Original Article

Giardia duodenalis genotypes among schoolchildren and their families and pets in urban and rural areas of Sinaloa, Mexico

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Abstract

Introduction: Giardiasis is a human health concern worldwide, especially among schoolchildren. *Giardia duodenalis* genotypes A and B are infective to humans, but their zoonotic potential remains controversial. In Mexico, the most prevalent genotype is A, but B was also detected in southeastern Mexico. In Sinaloa state, northwestern Mexico, giardiasis is highly prevalent, but *Giardia* genotypes have been poorly studied.

Methodology: This study aimed to investigate the distribution and clinical-epidemiological correlation of *G. duodenalis* genotypes in schoolchildren and their families and pets in urban and rural areas of Sinaloa state, Mexico.

Results: Among 395 schoolchildren (274 urban, 121 rural), 76 (49 urban, 27 rural) were infected with *G. duodenalis*. In total, 22 families (15 urban, 7 rural) of infected schoolchildren, consisting of 60 family members (41 urban, 19 rural) and 21 pet dogs (15 urban, 6 rural) were examined; 10 family members (5 urban, 5 rural) and 5 pet dogs (3 urban, 2 rural) of 10 families (6 urban, 4 rural) were infected. After PCR-RFLP analyses of *vsp417* and *gdh* genes, genotype prevalence among infected urban schoolchildren was 79.5% AI, 12.8% AII, and 7.7% mixed AI+B. However, only AI genotype was found in family members and pets. In the rural area, only the AI genotype was detected. Genotypes were not correlated with clinical manifestations.

Conclusions: This paper shows the presence of B genotype in northwestern Mexico for the first time. Detection of AI genotype in dogs suggested the possible role of dogs as the reservoir for human giardiasis in Sinaloa, Mexico.

Key words: *Giardia* genotypes; variant-specific surface protein 417; *vsp417*; glutamate dehydrogenase.

Introduction

Giardia duodenalis (synonym *G. intestinalis* and *G. lamblia*) is a cosmopolitan enteropathogenic protozoon that infects not only humans but also a wide range of domestic and wild mammals [1-3]. This parasite is more prevalent in children, causing chronic diarrhea, abdominal pain, malabsorption syndrome, and resultant impairment of growth and development [4]. Giardiasis is the main cause of epidemic enteritis worldwide and is considered a re-emerging infectious disease of public health concern [5]. In 2004, the World Health Organization (WHO) included giardiasis in the group of neglected tropical diseases [6].

About 200 million people are infected with *G. duodenalis* in Asia, Africa, and Latin America, and 500,000 new cases are registered every year [7]. It is the most common human intestinal parasite in developed countries, with prevalence rates ranging from 2% to 7%

[3]. In developing countries, the prevalence rates are > 30% [8]. In Mexico, the prevalence rates range between 3% and 50% in various states (Sinaloa, Sonora, Mexico D.F., Oaxaca, and Yucatan), and the prevalence rates in some states are much higher than those of other developing countries [9-12].

G. duodenalis is also a common protozoon in dogs, with various prevalence rates depending on the region and/or country [1]. Cysts and trophozoites of *G. duodenalis* in fecal samples of humans and pet dogs are morphologically indistinguishable from each other. Thus, genotyping is essential to determine the zoonotic potential of this parasite. Until now, eight genotypes have been identified, but only genotypes A (AI, AII) and B (BIII, BIV) have been proven to be human pathogens [3]. The *Giardia* genotypes in humans have also been detected in different hosts such as canines, felines, and artiodactyls [13-17].

Sinaloa state is located in northwest Mexico, on the Pacific coast, and is known as a high prevalence area of giardiasis (31%), which affects mainly children [10]. In spite of the public health concern of giardiasis in Sinaloa state, only one preliminary research study has been published on genotyping with a small number of samples (12 children and 19 dogs); this study showed only genotype A in humans and dogs, with comparable prevalence rates of subgenotypes AI and AII in dogs (AI 52.6% and AII 47.4%) and children (AI 41.7% and AII 58.3%) [18].

The aim of this study was to analyze the genotypic prevalence of *G. duodenalis* in symptomatic and asymptomatic schoolchildren together with their families and pets in urban and rural areas of Sinaloa state, Mexico. The relationship between the genotypes/subgenotypes and the hygienic habits of schoolchildren was analyzed to generate information about the mode of giardiasis transmission.

