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Correlation of *Giardia duodenalis* assemblages with clinical and epidemiological data in Cuban children

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ABSTRACT

Giardia duodenalis is one of the most frequent intestinal parasitic infections in children worldwide. To date, eight main assemblages of *G. duodenalis* have been described, but only A and B genetic groups are known to infect humans. In Cuba, this parasite has most clinical impact on children. The aim of this investigation was genetic characterization of *G. duodenalis* isolated from children with giardiasis diagnosed at the Paediatric Hospital "William Soler" between 2010 and 2011, and to compare the genetic results with clinical and epidemiological data. A total of 103 stool samples from 452 children were positive for *G. duodenalis* and co-infections with other parasites were noted in 5 cases. Assemblage identification was carried out by the amplification of a fragment of the triosephosphate isomerase (*tpi*) gene. Sub-assemblages of assemblage A (AI and AII) were identified by a nested PCR using the intergenic spacer (IGS) region of ribosomal deoxyribonucleic acid gene as a target. DNA from 90 of 103 (87.4%) samples was successfully amplified by PCR–*tpi*. The prevalence of assemblages A and B was 40% and 42%, respectively. Infections with both assemblages were reported in 16 cases. No associations between epidemiological information and assemblage was detected, but assemblage B was significantly ($P < 0.01$) more frequently found in children with diarrhea, flatulence or abdominal pain than assemblage A. Sub-assemblage AII accounted for the majority of cases (86.5%).

1. Introduction

Giardia duodenalis (syn. *G. intestinalis*, *G. lamblia*) is the only species of *Giardia* found in humans, although it is also found in other mammals, including pets and livestock (Thompson, 2004). Giardiasis is one of the most common human intestinal protozoal infections reported worldwide. It has been estimated that there are 280 million symptomatic cases of giardiasis each year (Feng and Xiao, 2011).

Children are one of the high risk groups for infection, particularly those in daycares settings, orphanages and primary schools (Feng and Xiao, 2011; Muhsen and Levine, 2012). In Cuba, the overall prevalence of this protozoal infection in Cuban population is about 7.2% (Escobedo et al., 2011); however, higher prevalence rates have been found among young children attending day-care centers and primary schools (Cañete et al., 2012).

G. duodenalis consists of a highly heterogeneous group of organisms and is referred to as a species complex divided at the genetic level into assemblages or genetic groups (A–H) with different host specificities. Of these, human infections are mainly caused by assemblage A and B (Monis et al., 2003; Al-Mohammed, 2011). *G. duodenalis* assemblage A was subdivided commonly into sub-assemblages AI which is mainly zoonotic and AII which is commonly anthroponotic, although it was reported by a few studies in animals (Cacciò and Ryan, 2008; Sprong et al., 2009).

A number of molecular genetic techniques have been employed successfully to characterize *Giardia* from stool samples. The vast majority of these studies have relied on the analysis of some genes such as, the small subunit ribosomal RNA (ssu-rRNA) (Hopkins et al., 1997), the β-giardin (*bg*) (Lalle et al., 2005), the glutamate dehydrogenase (*gdh*) (Read et al., 2004), and the triosephosphate isomerase (*tpi*) (Bertrand et al., 2005). The intergenic spacer (IGS) regions of rDNA have also been successfully used for molecular characterization of *G. duodenalis*, including sub-genotyping (Healey et al., 1990; Lee et al., 2006; Al-Mohammed, 2011).

The development of tools to dissect the molecular biology of different *Giardia* isolates, and the knowledge of the spectrum of symptoms associated with giardiasis, has led to the hunt for associations between particular assemblages and defined symptom patterns.

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The current assimilation of results is inconclusive, with both assemblages associated with diarrheal disease. Different symptom spectra are apparently associated with different assemblages in different populations (Robertson et al., 2010). In a previous study made by Pelayo et al. (2008) in a small group of children from La Habana, the prevalence rates in the positive cases of children in the study were 55% for assemblage B and 45% for assemblage A, using the *bg* and *gdh* genes as molecular markers, although the successful rate of genotyping was low. In that study children harbouring assemblage B of *Giardia* were more likely to have symptomatic infections than children with isolates from assemblage A.

The purpose of this study was to determine the prevalence of different *G. duodenalis* assemblages and subtypes among children with giardiasis, and investigate associations with clinical and epidemiological data collected from children attending a Paediatric Hospital from La Havana, Cuba.

