Molecular identification of *Giardia duodenalis* in Ecuador by polymerase chain reaction-restriction fragment length polymorphism

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The aim of this study was to determine the genetic diversity of Giardia duodenalis *present in a human population living in a northern Ecuadorian rain forest. All* Giardia *positive samples (based on an ELISA assay) were analysed using a semi-nested polymerase chain reaction-restriction fragment length polymorphism assay that targets the glutamate dehydrogenase (*gdh*) gene; those amplified were subsequently genotyped using NlaIV and RsaI enzymes. The* gdh *gene was successfully amplified in 74 of 154 ELISA positive samples; 69 of the 74 samples were subsequently genotyped. Of these 69 samples, 42 (61%) were classified as assemblage B (26 as BIII and 16 as BIV), 22 (32%) as assemblage A (3 as AI and 19 as AII) and five (7%) as mixed AII and BIII types. In this study site we observe similar diversity in genotypes to other regions in Latin America, though in contrast to some previous studies, we found similar levels of diarrheal symptoms in those individuals infected with assemblage B compared with those infected with assemblage A.*

Key words: Ecuador - *Giardia duodenalis* - genotypes - PCR-RFLP - glutamate dehydrogenase

A variety of molecular tools including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), isoenzymes analysis and sequence analysis of several genes have provided genotypic information about *Giardia duodenalis* (Read et al. 2004, Cordón et al. 2008, *Tungtrongchitr* et al. 2010, Feng & Xiao 2011, Pestehchian et al. 2012). Based on these analyses, isolates of *G. duodenalis* have been classified into seven genetic assemblages, A-G (Thompson et al. 2000, Read et al. 2004). Assemblages A and B, infect humans as well as a number of other hosts, whereas the other genetic assemblages C-G are restricted to domestic animals, livestock and wild animals (Bertrand et al. 2005). Assemblage A, in humans has been classified into two groups, I and II and assemblage B has been classified into groups, III and IV (Thompson et al. 2000). PCR-RFLP has been used previously to target the glutamate dehydrogenase (*gdh*) *locus*. This gene has been shown to be a reliable, easy and cost-effective method to identify *G. duodenalis* isolates directly from faeces (Thompson et al. 2000, Cordón et al. 2008, Fallah et al. 2008, Tungtrongchitr et al. 2010, Pestehchian et al. 2012). Studies on the relationship between parasite genotype and symptoms have been mixed, though a genotypic analysis using PCR-RFLP amplification of the *gdh* gene did find a strong genetic link with clinical symptomatology in 18 Dutch patients (Homan & Mank 2001).

G. duodenalis is a common parasite in Ecuadorian children living in rural communities, with prevalence measures of approximately 20% (Rinne et al. 2005, Jacobson et al. 2007). This is in agreement with five years of case control data collected from the communities included in this study (Bhavnani et al. 2012).

We report here for the first time the genotypes of *G. duodenalis* present in humans in rural communities in the northern coastal rainforests of Ecuador.

The study was conducted in North-western Ecuador within the province of Esmeraldas and the canton Eloy Alfaro. The study region comprises approximately 150 villages located along the three rivers, Cayapas, Santiago and Onzole. Between November 2004-July 2005, 595 stool samples were collected from 152 cases (diarrhoea) and 443 controls and frozen in liquid nitrogen before being transported to the lab in Quito. In Quito, the presence of *Giardia* antigen was tested using an ELISA kit (RIDASCREEN *Giardia*, R-Biopharm, USA).

