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ScienceDirect
Acta Tropica 106 (2008) 44–53
ACTA TROPICA
www.elsevier.com/locate/actatropica

Dominance of *Giardia* assemblage B in León, Nicaragua

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Received 19 April 2007; received in revised form 9 December 2007; accepted 17 January 2008
Available online 31 January 2008

Abstract

Giardiasis is a major problem in León, Nicaragua, yet despite this no data are available regarding the prevalence of different *Giardia* genotypes in this area. To address this question, a molecular analysis of *Giardia* isolates from humans and dogs living in the same area in León, Nicaragua was performed. *Giardia* isolates from 119 Nicaraguan patients and 8 dogs were successfully genotyped using single and/or nested β-giardin PCR with subsequent restriction length fragment polymorphism (RFLP) analysis. The analyses of human samples yielded 94 (79%) assemblage B isolates and 25 (21%) assemblage A isolates. Only the non-human-associated assemblages C and D were found in the dog samples. Sixteen isolates with assemblage A pattern, 26 isolates with assemblage B pattern and all dog isolates were further characterized by sequencing the nested β-giardin PCR product and by molecular analyses of the glutamate dehydrogenase (gdh) gene. Within the study area the assemblage A isolates were highly genetically homogenous, showing only sub-genotypes A2 ($n = 3$) or A3 ($n = 13$) at the β-giardin locus and AII only at the gdh locus while assemblage B showed a high genetic polymorphism at both loci. Seven different sub-genotypes were identified within 13 of the sequenced assemblage B β-giardin isolates. The remaining 13 sequenced assemblage B-isolates appeared to contain several different variants of the β-giardin gene since the chromatograms displayed one to seven double peaks. The gdh sequences showed an even higher polymorphism since only 2 of 26 assemblage B isolates were without double peaks. Two mixed infections between assemblage A and B were found when the gdh gene was analyzed. Polymorphisms were also observed in the dog-associated assemblages C and D, but to a lesser extent than in assemblage B.

Keywords: Nicaragua; *Giardia intestinalis*; (-Giardin gene; Glutamate dehydrogenase gene; Genotyping; Diarrhea

1. Introduction

The parasitic protozoa *Giardia intestinalis* (syn. *G. lamblia, G. duodenalis*) can infect a wide range of vertebrates including humans, pets and livestock (Thompson, 2000). *G. intestinalis* has a global distribution and is one of the most frequent intestinal parasites in humans, where infection ranges from asymptomatic to acute or chronic disease. The factors determining the variability in clinical infection in giardiasis are still poorly understood (Gardner and Hill, 2001). About 200 million people in Asia, Africa and Latin America have symptoms of intestinal *Giardia* infection and approximately 500 000 new cases occur annually (WHO, 1996). In Nicaragua, like in other South American countries, the prevalence of giardiasis is high, especially in children (Moya-Camarena et al., 2002; Prado et al., 2005; Tellez et al., 1997). Although this disease is usually self-limiting and not life threatening, *Giardia* infection in young children has been correlated to stunted growth and poor cognitive function (Berkman et al., 2002; Celiksoz et al., 2005).

G. intestinalis has been suggested to comprise a species complex, whose members, although morphologically identical, consists of seven genetically distinct assemblages (Monis et al., 2003). Only assemblage A and B parasites have so far been detected not only in humans but also in a wide range of other mammals, while the other assemblages (C–G) appear to have a narrower host-range. The role of animals in the transmission of

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doi:10.1016/j.actatropica.2008.01.004

the infection to humans is still uncertain (Monis and Thompson, 2003). A few studies have shown a link between the severity of infection and certain assemblages but the results are controversial (Aydin et al., 2004; Haque et al., 2005; Homan and Mank, 2001; Read et al., 2002). While a rather large number of human and animal *Giardia* isolates from Europe, North America, Australia and Asia have been characterized genetically, little information is available from other parts of the world. Most data from South and Central America arrive from Mexico where four different studies demonstrated assemblage A exclusively in human isolates (Cedillo-Rivera et al., 2003; Eligio-Garcia et al., 2005; Lalle et al., 2005a; Ponce-Macotela et al., 2002). Surveys of intestinal parasites in dogs from Brazil, Argentina and Mexico have demonstrated high prevalence of *Giardia* both in stray and domestic dogs (Oliveira-Sequeira et al., 2002; Ponce-Macotela et al., 2005; Taranto et al., 2000). A study from India suggests that zoonotic transmission of *Giardia* can occur between humans and dogs living in the same community (Traub et al., 2004).

Giardiasis is a major problem in León, Nicaragua (Tellez et al., 1997). There is however, no data currently available concerning the prevalence of different *Giardia* genotypes (assemblages) in humans or animals in this region. To address this question a molecular analysis of *Giardia* isolates from humans and dogs living in the same area in León, Nicaragua was performed.