Methodology

Application of a clinical-epidemiological questionnaire

Elementary schoolchildren of the urban and rural areas in Sinaloa state, Mexico, were randomly selected. A clinical-epidemiological questionnaire was applied to *G. duodenalis*-positive schoolchildren to obtain data about clinical manifestations, hygienic habits, and contact with domestic animals. Additionally, the fecal samples of their family members and pets were examined (Table 1). Correlation between clinical features and the genotypes of *Giardia* detected were examined. The ethics committee of the Autonomous University of Sinaloa approved the experimental protocol for this research, and all individuals involved provided their signed informed consent for their data to be used in this research.

Sampling

School authorities and parents were informed about the objectives and methods of this study, and written informed consent was obtained. Fecal samples of schoolchildren in urban (274) and rural (121) areas were collected from June 2013 to June 2014. All participants provided three consecutive samples so that

Table 1. Number and distribution of the schoolchildren, families, and pets studied.

Area	Schoolchildren	Families (number of family members)	Pets
Urban	274	15 (41)	15
Rural	121	7 (19)	6
Total	395	22 (60)	21

the total number of samples examined was 1,185. *Giardia*-positive children were identified by copro-parasitological examinations, and the fecal samples of their families and pets (three samples per family member and pet dog) were collected. The number of family members and pet dogs studied were 41 and 15 in the urban area and 19 and 6 in the rural area, respectively (Table 1). *G. duodenalis* genotyping was carried out for 63 schoolchildren (39 urban and 24 rural areas), 10 family members, and 5 pet dogs.

Coproparasitological examination

Presence of *G. duodenalis* in fecal samples was determined by the concentration methods of Faust *et al.* [19] and Ritchie [20]. All feces with the parasite were stored at 4°C. The cysts in fecal samples were concentrated and purified as previously described [21] and stored in 70% ethanol at -20°C until use.

DNA extraction from G. duodenalis cysts

DNA extraction was carried out as previously described [22] with slight modification. Briefly, concentrated cysts were incubated at 65°C for 30 minutes in lysis buffer pH 7.4 (100 mM Tris-HCl, 100 mM EDTA, 2% SDS, 0.2 M NaCl, 1 mM mercaptoethanol) and proteinase K (1 mg/mL) (Promega, Madison, USA). The mixture was stirred for 10 seconds every 10 minutes. DNA was purified with the Ultra Clean Fecal DNA Kit (MO BIO, Carlsbad, USA) according to the manufacturer's instruction. DNA was spectrophotometrically quantitated at 260 nm, and its quality corroborated in 1% agarose gel electrophoresis in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3).

Polymerase chain reaction (PCR)

G. duodenalis genotyping was done using fragment amplification of the variant-specific surface protein (*vsp*417) and glutamate dehydrogenase (*gdh*) genes [23]. PCR products were analyzed by agarose (1%) gel electrophoresis in TBE (90 minutes, 50 mA, 70 V, and 10 W). Gels were stained with ethidium bromide 0.5 µg/mL.

Amplification of the vsp417 gene

Two fragments of vsp417 gene were simultaneously amplified. The fragment for the A genotype was about 520 bp and for the B genotype was 350 bp. The primers used were 432, 5´ ACGACGGTACTAAGGGCACGTG 3´; and 433, 5' GTATCC(T/C)GGAGCTCAC(G/A)CCTTACATGTT GTAGCTGCTCCA 3´. The reaction mixture contained

Table 2. Prevalence of *Giardia duodenalis* and other intestinal parasites in schoolchildren (S) and family members (FM) in urban and rural areas of Sinaloa, Mexico.

