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Molecular diagnosis and genotype analysis of *Giardia duodenalis* in asymptomatic children from a rural area in central Colombia

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ABSTRACT

Giardiasis is a parasitic infection that affects around 200 million people worldwide. This parasite presents a remarkable genetic variability observed in 8 genetic clusters named as 'assemblages' (A–H). These assemblages are host restricted and could be zoonotic where A and B infect humans and animals around the globe. The knowledge of the molecular epidemiology of human giardiasis in South-America is scarce and also the usefulness of PCR to detect this pathogen in fecal samples remains controversial. The aim of this study was to conduct a cross-sectional study to compare the molecular targets employed for the molecular diagnosis of *Giardia* DNA and to discriminate the parasite assemblages circulating in the studied population. We analyzed 181 fecal samples from Children at La Virgen, Cundinamarca, Colombia that were DNA-extracted and analyzed by SSU rDNA, *tpi* and *gdh* loci. We observed positivity by microscopy of 13% and by PCR around 76–80% depending on the molecular marker. Additionally, a lack of statistical concordance between microscopy and PCR was detected. Regarding the genetic assemblages, we detected assemblage A (3%), assemblage B (90%) and mixed infections assemblages A + B (7%). Hence, the sub-assemblages were typed as AI, AII, BIII and BIV across the population. This study represents a reliable attempt to understand the molecular epidemiology of giardiasis in Colombia and the use of PCR to detect cryptic infections. The epidemiological implications are herein discussed.

1. Introduction

The protozoan parasite *Giardia duodenalis* (syn. *Giardia intestinalis*, *Giardia lamblia*) infects a wide range of vertebrates including humans, domestic and wild animals. This cosmopolitan parasite is re-emerging as one of the most frequent causes of diarrhea in humans from developing countries and is considered as one of the most common parasitic infection with 200 million people infected worldwide representing a serious public health concern (Ryan and Cacciò, 2013). In some cases the infection is asymptomatic and when symptoms occur they may range from chronic diarrhea, abdominal pain to severe malabsorption, having an adverse impact on growth and intellectual development (Feng and Xiao, 2011; Haque et al., 2005). Children, in special those living in developing countries and living in rural communities, are most at risk from the clinical consequences of *Giardia* infection. In September 2004, giardiasis was included in the 'Neglected Diseases Initiative' of the WHO (Savioli et al., 2006) demonstrating the epidemiological relevance of this infection. In Colombia, *Giardia* prevalence ranges from 11% to 61% in studies conducted in children, representing a concern in terms of public health (Giraldo-Gómez et al., 2005; Chaves Mdel et al., 2007; Lora-Suárez et al., 2002; Rodríguez et al., 2014).

Isolates of *G. duodenalis* are currently grouped into 8 recognized genotypic clusters or 'assemblages' (A–H), based on molecular genetic analyses at limited and highly conserved loci. Assemblages A and B are zoonotic, whereas C, D, E, F, G, and H are defined as host restricted (Thompson, 2000; Monis and Thompson, 2003; Monis et al., 2009; Andrews et al., 1998). Human infective assemblages (A and B) have been further sub-grouped using conserved loci such as glutamate dehydrogenase (*gdh*) and triose phosphate isomerase (*tpi*) accordingly: AI, AII, BIII, and BIV with no strict associations based on clinical presentation (Monis et al., 1998; Ryan and Cacciò, 2013; Thompson, 2002). *G. duodenalis* isolates obtained from children stools showed a possible association between the clinical signs and assemblage (Read et al., 2002; Cedillo-Rivera et al., 2003). For example, in

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Cuba children harboring assemblage B infection were more likely to have symptomatology than children infected with assemblage A (Puebla et al., 2014). Assemblage AI is associated with multiple host species and AII is more restricted to humans. In Colombia, two reports have demonstrated the prevalence of assemblages among humans in two areas of the country. In the northwest province of Bolivar, molecular characterization of *Giardia* has identified Assemblage A (5.1%) and Assemblage B (92.3%) (Arroyo-Salgado et al., 2013). Recently, another report in the center of the country (Tolima) suggests that the distributions of Assemblage A (48%) and B (52%) are similar. Regarding the sub-assemblages of A and B; AII, BIII and BIV were identified (Rodríguez et al., 2014).

The advent of molecular biology techniques has allowed the identification of target candidates for the molecular diagnosis of *Giardia* such as SSU rDNA, β-giardin, *tpi* and *gdh* (Yang et al., 2014; Amar et al., 2002; Almeida et al., 2010; Bertrand et al., 2005; Lalle et al., 2005). These markers have demonstrated that cryptic infection is evident and in some cases the prevalence of infection can change from 5.8% to 31.5% suggesting the need to implement molecular diagnosis instead of microscopy in *Giardia* (Mejia et al., 2013). The aim of the study was to determine the prevalence of *Giardia* infection by means of molecular methods via PCR targeting three molecular markers (*gdh*, SSU rDNA and *tpi*). Sensitivity, specificity and statistical concordance (kappa index) with microscopy was calculated. Finally, positive samples were analyzed in order to discriminate the genetic assemblages circulating in a geographically restricted population of children in Colombia. This is the first time that a Colombian cohort with *Giardia* infection is genotyped by means of DNA sequencing.