DNA extraction was performed on the 154 faecal samples that were positive for infection by ELISA and samples were stored at -20ºC until required for PCR and RFLP analysis. DNA extraction, PCR [using primers GDHeF, GDHiF and GDHiR to identify a fragment of approximately 432 base pairs (bp) of the *gdh*] and RFLP was exactly as described by Read et al. (2004) and Cordón et al. (2008) with the following changes: the concentration of the forward primers in the PCR reaction were changed to 25 pmol to improve sensitivity; RFLP digestion was increased to four units for 8 h at 37ºC to improve cleavage by the restriction enzymes NlaIV and RsaI. PCR amplification of samples included a known *Giardia* positive (courtesy of Baca Ortiz Hospital, Quito) and a negative control (PCR water instead of DNA). *Giardia* RFLP assemblages were identified using a diagnostic genotyping profile as shown previously by

TABLE I

A summary of ELISA test results in case and control stool samples

Infection status	*Giardia*-negative (n)	*Giardia*-positive (n)	Not tested (n)	Total (n)
Diarrhoea	85	66	1	152
No diarrhoea	353	88	2	443
Total	438	154	3	595

Read et al. (2004) and Babaei et al. (2008). One positive control identified as assemblage AII and another as assemblage BIII were used as references for the RFLP.

The estimated weighted prevalence of *Giardia* infection in our study population was 24%. All but three stool samples were analysed for *Giardia* using ELISA. Of the 592 samples analysed, 154 (26%) were positive for *Giardia* (Table I). Subsequently, all ELISA-positive samples were analysed by semi-nested PCR and 74 (48%) exhibited a band at 432 bp specific for the *gdh* gene (Fig. 1). Of these 74 samples, 69 were genotyped using RFLP (Table II), with the NlaIV restriction enzyme used to distinguish between assemblage A and assemblage B genotypes (Fig. 2A). Those identified as assemblage B were further processed using the RsaI restriction enzyme (Fig. 2B). Five (7%) samples that were PCR-positive could not be genotyped because the bands could not be clearly identified. These samples were excluded from our analysis.

In our study, all four genotypes were identified and the presence/absence of diarrhoea examined (Table III). Assemblage B was seen to be predominant (42 of 69 or 61%) with group BIII the most common of the four genotypes (26 or 38%). This is in agreement with previous studies (Amar et al. 2002, Bertrand et al. 2005, Lebbad et al. 2008, Minvielle et al. 2008, Aldana et al. 2011, Molina et al. 2011). Assemblage A was found in 22 of the 69 isolates (32%) with group II (19 of 69 or 28%) the most common. Assemblage BIV was found in 16 (23%) and only three individuals were infected with Group AI (4%); this low frequency is consistent with the literature (Amar et al. 2002, Bertrand et al. 2005, Babaei et al. 2008). Interestingly, five individuals (7%) in this study were co-infected with both genotypes AII and BIII, which also has been previously documented (Amar et al. 2002, Babaei et al. 2008, Molina et al. 2011). Overall, the odds ratio (OR) of diarrhoea in individuals infected with *Giardia,* compared to those uninfected, was 3.11 (95% CI = 2.11, 5.10). The OR of diarrhoea associated with assemblages B and A, each compared to no infection with *Giardia,* were 2.94 [95% confidence interval (CI) = 1.45, 5.81] and 3.46 (95% CI = 1.83, 9.24), respectively. The associations between diarrhoea and specific genotypes within assemblage A and B were similar to those reported above (Table IV).

Some prior studies have suggested that assemblage A infections are more likely to cause diarrhoea than assemblage B (Read et al. 2002, Haque et al. 2005, Cordón et al.
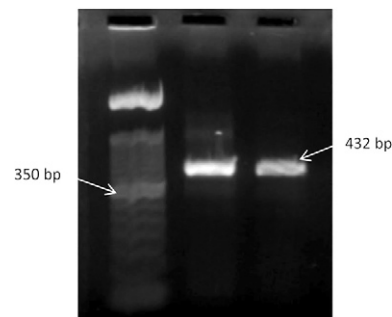

Fig. 1: amplification of the 432 base pairs (bp) band specific for the glutamate dehydrogenase gene of *Giardia duodenalis*. Lane 1: molecular weight marker 1,000 bp; 2: *Giardia* positive control 432 bp; 3: *Giardia* positive 432 bp.