Parasite	Number of positive cases (%)[1]			
	Urban		Rural	
	S (n = 274)	FM (n = 41)	S (n = 121)	FM (n = 19)
Protozoa				
Giardia duodenalis	49 (18)	5 (12.2)	25 (20)	5 (26.3)
Entamoeba histolytica/ Entamoeba dispar	4 (1.5)	0 (0)	0 (0)	0 (0)
Blastocystis hominis	2 (0.7)	0 (0)	1 (0.8)	0 (0)
Helminths				
Enterobius vermicularis	19 (6.9)	3 (3.7)	7 (5.8)	0 (0)
Hymenolepis nana	8 (2.9)	3 (3.7)	0 (0)	0 (0)
Strongyloides stercoralis	2 (0.7)	1 (2.4)	0 (0)	0 (0)
Polyparasitism				
G. duodenalis and Enterobius vermicularis	0 (0)	0 (0)	1 (0.8)	0 (0)
G. duodenalis and Hymenolepis nana	0 (0)	0 (0)	1 (0.8)	0 (0)
Entamoeba coli and Endolimax nana	0 (0)	3 (7.3)	4 (3.3)	0 (0)
Non-pathogenic protozoa				
Entamoeba coli	44 (16)	3 (7.3)	9 (7.4)	0 (0)
Endolimax nana	65 (23.7)	1 (2.4)	11 (9.1)	0 (0)
Iodamoeba bütschlii	1 (0.4)	0 (0)	0 (0)	0 (0)

[1] n is the total number of schoolchildren (S) and family members (FM) studied in the corresponding area.

2.5 mM $MgCl_2$, 200 µM dNTPs, 12.5 µM of each primer, 1.25 units of DNA Taq polymerase (Expand Long Template PCR System, Roche, Basilea, Switzerland), and 50 ng of DNA of *G. duodenalis* cysts. The final reaction volume was 50 µL. Amplification was for 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 2 minutes, 95°C for 30 seconds, 66°C for 30 seconds, starting from the initial DNA denaturation temperature of 95°C for 5 minutes and a final PCR extension of 72°C for 7 minutes [23].

Amplification of gdh gene

Nested PCR was used to amplify a fragment of 430 bp of the gdh gene. The primers for the first amplification were 578, 5´ GAGAGGATCCTTGARCCNGAGCGCGTNATC 3' and 579, 5´ ACCTTCTAGAANCCNGCDATGTTNGCGCC 3´. The reaction mixture contained 4 mM $MgCl_2$, 200 µM dNTPs, 12.5 µM of each primer, 1.25 units of DNA Taq polymerase (Expand Long Template PCR System), and 50 ng of DNA of *G. duodenalis* cysts. The final volume of the reaction mixture was 50 µL. Amplification was done by 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 2 minutes, starting with an initial DNA denaturation temperature of 95°C for 4 minutes

and a final PCR extension of 72°C for 6 minutes [24]. The primers for the second amplification were gdhIF (5´ CAGTACAACTCYGCTCTCGG 3´) and gdhIR (5´GTTRTCCTTGCACATCTCC 3´). Samples were amplified for 40 cycles of 94°C for 30 seconds, 58°C for 1 minute, 72°C for 2 minutes, 94°C for 30 seconds, 56°C for 30 seconds, and 72°C 45 seconds, starting with an initial DNA denaturation temperature of 94°C for 2 minutes and a final PCR extension of 72°C for 7 minutes. The reaction mixture contained 1.5 mM $MgCl_2$, 200 µM dNTPs, 12.5 µM of each primer, 1.25 units of DNA Taq polymerase (Expand Long Template PCR System), and 3 µL of the first amplification reaction. The final volume of the reaction mixture was 50 µL [25].

Restriction analyses of the PCR products

The amplified product of 520 bp of the v*sp417* gene was digested with PstI (Promega, Madison, USA) for 3 hours at 37°C in a reaction volume of 20 µL. The expected bands for the AI subgenotype were about 520, 330, and 190 bp, and for the AII were 520, 370, 330, 190, and 150 bp [23]. Digested fragments were analyzed on 1% agarose gel electrophoresis (90 minutes, 50 mA, 70 V, and 10 W) in TBE and stained with 0.5 µg/ mL ethidium bromide.

Table 3. *Giardia duodenalis* prevalence in family members and pets of infected schoolchildren in urban and rural areas of Sinaloa, Mexico.