2. Materials and methods

2.1. Study-area

The study material was collected in different areas of León, Nicaragua, which has a population of approximately 200 000 inhabitants. The climate is tropical with a dry and a rainy season. Sanitary conditions are often insufficient in large areas of the city, mainly in its surroundings, where poverty is more evident. Both stray and domesticated dogs are common in the area investigated.

2.2. Source of isolates

Human fecal samples containing *Giardia* cysts and/or trophozoites ($n = 136$) were collected on four different occasions during an 18 month period, starting February 2002; 22 samples originated from an *Entamoeba* study on diarrheal patients (Leiva et al., 2006), 72 samples from a study of intestinal pathogens in children, 32 samples from a parasite prevalence study of healthy individuals and 10 samples from children seeking medical advice at a local health center in León. Study participants ranged in age from 1 to 53 (median 5) years, where 56 were female and 70 were male. For the 10 children seeking medical advice from a local health centre, gender was not recorded. Thirty-four of 136 participants complained of current clinical symptoms of diarrhea. Fecal samples from 100 dogs were collected during 2004. One sample originated from a dog belonging to the Veterinary school, the other samples were collected from domestic dogs in the study area. A wild-trapped rat and a dog from an ongoing study of *Giardia* genotypes in Swedish animals (manuscript in preparation) were included for phylogenetic analyses.

Informed consent was obtained from all participants, and regional ethical committees at UNAN, Leon, Nicaragua, and the Karolinska Institute, Stockholm, Sweden (Dnr.2006/911-31/3) approved the study.

2.3. Microscopy and DNA isolation

The presence of *Giardia* cysts and trophozoites in human samples was determined by light microscopy on wet smears before and after formol-ethyl acetate concentration. Fecal samples were fixed in ethanol as described elsewhere (Lebbad and Svard, 2005) shortly after collection and kept at 4 °C prior to extraction.

Dog samples were screened for *Giardia* parasites by light microscopy using the same methods as for human samples. In addition, microscopy was performed after a sucrose gradient isolation. Fresh fecal material was suspended in distilled water and filtered through two layers of gauze. The suspension was centrifuged at 400 × *g* for 5 min, the supernatant was decanted and the sediment washed once with distilled water. The pellet was re-suspended in 3 mL of distilled water and gently administered over 3 mL of cold 1 M sucrose solution followed by centrifugation at 600 × *g* for 5 min. The interface and upper layer of the supernatant were transferred to a new tube, diluted with water and re-centrifuged at 600 × *g* for 5 min. The supernatant was decanted, and the pellet suspended in ethanol prior to extraction.

DNA from 33 samples was extracted in Nicaragua shortly after collection using the QIAamp™ DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. An initial washing step was added, where approximately 0.5 mL of the stool–ethanol suspension was mixed with 1 mL saline and centrifuged for 3 min at 6000 × *g*. The sediment was re-suspended in an equal amount of saline and 20 μL was used for extraction.

The remaining samples, including the cyst concentrates from dog isolates, were kept at 4 °C and extracted after 3–10 months. An initial disruption of cysts using a Bead-Beater was added to the extraction procedure. Briefly, 70 μL of the washed stool–ethanol suspension was added to a tube containing 600 μL ASL buffer (Qiagen, Hilden, Germany) and approximately 800 mg of 0.5 mm zirconica silica beads (Biospec Products Inc., Bartlesville). The tube was agitated in a Mini-BeadBeater (Biospec Products Inc.) for 60 s at 5000 rpm, the beads were allowed to sediment for 15 min and 200 μL of the supernatant was finally withdrawn. Twenty microliters of proteinase *K* was added and the extraction was performed using the QIAamp™ DNA mini kit.

2.4. PCR-RFLP and sequencing

A 753 bp fragment of the β-giardin gene was amplified using the single-step PCR method (forward primer G7 and reverse primer G759) as described by Caccio et al. (2002). A 384 bp fragment of the same gene was amplified using the forward primer G376 and the reverse primer G759 on all samples show-