2. Materials and methods

2.1. Population study

We obtained 181 fecal samples from asymptomatic children under 16 years old from the hamlet La Vírgen, Cundinamarca (Colombia) that voluntarily accepted to participate in the study. The children were physically examined to verify their asymptomatic status. The ethical clearance of this study was followed by the ethics of Helsinki declaration and resolution No. 008430 of 1993 from the Ministry of Health from Colombia and "El Código del Menor". The study protocol was approved by the ethics committee from the faculty of Medicine of the Universidad Nacional de Colombia under the Number 0045763. La Virgen hamlet is located in the department of Cundinamarca at 4° 45′ north latitude and 74° 32′ west longitude with an altitude of 1050 m above sea level. The overall percentage of unsatisfied basic needs (UBS) is 58%.

2.2. Fecal samples collection, microscopic diagnosis and DNA extraction

The fecal samples were collected in plastic recipients, labeled and conserved in refrigerated boxes. The samples were divided in two parts: one part was fixed in a proportion (1:4) in ethanol 70% and stored at −20 °C for DNA extraction. The other part was used for conducting Kato-katz, modified Richie–Frick method and direct microcopy examination for diagnosis of intestinal parasites (Chaves et al., 1979). For the microscopic diagnosis of intestinal parasites, fecal samples were examined by experimented microscopists using wet mount smears in saline solution and by triplicate. All slides were examined by three experienced parasitologists for quality check. From each sample, 250 mg were submitted to DNA extraction using the QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to manufactureŕs instructions. Genomic DNA was preserved at −20 °C until analysis.

2.3. Molecular diagnosis

Detection of *G. duodenalis* DNA was carried out using SSU rDNA, *gdh* and *tpi*. For SSU rDNA subunit, a nested PCR was done using specific primers RH11 and RH4 in the first round, and GiarF and GiarR in the second round. For *gdh* locus, a semi-nested PCR was done using primers *GDH*eF, *GDH*iF and *GDH*iR. For the *tpi* locus, a nested PCR was done using primers AL3543 and AL3546 in the first round, and AL3544 and AL3545 in the second round. PCR assays were performed on a SensoQuest 96 Well Thermal-cycler (LabCycler) using the GoTaq Green Master Mix (Promega). Concentration of primers in PCR reactions was 10 μM each and we added 5 μL of DNA, the samples were run in duplicate. All reactions started with an activation-denaturation step at 94 °C for 5 min and then were carried out for 40 cycles, each consisting of 94 °C for 30 s, an annealing step of 30 s at a temperature that varied from 50 to 58 °C depending on the marker as reported elsewhere (Feng and Xiao, 2011), and of 72 °C of extension for 30 s. A final extension step was carried out at 72 °C for 7 min. PCR products were electrophoresed on GelRed-stained 2% agarose gels in TAE 1× at 100 V for 30 min. Positive (WB and GS strains) and negative (water) controls were always included in the reactions.

2.4. Molecular characterization

For the discrimination of *G. duodenalis* genetic assemblages (A and B) we employed five strategies: (i) PCR-RFLP of *gdh*, (ii) assemblage specific PCR primers of *tpi* as reported by Bertrand et al., 2005, (iii) assemblage specific PCR primers of GL50581_3242 Hypothetical protein, (iv) assemblage specific PCR primers of GL50581_725: Protein 21.1 as reported elsewhere (Vanni et al., 2012) and (v) Direct Sanger sequencing of *gdh* locus (Read et al., 2004; Bertrand et al., 2005). For the discrimination of sub-assemblages AI, AII, BIII and BIV we employed a PCR-RFLP targeting the *gdh* locus. The sequences of the primers employed are listed in Table 1 and the algorithm employed for the discrimination of *G. duodenalis* assemblages and sub-assemblages is shown in Fig. 1.

Table 1
List and sequence of primers used for the molecular diagnosis and characterization of *G. duodenalis*.

Gene	Primer (5′–3′)	Reference
SSU rDNA	RH11 (CATCCGGTCGATCCTGCC)	Hopkins et al. (1997)
	RH4 (AGTCGAACCCTGATTCTCCGCCCAGG)	Hopkins et al. (1997)
	GiarF (GACGCTCTCCCCAAGGAC)	Hopkins et al. (1997)
	GiarR (CTGCGTCACGCTGCTCG)	Hopkins et al. (1997)
gdh	GDHeF (TCAACGTYAAYCGYGGYTTCCGT)	Read et al. (2002)
	GDHiF (CAGTACAACTCYGCTCTCGG)	Read et al. (2002)
	GDHiR (GTTRTCCTTGCACATCTCC)	Read et al. (2002)
tpi	AL3543 (AAATIATGCCTGCTCGTCG)	Sulaiman et al. (2003)
	AL3546 (CAAACCTTITCCGCAAACC)	Sulaiman et al. (2003)
	AL3544 (CCCTTCATCGGIGGTAACTT)	Sulaiman et al. (2003)
	AL3545 (GTGGCCACCACICCCGTGCC)	Sulaiman et al. (2003)
4E1-HP	A4E1F (AAAGAGATAGTTCGCGATGTC)	Vanni et al. (2012)
	A4E1R (ATTAACAAACAGGGAGACGTATG)	Vanni et al. (2012)
	B4E1F (GAAGTCATCTCTGGGGCAAG)	Vanni et al. (2012)
	B4E1R (GAAGTCTAGATAAACGTGTCGG)	Vanni et al. (2012)
5C1-P21	A5C1F (ATGCTAGCCGTAGTTAATAAGG)	Vanni et al. (2012)
	A5C1R (ACCGGCCTTATCTACCAGC)	Vanni et al. (2012)
	B5C1F (TTAATAGAAATGCTTTCGACACG)	Vanni et al. (2012)
	B5C1R (TTGCTACAGCAGAAAGGTGC)	Vanni et al. (2012)