Area	Family[1]	Pet[2]	Number of family members[3]	
			Total	Infected (%)
Urban	1	+	4	0 (0)
	2	+	4	0 (0)
	3	-	5	2 (40)
	4	+	5	1 (20)
	5	-	3	1 (33.3)
	6	-	3	1 (33.3)
Rural	1	-	5	2 (40)
	2	-	3	2 (66.6)
	3	+	4	1 (25)
	4	+	4	0 (0)

[1] Of the 22 families (15 urban and 7 rural) studied, only 6 urban and 4 rural families had *Giardia*-positive family members or pets; [2] Total number of studied pets were 21 (15 urban and 6 rural). Families included in this table had one pet (adult dog). Pet was infected (+) or not (-); [3] Total number of studied individuals were 60 family members (41 urban and 19 rural).

The PCR product of *gdh* was digested with *Nla IV* (Promega, Madison, USA) for 3 hours at 37°C in a reaction volume of 20 µL. The expected bands for the *G. duodenalis* genotypes were for AI, 150, 120, and 90 bp; for AII, 120, 90, 80, and 70 bp; and for B, 290 and 120 bp [25]. Digested fragments were analyzed on 1.5% agarose gel electrophoresis (90 minutes, 50 mA, 70 V, and 10 W) in TBE and stained with 0.5 µg/ mL ethidium bromide.

Statistical analysis

This research was a cross-sectional, prospective, observational, descriptive, and correlational study. Unrestricted random sampling was carried out for the elementary schoolchildren in the urban and rural areas in Sinaloa state, Mexico. Genetic analyses were carried out in triplicate. Data were analyzed by descriptive statistics, and statistical differences were determined using Pearson's Chi-square test with the software Stata Intercooled, version 13.1.

Results

Prevalence of G. duodenalis

G. duodenalis prevalence among schoolchildren in urban (18%) and rural (20%) areas was similar, without statistical difference ($p = 0.303$). The schoolchildren studied were infected with other parasites and/or non-pathogenic protozoa, but the prevalence of these was far lower than that of *G. duodenalis* (Table 2).

Table 3 shows the prevalence of giardiasis in families in which at least one relative or pet dog was *Giardia* positive. As shown in Table 3, 6 families in the urban area and 4 families in the rural area had at least one relative or pet dog positive for giardiasis. The infection rate of the family members of *G. duodenalis*-infected schoolchildren was 5/41 (12.2%) in the urban area and 5/19 (26.3%) in the rural area (Table 3), similar to the prevalence among the schoolchildren in the present study (Table 2). Infection rates of the pet dogs were 3/15 (20%) and 2/6 (33.3%) in the urban and rural areas, respectively.

Hygienic habits of schoolchildren in relation to Giardia transmission

Schoolchildren in urban and rural areas showed basically similar hygienic habits. The most common one was playing with soil, although no significant difference was seen between rural and urban areas. Significant correlation was seen only between giardiasis and not washing hands after going to the bathroom ($p = 0.049$) (Table 4).

G. duodenalis genotypes / subgenotypes in schoolchildren

Genotyping results revealed that *Giardia*-positive schoolchildren in the urban area were infected with *G. duodenalis* genotypes AI, AII, and AI+B (Table 5). Among 49 isolates from schoolchildren in the urban

Table 4. Hygienic habits of schoolchildren from urban and rural area of Sinaloa, Mexico.

Hygienic habits of schoolchildren	Frequency in the area (%)		
	Urban	Rural	P value
Play with soil	71.8	62.5	0.441
Do not wash their hands before eating	43.6	58.3	0.256
Do not wash their hands after going to the bathroom	25.6	50.0	0.049
Do not wash fruits and vegetables	7.7	4.2	0.577

Table 5. Distribution and prevalence of *Giardia duodenalis* genotypes/ subgenotypes of the isolates from the studied individuals or pets.

Area	Sampled population	Number of positive cases (%)		
		AI	AII	AI, B
Urban	Schoolchildren	31 (79.5)	5 (12.8)	3 (7.7)
	Females	16	3	2
	Males	15	2	1
	Family members	5 (100)	0 (0)	0 (0)
	Pets	3 (100)	0 (0)	0 (0)
Rural	Schoolchildren	24 (100)	0 (0)	0 (0)
	Females	11	0	0
	Males	13	0	0
	Family members	5 (100)	0 (0)	0 (0)
	Pets	2 (100)	0 (0)	0 (0)