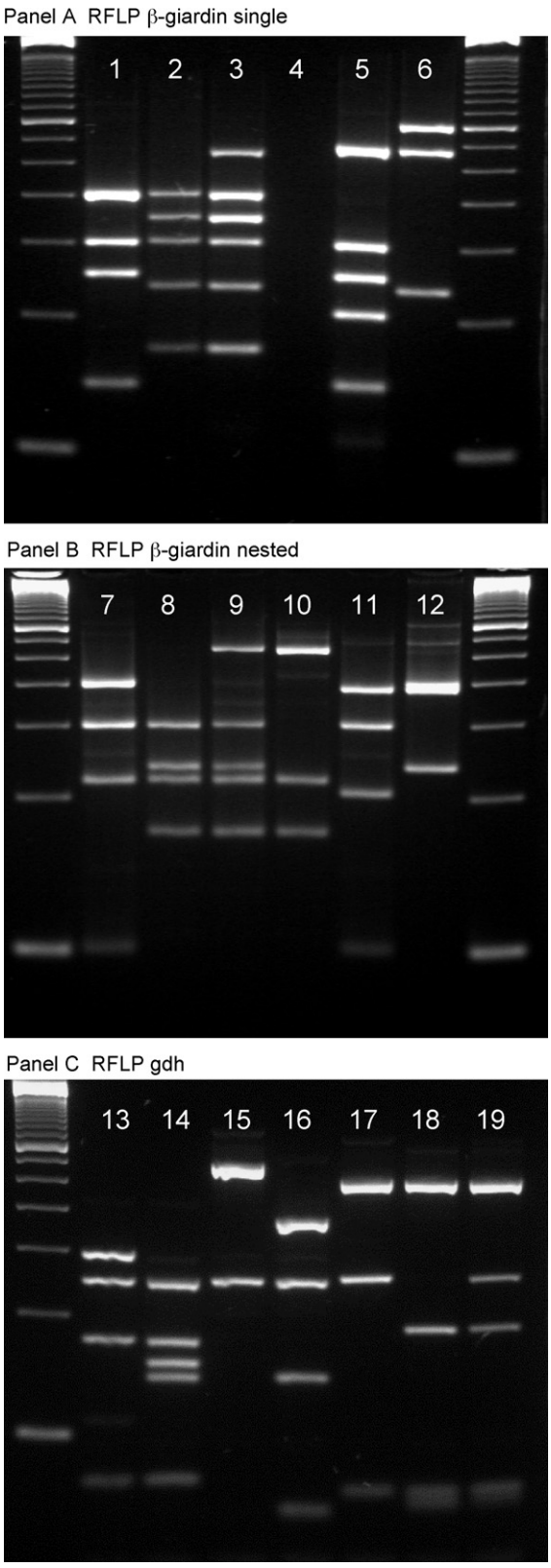

Fig. 1. Panel A: Gelred (Biotium) stained 4% MetaPhor agarose gel (Cambrex) showing electrophoretic separation of single β-giardin PCR products (753 bp) after digestion with HaeIII. Lane 1, assemblage A; lane 2, assemblage B; lane 3, assemblage B mixed sub-genotypes (NicD059.1); lane 4, empty, lane 5, assemblage C (Nicdog7); lane 6, assemblage D (Nicdog1). Panel B: Electrophoretic separation of nested β-giardin PCR products (511 bp) after digestion with HaeIII. Lane 7, assemblage A; lane 8, assemblage B; lane 9, assemblage B mixed sub-genotypes (NicD059.1); lane 10, assemblage B new pattern (NicD059.2), lane 11, assemblage C (Nicdog7); lane 12, assemblage D (Nicdog1). Panel C: Electrophoretic separation of semi-nested gdh PCR products (432 bp) after digestion with BspL1. Lane 13, assemblage A sub-genotype AI; lane 14, assemblage A sub-genotype AII; lane 15, assemblage B; lane 16, assemblage C (Nicdog7); lane 17, assemblage D, lane 18, assemblage D new pattern (Nicdog3), lane 19, assemblage D mixed pattern (Nicdog1). Molecular size markers are 50-bp ladders (Invitrogen).

ing assemblage A pattern in RFLP (Caccio et al., 2002). A subset of the human samples and all dog samples were studied using a nested β-giardin PCR as described by Lalle et al. (2005b) and a semi-nested glutamate dehydrogenase (gdh) PCR as described by Read et al. (2004). The cycling conditions for the gdh PCR were modified; instead of 55 cycles, 35 cycles were used both in primary and in sequential nested PCR-reactions. Positive controls consisting of DNA extracted from WB-C6 (assemblage A) ATCC50803 and GS-H7 (assemblage B) ATCC50581 were included in each PCR-run.

Aliquots of the PCR products from both single and nested β-giardin PCR and from the semi-nested gdh PCR were analyzed by RFLP using the conditions described elsewhere (Caccio et al., 2002; Lalle et al., 2005b; Read et al., 2004). The PCR-products from the nested β-giardin PCR and the semi-nested gdh PCR were sequenced in both directions at AGOWA (Berlin, Germany). A 292 bp fragment of the small subunit rRNA gene was amplified from all dog samples and sequenced as previously described (Appelbee et al., 2003). Chromatograms and sequences were examined using BioEdit (http://www.mbio.ncsu.edu/BioEdit/page2.html). Sequence searches were conducted using BLAST (http://www.ncbi.nlm.nih.gov/blast/).