2. Materials and methods

2.1. Clinical samples and surveillance data

A descriptive cross-sectional study in children attending at the Pediatric Hospital "William Soler" was conducted from 2010 to 2011. Stool samples from 452 symptomatic and asymptomatic children were examined for intestinal parasites by a wet smear stained with Lugol's iodine and followed by formalin ethyl acetate concentration technique. All the stool samples were stained by modified acid-fast for *Cryptosporidium* spp., *Cyclospora*, and *Cystoisospora* (Garcia, 2001), and were tested for common bacterial pathogens using standard culture methods (Murray and Baron, 2007).

Our samples came from a mixture of clinical cases as well as asymptomatic children enrolled in a program of school-based surveillance. These samples were divided in 163 asymptomatic children from day care centers corresponding to local surveillance to intestinal protozoa and 289 symptomatic children from primary schools and day care assistants attending at the Pediatric Hospital "William Soler" due to gastrointestinal symptoms.

All stool samples positive to *G. duodenalis* and in which bacterial pathogens were excluded were included in this research. Cases in which coinfection with other parasites, were not included in the investigation between infecting assemblages and clinical signs.

A questionnaire to obtain epidemiological and clinical data was 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 clinical symptoms (fever, flatulence, nausea, vomiting, headache, anorexia, fatigue, and loss of weight).

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

2.2. Purification of G. duodenalis cyst and DNA extraction

Giardia cysts were purified and concentrated from stool samples in a sucrose gradient with a specific gravity of 0.85 M and then washed with distilled water, following the protocol described by Babaei et al. (2011). The cyst wall was disrupted by 8–10 freeze–thaw cycles in liquid nitrogen alternated with a 95 °C water bath. After that 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 sulfate (SDS)) and vortexed. 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 40 μL distilled water and used as a template for PCR.

2.3. PCR amplification

Amplification of the *tpi* gene was performed as a single PCR with a forward 5′GGAGACCGACGAGCAAAGC3′, and reverse 5′ CTTGCCAAGCGCCTCAA3′ primers for assemblage A (PCR–*tpi* A), and a forward 5′AATAGCAGCACARAACGTGTATCTG3′ and reverse 5′CCCATGTCCAGCAGCATC 3′ for assemblage B (PCR–*tpi* B), as described by Bertrand et al. (2005). The PCR reaction mixture was done using a TopTaq Master Mix Kit (Qiagen, Germany) in a total volume of 25 μL, and comprised 10 μL of 10× PCR buffer (Qiagen, Germany), 0.2 mM of each deoxynucleoside triphosphate (dNTPs) (Applied Biosystems), 1U of Taq polymerase (Qiagen, Germany), 0.4 μM of each primer, 5 μL of Q solution, and 5 μL of DNA template, with ultrapure water used as a negative control.

The DNA was amplified using a thermocycler (MJ Research, USA) following conditions described by Bertrand et al. (2005). The PCR products were visualized on 0.5 μg/mL of ethidium bromide-staining 2% agarose gels.

To amplify the IGS of rDNA, a nested PCR was done (Table 1) on all samples previously identified as being of assemblage A, using a nested PCR as described by Lee et al. (2006), with 5 μL of template used in the first PCR, and 2 μL of template (product from the first PCR) in the second PCR. Primers are described in Table 1.

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

2.4. Statistical analysis

All data were analyzed using EPINFO 6.04 and EPIDAT 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.

After normality tests described for Shapiro–Wilk and D'Agostino and Pearson, the medians of age in years, and duration of diarrhea in days were compared using the Kruskal–Wallis non parametric test. The *P* values less than 0.05 were considered as statistically significant for all test. The odds ratio (OR) with 95% confidence interval (CI) were performed as measures of association.

3. Results

Of the 452 stool samples examined, 103 were positive to *G. duodenalis* and co-infection with other parasites was found in 5 symptomatic children (*Entamoeba histolytica*/*E. dispar*, *Ascaris lumbricoides*, *Enterobius vermicularis*, *Cyclospora cayetanensis,* and *Trichuris trichiura*). No bacterial pathogens were found in those cases. These samples were not included in the investigation of associations between assemblages and clinical characteristics. Among 163 asymptomatic children investigated from day care centers only 8 individuals were infected by *Giardia,* the other 95 were represented by symptomatic children from day care centers or primary schools.

Of the samples from 103 children with giardiasis, PCR products of the expected size were generated from 90 of them by PCR–*tpi* (87.4%). The prevalence of assemblages showed that 36 (40%) isolates belonged to assemblage A and 38 (42.2%) to assemblage B. The remaining 16 (17.8%) isolates demonstrated mixed infections of both assemblages. Among the 52 positive samples classified as assemblage A (including those in mixed infections), 45 (86.5%)