalence of mixed infections[25].

In this study we could not genotype 6 samples and this would be expected due to intermittent and/or low parasite shedding, DNA polymerase inhibitors in faecal material, and differences in gene copy number for the *gdh*[22].

As to the clinical significance of *G. duodenalis* among children, we herein described associations of symptoms with the presence of the parasite. *G. duodenalis* infection is known to vary widely in clinical manifestation including acute, chronic, and asymptomatic courses.

We compared symptoms and sub-assemblages in 36 children with pure sub-assemblages AII or BIII and BIV and AII/BIII infections. The only apparent correlation was that abdominal pain was significantly more common in children who were infected with sub-assemblage BIII parasites. We did not find any international report that had correlated symptomatology with sub-assemblage B as we identified herein, perhaps due to the fact that according to some authors, sequences from known subgroup BIII or BIV isolates are insufficient to assess the existence of subgroup BIII/BIV-specific sequence polymorphisms, and subtyping analyses of field isolates produced inconsistent sub-assemblages among different loci[1]. Nonetheless, a recent study focused on DNA sequence analysis of the *gdh* gene of *Giardia* had revealed that two assemblage B genotypes, consistent with previous *gdh* BIII/BIV descriptions were detected in one Australian community, and both genotypes were detected separately, and as mixed samples[25], supporting the former classification of BIII/BIV sub-assemblages. We were unable to supporting these results by sequencing data, which is one limitation of this study.

In previous studies made in our country by Pelayo *et al*. and Puebla *et al*.[14,15], they found a close association between assemblage B (more frequently found in children with diarrhoea, flatulence or abdominal pain) and symptomatic school children. There was no difference in features of diarrhoea or other associated symptoms, such as vomiting and fever between children with sub-assemblages A and B infection.

The majority of infections among the symptomatic cases genotyped were a result of assemblage BIII infection (8 of 21, 38.1%). According to the European network of public and veterinary health Institutions from 9 European countries that focused on zoonotic protozoan parasites, the geographic distribution of sub-assemblages BIII and BIV in humans showed marked differences between continents. In Africa, infection with *G. duodenalis* assemblage B, sub-assemblage BIII is more prevalent (81%) than infection with sub-assemblage BIV (19%), whereas the opposite is found in North America where 86% of infections are associated with sub-assemblage BIV and only 14% with sub-assemblage BIII. Finally, a more balanced distribution is found in Europe and Australia[7].

In our setting, this technique has the advantage of being able to detect and identify the presence of sub-assemblages and allows the differentiation of *Giardia* of both animal and human origin issue of importance in epidemiological studies[26].

To the best of our knowledge, this is the first study done in our country which compares results from sub-assemblages A and B of *G. duodenalis* with clinical symptoms in children. Further studies on the epidemiology of giardiasis especially the risk factors associated with sub-assemblages and genotypes would help to understand the nature of this disease, which will benefit the development of prevention and control strategies

in this population. Also the use in further studies of different loci in identifying genotypes could bring more exact information about the genetical variability in this enigmatic intestinal protozoan.

G. duodenalis remains as one of the main intestinal parasitic infections in Cuban children. The use of molecular epidemiological tools, and particularly subtyping tools are important in understanding the epidemiology and dynamic transmission of *Giardia* infection. Sub-assemblage BIII was the most commonly identified in the population of children studied, and was significantly associated with abdominal pain in the symptomatic children. Mixed sub-assemblage infections had a high frequency. Studies carried out in a higher number of samples, human and animals, with several molecular markers and from different areas will increase our understanding of the epidemiology of giardiasis. Further studies by using additional, more sensitive subtyping tools will undoubtedly gain a more comprehensive understanding of the host-specificity interaction of *Giardia* in children.

Conflict of interest statement

We declare that we have no conflict of interest.

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