2.5. Phylogenetic analyses

A dataset was assembled containing 9 novel and unambiguous β-giardin nucleotide sequences obtained in this study, and 28 sequences retrieved from GenBank at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) representing the diversity of β-giardin sequences. No insertions, deletions or ambiguously aligned positions were identified in any of the 37 sequences within the 475 bp (after reduction of primers) homologous region. TN + G was identified as the optimal nucleotide substitution model for the dataset using the program MODELGENERATOR (Keane et al., 2006). A nucleotide maximum likelihood phylogenetic tree based on all three codon positions was created with PHYML V2.4.4. (Guindon and Gascuel, 2003) using the optimal substitution model. In addition, a bootstrap analysis with 500 replicates was performed with the same parameters. A *Giardia muris* sequence was used to root the tree. Using the same approach a phylogenetic analysis was performed on the obtained gdh sequences together with representatives from GenBank (data not shown). For comparisons, bootstrap analyses using a simpler, but sub-optimal, nucleotide substitution model (JC) were performed. These analyses resulted in topologies similar to the analyses using the TN + G model where the major assemblages

are resolved, but with little resolution within them (data not shown). Thus, the results of the phylogenetic analyses appear robust against nucleotide substitution model specifications.

2.6. Nucleotide sequence accession numbers

The new β-giardin nucleotide sequences obtained in this study have been deposited in the GenBank under the Accession numbers EF455591–EF455599.

3. Results

3.1. Molecular analysis of Giardia isolates from humans

3.1.1. PCR-RFLP of the β-giardin gene

Of the molecular analyses performed on 136 *Giardia*-positive human samples collected in the León area, single-step PCR using the β-giardin gene, yielded the expected 753 bp fragment on 112 of the samples. RFLP analysis of the PCR products using the restriction enzyme HaeIII (Fermentas) identified 88 assemblage B and 24 assemblage A isolates. All isolates showed the expected restriction fragments, however, in three of the assemblage B isolates (NicD059, NicD156, NicIS089) an additional band at about 270 bp was seen (Fig. 1, panel A). No mixture between assemblage A and B was observed in the samples using this method. Samples with assemblage A patterns were submitted to a new PCR, amplifying a 384 bp fragment of the β-giardin gene. RFLP analysis of the PCR products, using restriction enzyme HhaI, showed a fragment pattern identical to sub-genotype A2/A3 in all cases (Caccio et al., 2002).

To further characterize the *Giardia* isolates, a nested PCR of the β-giardin gene with subsequent RFLP using the restriction enzyme HaeIII was performed on 26 samples with assemblage B pattern and 16 samples with assemblage A pattern. This analysis revealed the same assemblages as the original single-step PCR-RFLP analysis in all cases. Additional bands in the RFLP analysis were seen as earlier in the three assemblage B isolates (Fig. 1 panel B).

DNA from 24 human samples did not produce any bands in the single-step β-giardin PCR. The stool samples had originally been considered positive for *Giardia* cysts and/or trophozoites by light microscopy in León, Nicaragua. Further confirmation of the presence of *Giardia* cysts was undertaken using an immunofluorescence (IF) test (Agua-Glo, Waterborne Inc., New Orleans, LA) and the light-microscopy results were confirmed in 16 cases, while no cysts were detected in eight samples. The number of cysts varied from one cyst per slide to >100 cysts per slide. Nested β-giardin PCR, performed on all samples previously negative in single-step PCR, yielded seven additionally positive cases, all from samples containing cysts in IF. RFLP analysis identified six assemblage B and one assemblage A isolate. The remaining 17 negative samples were spiked with highly diluted *Giardia* DNA and checked for inhibition. All samples produced the expected bands (data not shown), thus, no major inhibition occurred. To conclude, the combined result of single and nested β-giardin PCR-RFLP analysis had a yield of 94 (79%) isolates that belonged to assemblage B and 25 (21%) isolates that belonged to assemblage A.

3.1.2. Sequencing of the β-giardin gene

The nested β-giardin PCR products from 16 human assemblage A isolates and 26 assemblage B isolates were analyzed in both directions by direct sequencing. Within assemblage A, three sequences were identical to genotype A2 (GenBank accession no. AY072723) and 13 were identical to genotype A3 (GenBank accession no. AY072724). No double peaks or divergences were observed while analyzing the assemblage A chromatograms. The results of sequencing of the 26 assemblage B isolates are presented in Table 1. Nine of the assemblage B isolates showed 100% homology with published sequences; six isolates were identical to genotype B3, two to isolate BG-Ber2 and one to genotype B4 (Caccio et al., 2002; Lalle et al., 2005b; Robertson et al., 2006). Three sequences (NicD069, NicD133, and NicD144) differed with a single-nucleotide polymorphism (SNP) from published sequences B3 or BG-Ber2 and one sequence (NicIS086) with two SNPs from B3 (Fig. 2 and Table 1). All the substitutions occurred at the third codon position, causing no amino acid alterations in β-giardin. The chromatograms of the remaining 13 assemblage B sequences showed between one to seven double peaks, which occurred at 16 different positions of the sequenced β-giardin gene fragment (Table 1).