area, the *vsp417* gene was successfully amplified from 39 (79.6%) samples. After restriction fragment length polymorphism (RFLP), 36 were identified as genotype A (92.3%) and, remarkably, 3 were identified as mixed infections of A and B genotypes (7.7%). The *PstI* restriction of the amplicons of genotype A showed that subgenotype AI was detected in 31 schoolchildren (79.5%), AII in 5 (12.8%), and AI+B mixed infections in 3 (7.7%). All 5 isolates from family members and 3 pets in the urban area were AI subgenotype. The amplification of the *gdh* gene and *Nla IV* restriction of the amplified fragments gave the same detection rates. In contrast, only subgenotype AI was found in the isolates from the rural area (Table 5).

Relationship between G. duodenalis genotype and the gender and age of infected schoolchildren

In the urban area, the AI subgenotype was most prevalent in both genders and comprised 79.5% of the total isolates. Two of three mixed infections (AI+B) and three of five AII infections were in females. Consequently, distribution of *Giardia* genotypes was not associated with gender (p = 0.768) (Table 5).

In the urban area, the age range of studied schoolchildren was 6–12 years. Mixed infections (A+B) were found among schoolchildren 7–8 years of age, and AII infections were seen in the 8, 10, and 12- year-old children. Statistical analysis revealed that genotype AI was significantly prevalent among the 8-year-old schoolchildren (6/8), followed by 10-year-old children (1/3).

In the rural area, AI was the only subgenotype found, and the frequencies among males and females were almost comparable (Table 5).

Relationship between G. duodenalis genotypes and clinical features of infected people

In total, 26 (66.7%) of 39 *Giardia*-positive schoolchildren in the urban area were symptomatic and 13 (33.3%) were asymptomatic. The most frequent symptom was abdominal pain alone (61.5%) or accompanied with diarrhea (34.6%); other symptoms such as nausea/vomiting and/or diarrhea were less prevalent. Two of the three cases with mixed infections (AI+B) and four of the five cases with AII subgenotype showed symptoms compatible with giardiasis (abdominal pain and diarrhea) (Table 6). In general, symptomatology and *G. duodenalis* genotypes/subgenotypes were not correlated (p = 0.498).

In the rural area, all 24 isolates from schoolchildren were identified as subgenotype AI, and 14 (58.3%) of them showed giardiasis symptoms (Table 6).

Table 6. *Giardia* genotypes and clinical features of 63 schoolchildren (39 in urban and 24 in rural areas) from Sinaloa, Mexico.

Clinical features	Area			
	Urban			Rural
	AI	AII	AI, B	AI
Asymptomatic	11	1	1	10
Symptomatic	20	4	2	14
Diarrhea	1	0	0	0
Abdominal pain	12	2	2	11
Abdominal pain and vomiting	0	0	0	1
Abdominal pain and diarrhea	7	2	0	2

Discussion

G. duodenalis is the most prevalent intestinal protozoon associated with chronic diarrheal disease worldwide; it impairs the growth, learning, and nutritional status of infected people, particularly children [3]. This illness is considered to be a marker of socio-cultural backwardness [26]. In Mexico, the prevalence of giardiasis varies from 3% to 50% depending on the regions [27]. In Sinaloa state, Quihui et al. [10] registered a *G. duodenalis* prevalence of 31% for children in 2006. The present results showed a similar high prevalence rate of *G. duodenalis* infection among schoolchildren, and it was the most prevalent enteropathogenic parasite among the schoolchildren studied. This protozoon is the most prevalent parasite in northwestern Mexico [11], and our results strongly suggest the requirement of rapid improvement of sanitation and health conditions in the studied area.

Nowadays, information about epidemiology and transmission of *G. duodenalis* is supported by genotyping. However, despite the high prevalence of giardiasis in Latin America, molecular epidemiological studies are still scarce. Lindarte et al. [28] reviewed *G. duodenalis* prevalence in several countries of the Americas (i.e. Canada, United States, Mexico, Brazil, Argentina, Colombia, Nicaragua, and Peru) during 1990–2009, and found that most of *G. duodenalis* isolated from human cases were identified as genotype A. However, *Giardia* genotypes in 181 individuals were recently studied in Colombia, with genotype B (90%), A (3%), and mixed infections A+B (7%) detected [29]. Although genotype B and mixed A and B infections have been reported in many countries of the world [30-36], previous studies in Mexico reported only the presence of genotype A (AI and AII) [9,18,37-40] until the recent discovery of genotype B in humans in southwestern Mexico [12].