TABLE II

Summary of expected band sizes from *Giardia duodenalis* glutamate dehydrogenase polymerase chain reaction-restriction fragment length polymorphism

Assemblage and group	Restriction enzyme	Diagnostic band size (base pairs)
AI	N1aIV	90, 120, 150
AII	N1aIV	70, 80, 90, 120
BIII	RsaI	130, 300
BIV	RsaI	430
AII, BIII	NlaIV and RsaI	90, 120, 290, 130, 300

2008, Sahagún et al. 2008); we, however, found no evidence of differential pathogenicity between assemblage A and B, which was in agreement with findings by García et al. (2002) and Ceu Souza and Poiares da Silva (2004).

Of the 154 ELISA-positive samples analysed for the presence of the *gdh* gene, only 74 (48%) were positive, likely due to low numbers of cysts in the faeces of some of our samples. This lack of PCR sensitivity for the *gdh* gene has been noted by other groups, such as Amar et al. (2002), Cordón et al. (2008) and Tungtrongchitr et al. (2010), who also suggested that absence of this band was

TABLE III

Frequency of symptomatic individuals (diarrhoea) across *Giardia duodenalis* genotypes

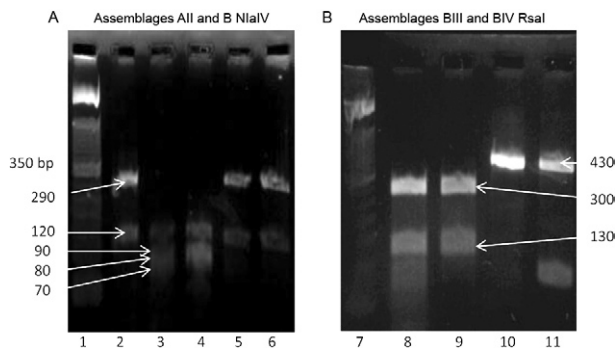| Infection status	AI (n)	AII (n)	BIII (n)	BIV (n)	Not determined (n)	Mix (n)	*Giardia*-negative (n)
Diarrhoea	1	9	10	7	3	2	85
No diarrhoea	2	10	15	9	2	3	353
Unknown	0	0	1	0	0	0	0
Total	3	19	26	16	5	5	438

Fig. 2: ethidium bromide stained 2% high-resolution agarose gel showing DNA amplified at the glutamate dehydrogenase for genetic assemblages AII and B digested with Nla IV (A) and genetic assemblages BIII and BIV digested with RsaI (B). Lane 1: molecular weight marker 1,000 base pairs (bp); 2, 5, 6: assemblage B (120 bp, 290 bp); 3, 4: assemblage AII (70 bp, 80 bp, 90 bp, 120 bp); 7: molecular weight marker 1,000 bp; 8, 9: assemblage BIII (130 bp, 300 bp); 10, 11: assemblage BIV (430 bp).

TABLE IV

Odds ratios (OR) of diarrhoea associated with infection status and bootstrapped 95% confidence intervals (CI)

Infection status	OR	95% CI
Giardia infection (ELISA-positive)	3.11	2.11, 5.10
Assemblage A	3.46	1.83, 9.24
Assemblage A, group I	NA	NA
Assemblage A, group II	3.74	1.93, 11.74
Assemblage B	2.94	1.45, 5.81
Assemblage B, group III	2.77	1.28, 6.34
Assemblage B, group IV	3.23	0.98, 10.66

NA: not available.

proteases etc. Freeze-thawing of the samples may also be a reason for degradation, suggesting that ELISA and DNA extraction should be performed on fresh samples.

Recent studies (Traub et al. 2004, Sprong et al. 2009) have shown the presence of human genotypes, such as AI and AII, in livestock and pets. Future research on *Giardia* infection within animal populations in close proximity to humans could address whether or not those *Giardia* genotypes found in humans are being transmitted zoonotically.

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due to insufficient densities of cysts within the sample. Nested PCR analysis requires a high cyst density and *Giardia* cysts are susceptible to degradation (Amar et al. 2002). To avoid decreases in cyst density, samples should be frozen soon after collection to prevent degradation by

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