3.1.3. Investigation of atypical β-giardin RFLP-patterns

Three assemblage B isolates (NicD156, NicD059.1, NicIS089) showed an atypical pattern with an extra band in RFLP in both single and nested PCR (Fig. 1 panels A and B) and double peaks at two to seven positions in the chromatogram (Table 1). The three isolates had only one double peak in common at position 408, showing a substitution of G to A. In order to investigate the atypical RFLP-pattern further, nested PCR-RFLP and sequencing analyses were repeated four times on one of the samples (NicD059). Twice the RFLP patterns and sequences were identical to those first obtained on this isolate (NicD059.1). However, two of the repeated analyses demonstrated a new RFLP pattern (Nic059.2, Fig. 1 panel A, Table 1) and sequences without any double peaks, but with the same substitution G to A at position 408 as described earlier. This position is one of the restriction sites for the enzyme HaeIII used in the RFLP, thus the new RFLP-pattern with fragments of 267, 110, 84, 26 and 24 bp instead of the originally described 150, 117, 110, 84, 26 and 24 bp for assemblage B, was explained (Lalle et al., 2005b). The atypical pattern with extra bands seen originally in the three samples was apparently a mixture of these two patterns.

3.1.4. PCR-RFLP and sequencing of the gdh gene

The same subset of samples that were further characterized by nested PCR-RFLP and sequencing of the β-giardin gene was also analyzed with a semi-nested gdh PCR-RFLP using the restriction enzyme BspL1 (NlaIV) (Fermentas). The gdh gene fragment of 432 bp was successfully amplified for all 42 isolates. The RFLP revealed the same assemblages as the β-

Table 1
Nucleotide changes in assemblage B at the β-giardin gene locus

GenBank # no.	Isolate/sub-genotype	Position[a]															
		117	141	162	171	186	189	315	408	414	429	435	438	447	501	525	570
AY072727	BAH 8 (B3), NicD135, NicD143, NicD147, NicIS012, NicIS177, NicSS008	c	g	c	c	g	a	c	g	c	c	g	a	c	a	t	c
DQ090523	BG-Ber2, NicD157, NicIS137	c	g	c	c	g	a	T	g	c	c	g	a	c	a	t	c
AY072728	ISSGF4 (B4), NicIS117	c	g	c	c	g	G	T	g	c	c	g	a	c	a	C	T
EF455592	NicD069	c	g	c	T	g	a	c	g	c	c	g	a	c	a	t	c
EF455593	NicD133	c	g	c	T	g	a	T	g	c	c	g	a	c	a	t	c
EF455594	NicD144	c	A	c	c	g	a	T	g	c	c	g	a	c	a	t	c
EF455595	NicIS086	c	g	c	c	g	a	c	g	c	c	A	G	c	a	t	c
	NicD019	c	g	Y	Y	g	a	Y	g	c	c	g	a	c	a	t	c
	NicD059.1	c	g	c	Y	g	a	c	R	c	Y	g	a	c	a	Y	c
EF455591	NicD059.2	c	g	c	c	g	a	c	A	c	c	g	a	c	a	t	c
	NicD082	c	R	Y	c	g	a	Y	g	c	c	g	a	c	a	t	c
	NicD110	c	R	c	c	g	R	Y	g	c	c	g	a	c	a	t	c
	NicD152	c	g	Y	c	g	a	c	g	c	c	g	a	c	a	t	c
	NicD156	c	g	c	c	g	a	Y	R	c	c	g	a	c	a	t	c
	NicD158	c	g	c	c	g	a	Y	g	Y	c	g	a	c	a	t	c
	NicIS021	c	g	c	c	g	a	Y	g	c	c	g	a	c	a	t	c
	NicIS052	Y	g	c	c	g	G	Y	g	c	c	g	a	c	a	Y	Y
	NicIS089	c	g	c	c	g	R	Y	R	c	c	R	R	Y	a	Y	c
	NicIS171	c	R	c	c	g	a	Y	g	c	c	g	a	c	a	t	c
	NicSS002	c	g	c	Y	R	a	T	g	c	c	g	a	c	a	Y	c
	NicSS057	c	g	c	Y	g	a	Y	g	c	c	g	a	c	R	Y	c

The nucleotide substitutions at each position are in capital letters.
[a] Numbering represents distance from the ATG codon of the Portland-1 Reference strain (GenBank accession number X14185).