The present results show the predominance of genotype A, particularly subgenotype AI and, remarkably, the first report of mixed infections (AI+B) in humans in northwestern Mexico.

Giardiasis symptomatology is highly variable with its association to host and parasite factors, although the precise mechanism remains unknown [3]. The relationship between genotypes and clinical features remains controversial; some researchers have concluded that asymptomatic cases were caused by B genotype [41], whereas others reported the association of this genotype with recurrent diarrhea [34] or abdominal pain [22]. Various factors, such as biological, geographic, human activities, and wildlife of certain region, among others, have been proposed for higher prevalence of one genotype over another [21,42]. In Mexico, published studies about human giardiasis are lacking clinical data and, as mentioned above, this pathology is mainly associated with genotype A (AI, AII or AI-AII) [9,18,37-40]. Only one report showed six genotype B cases in southeastern Mexico [12]. In the present results, most of the *Giardia*-positive schoolchildren were symptomatic, with a high prevalence of abdominal pain. Abdominal pain and diarrhea were common symptoms in individuals with *Giardia* mixed infections AI+B (66.7%) and with AII (80%). Moreover, although differences were not significant (p > 0.05), most of the schoolchildren with abdominal pain were infected with subgenotype AI (Table 6), which is consistent with previous reports [37,43,44].

In dogs, *G. duodenalis* is one the most common causes of subclinical infections, and the risk of infection is associated with the environmental hygienic status [1]; the prevalence of *Giardia* infection in dogs in this study (urban zone 20% and rural zone 33.3%) agreed with this information. Prevalence of *Giardia*-positive dogs is highly variable among Latin American countries (1% to 46.5%) and among the regions of a country [1,45].

Keeping *G. duodenalis*-infected dogs in the home increases the risk of human infection, and if contact occurs more than once per week, the risk is 2.3 times higher [46,47]. Although *G. duodenalis* isolated from dogs typically belong to C or D genotypes, A and B also have been identified in the regions with high endemic areas of genotypes A and B, such as in Argentina [21], Brazil [42], Peru [48], and Mexico [18,38-40]. Sharing of the same *G. duodenalis* genotype between dogs and their owners has been reported [49]. Our study showed that pets (adult dogs), schoolchildren, and their family members (parents and siblings) share the subgenotype AI, suggesting the zoonotic transmission of this subgenotype in Sinaloa. Related to this, Eligio-García et al. [18] reported subgenotypes AI (52.6%) and AII (47.4%) in puppies in this area.

Conclusions

In this work, 395 schoolchildren (274 urban, 121 rural) were studied, and 76 (49 urban, 27 rural) of them were infected with *G. duodenalis*. The PCR-RFLP analyses of *vsp417* and *gdh* genes showed that genotype prevalence was 79.5% AI, 12.8% AII, and 7.7% mixed AI+B. In rural areas, only AI genotype was detected. A total of 22 families (15 urban, 7 rural) of infected schoolchildren, consisting of 60 family members (41 urban, 19 rural) and 21 pet dogs (15 urban, 6 rural) were examined, and 10 family members (5 urban, 5 rural) and

5 pet dogs (3 urban, 2 rural) of 10 families (6 urban, 4 rural) were infected only with AI *G. duodenalis* genotype. Genotypes were not correlated with clinical manifestations, and the most frequent symptom was abdominal pain. *G. duodenalis* subgenotype AI was the most prevalent in schoolchildren, their family members, and pets in Sinaloa state, located in northwestern Mexico, suggesting the possible zoonotic transmission of *G. duodenalis* in this area. In addition, this is the first record of B genotype (mixed infections AI+B) in northwestern Mexico. These results are very important because they were obtained from a Mexican endemic area of giardiasis where *Giardia* genotypes have been poorly studied. In the future, more studies including a higher number of areas, cases, and potential reservoir species are necessary for the design and implementation of effective strategies to control this intestinal parasitosis.

Acknowledgements

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Conflict of interests: No conflict of interests is declared.