giardin PCR-RFLP except in one case; sample NicD068 which was characterized as assemblage A at the β-giardin locus showed assemblage B at the gdh locus. Sub-genotype AII was demonstrated with RFLP for all A isolates (Fig. 1 panel C). The RFLP for discrimination between sub-genotype BIII and BIV using the restriction enzyme Rsa1 (Fermentas) was also performed on all assemblage B isolates and showed nine BIII, two BIV and 16 mixtures of BIII and BIV. The gdh PCR products were sequenced in both directions. All assemblage A sequences were identical to sub-genotype AII, GenBank accession no L40510. In one A isolate, small double peaks observed in the chromatogram were in agreement with a mixed infection with assemblage B. All other assemblage A sequences were without double peaks or divergences. In contrast, only two assemblage B sequences had chromatograms without double peaks (Table 2). Sample NicSS008 was identical to isolate gd-fox-03-132, which is 1 SNP from BAH-1, a BIII isolate, and sample NicIS171 was identical to isolate gi-hum1, which differs 1 SNP from NHL28, a BIV-like isolate according to the classification by Wielinga and Thompson (2007). The remaining 24 B isolates had 1–11 double peaks in their chromatograms. The double peaks occurred at 24 different positions in the sequenced gdh fragment, and none of the sequences showed identical patterns (Table 2).

One sample, NicD068, which yielded assemblage A at the β-giardin locus and assemblage B at the gdh locus, was investigated further. Both β-giardin and gdh PCR-RFLP were repeated three times and showed consistently the same result, assemblage A with β-giardin RFLP and assemblage B with gdh RFLP. The gdh RFLP showed some background bands and careful re-examination of the gdh sequence revealed very small double peaks consistent with a mixed infection with assemblage A. This isolate was not included in Table 2.

3.2. Molecular analysis of Giardia isolates from dogs in León

Eight of the 100 dog samples were shown to be *Giardia* positive by microscopy. The combined results of genotyping/sequence analysis at the small subunit rRNA gene, the β-giardin gene and the gdh gene are depicted in Table 3. There were no discrepancies between assemblages, except one sample, which showed a mixed infection C and D at the β-giardin locus and single C infection at the two other loci. Of the remaining

Table 2
Nucleotide changes in assemblage B at the gdh gene locus

GenBank # nr	Isolate	Position[a]																							
		264	297	309	315	342	351	357	360	384	414	429	447	462	480	519	540	546	561	570	582	585	597	606	612
AF069059	BAH12 BIII	c	c	c	c	c	c	t	g	c	c	t	t	c	c	c	c	c	c	c	g	c	c	c	g
DQ904425	NicSS008	c	T	c	c	c	c	t	g	c	c	t	t	c	c	c	c	c	c	c	g	c	c	c	g
DQ840541	NicIS171	c	c	T	c	c	c	t	g	c	c	t	C	c	c	c	T	c	c	c	g	c	c	c	A
	NicD019	c	Y	Y	c	c	c	Y	g	c	c	Y	Y	c	c	Y	c	c	c	c	R	c	c	c	g
	NicD059	c	c	T	c	c	c	Y	g	c	c	C	Y	c	c	Y	Y	c	c	c	g	c	c	c	g
	NicD069	c	c	Y	c	c	c	Y	g	c	c	Y	Y	c	c	Y	Y	c	c	c	g	c	c	c	R
	NicD082	c	Y	Y	c	c	c	Y	g	c	c	Y	Y	c	c	Y	Y	c	c	Y	R	c	c	c	A
	NicD110	c	Y	Y	c	c	c	Y	R	c	c	t	t	c	c	c	c	c	c	c	g	c	c	c	g
	NicD133	c	T	c	c	c	c	t	g	c	c	t	t	c	c	T	c	c	c	c	g	c	c	c	R
	NicD135	c	c	Y	c	c	c	Y	R	c	c	Y	Y	c	c	Y	c	Y	c	c	g	c	c	c	g
	NicD143	c	c	c	Y	c	Y	t	g	c	c	t	t	c	c	Y	c	c	c	c	A	Y	c	c	g
	NicD144	c	T	c	c	c	c	t	g	c	Y	t	t	c	Y	c	c	c	c	c	g	c	c	c	g
	NicD147	c	T	c	c	c	c	Y	g	c	c	t	t	c	c	Y	c	Y	c	c	g	c	c	c	g
	NicD152	c	Y	Y	c	Y	c	Y	R	Y	c	Y	Y	c	c	Y	Y	c	c	c	R	c	c	c	R
	NicD156	Y	c	T	c	c	c	C	g	c	c	C	C	c	c	c	T	c	c	Y	g	c	c	c	R
	NicD157	c	Y	Y	c	c	c	t	g	c	c	t	Y	c	c	Y	Y	c	c	Y	g	c	Y	Y	R
	NicD158	c	Y	Y	c	c	c	Y	g	c	c	Y	Y	c	c	c	Y	c	c	c	g	c	c	Y	R
	NicIS012	c	c	Y	c	c	c	Y	g	c	c	Y	Y	c	c	c	Y	c	c	c	R	Y	c	c	R
	NicIS021	c	c	Y	c	c	c	Y	g	c	c	Y	Y	c	c	c	Y	c	Y	c	g	c	c	c	R
	NicIS052	c	c	c	c	Y	c	t	g	c	Y	t	t	c	c	T	c	c	c	c	g	c	c	c	g
	NicIS086	c	Y	c	c	c	c	C	A	c	c	t	t	c	c	T	c	Y	c	c	g	c	Y	c	R
	NicIS089	c	Y	Y	c	c	c	Y	g	c	c	Y	Y	c	c	Y	Y	Y	c	c	g	c	c	Y	R
	NicIS117	c	c	Y	c	c	c	Y	g	c	c	C	C	c	c	c	Y	c	c	c	g	c	c	c	R
	NicIS137	c	c	Y	c	c	c	Y	R	c	c	C	C	c	c	c	Y	c	Y	c	g	c	c	c	R
	NicIS177	c	Y	c	c	c	c	Y	R	c	c	Y	Y	Y	c	Y	Y	c	c	c	g	c	c	c	R
	NicSS002	c	c	T	c	c	c	C	R	c	c	Y	Y	c	c	Y	Y	c	Y	c	g	c	c	c	R
	NicSS057	c	Y	c	c	c	c	t	g	c	c	t	t	c	c	c	Y	c	Y	c	g	c	c	c	R

The nucleotide substitutions at each position are in capital letters.
[a] Numbering represents distance from the ATG codon of the WB Reference strain (GenBank accession number XM_773614).

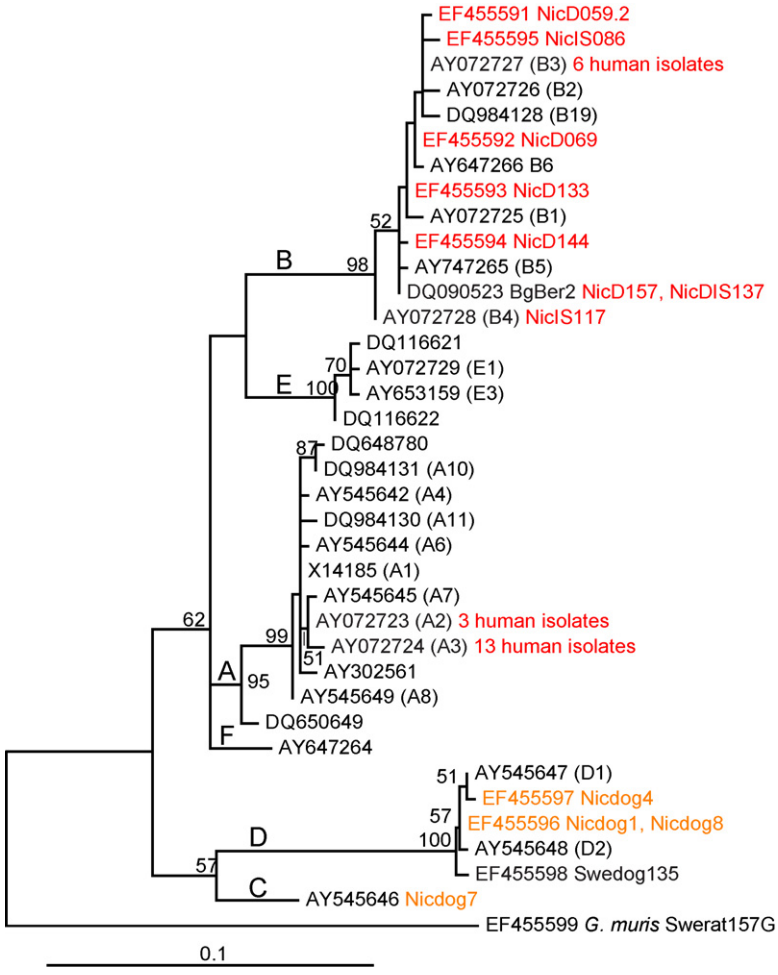

Fig. 2. Nucleotide maximum likelihood tree of β-giardin gene sequences. The phylogenetic tree is based on 475 unambiguously aligned positions. Sequences obtained from *G. intestinalis* isolates from humans and dogs in Nicaragua in this study are shown in red and orange fonts, respectively. Two additional novel sequences are included obtained from *Giardia* isolates from a Swedish dog and rat, respectively (Swedog135 and Swerat157). Sub-genotypes according to Caccio et al. (2002) and Lalle et al. (2005b) are given in parenthesis after certain GenBank accession numbers. Analysis of 500 bootstrap replicates was performed and only values greater than 50% for bipartitions are shown. The tree is rooted on the branch leading the *Giardia muris* sequence obtained from a Swedish rat. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

seven isolates, three had double peaks in the β-giardin sequence and five in the gdh sequence, which made comparisons of sub-genotypes difficult. Only one C isolate, NicDog7 was without double peaks in chromatograms from both genes and two published sequences could be compared (see Table 3). The two dogs living in house number 2 shared the same assemblages but not the same sub-genotypes, while the two dogs living in house number 4 had assemblages C and D respectively. The RFLP-patterns for assemblages C and D in nested β-giardin PCR were identical to those already published (Lalle et al., 2005b), while

Table 3
Comparison of results from genotyping of dog isolates at three genes

Isolate	Location	SSUrRNA	β-giardin			gdh		
		Sequence	RFLP	Sequence	GenBank # no	RFLP	Sequence	GenBank # no
Nicdog1	Vet school	D	D	D, 0 dp[a]	EF455596	D mixed pattern	D, 3 dp	
Nicdog2	House 1	C	C + D	C + D		C	C, 1 dp	
Nicdog3	House 2	D	D	D, 3 dp		D new pattern	D, 0 dp	DQ417372
Nicdog4	House 2	NS[b]	D	D, 0 dp	EF455597	D new pattern	D, 2 dp	
Nicdog5	House 3	D	D	D, 5 dp		D new pattern	D, 3 bp	
Nicdog6	House 4	C	C	C, 2 dp		C	C, 1 dp	
Nicdog8	House 4	NS	D	D, 0 dp	EF455596	D mixed pattern	D, 2 dp	
Nicdog7	House 5	C	C	C, 0 dp	AY545646	C	C, 0 dp	DQ417370

[a] Double peak.
[b] PCR not successful.

the corresponding patterns for single β-giardin products had not been described previously (Fig. 1 panel A). A restriction map for assemblage D was constructed from a published 753 pb fragment (GenBank accession no. AY545647) and for assemblage C from sequence data arrived from single β-giardin PCR on a Swedish dog C isolate (data not shown). The expected fragments were: assemblage C 286, 150, 126, 102, 70, 15 and 4 bp; assemblage D 346, 286, 117 and 4 bp. The RFLP patterns for assemblage C in the semi-nested gdh PCR corresponded to those published (Read et al., 2004) while RFLP of the five assemblage D isolates showed two unexpected patterns (Fig. 1 panel C). Sequencing showed a substitution T–C (three samples) and a double peak T/C (two samples) at the restriction site for BspL1 (NlaIV) at position 603. This explained both patterns, one new, and one mixed between the new and the pattern originally described by Read et al. (2004).

3.3. Genetic diversity of the β-giardin and the gdh gene

To assess the extent of genetic diversity of *Giardia* in the samples in this study a phylogenetic analysis was carried out. In this analysis, public *Giardia* β-giardin nucleotide sequences in GenBank were compared to the β-giardin sequences from this study (Fig. 2). Only sequences without double peaks were included in the analysis. The novel sequences grouped with previously identified assemblages in monophyletic groups with strong bootstrap support values (>95%). Discrepancies between the results from PCR-RFLP and phylogenetic analysis of the β-giardin gene sequences were not observed. The relationships within the assemblages could not be determined with any significant bootstrap support, most likely due to the limited number of sequence substitutions observed between the sequences (Fig. 2). A phylogenetic analysis was also performed based on the obtained gdh sequences together with previously characterized sequences, even though the high frequency of double peaks in the gdh data (Table 2) limits its value. Again, the major assemblages were identified while there was no support for the relationships within the groups (data not shown). The gdh sequences containing double peaks were nested within assemblage B, in agreement with origins from combinations of assemblage B gdh sequences present within the samples.

4. Discussion

The primary objective of this study was to determine the prevalence of different *Giardia* assemblages in humans and dogs living in the same area in León, Nicaragua. The combined result of single and nested PCR analyses of the β-giardin gene demonstrated a dominance of assemblage B in human samples, while the dog samples harbored only the dog-specific assemblages C or D.

Studies performed on DNA extracted directly from fecal samples from various countries confirm that only *Giardia* assemblages A and B are associated with human infections. The prevalence of each assemblage varies from country to country, and assemblage B seems to be more common overall, even though no strong conclusion can be drawn from current data (Caccio et al., 2005). Our results, with a predominance of assemblage B, differs from studies from Mexico and Brazil where assemblage A was found almost exclusively, both in humans and animals (Cedillo-Rivera et al., 2003; Eligio-Garcia et al., 2005; Lalle et al., 2005a; Ponce-Macotela et al., 2002; Volotão et al., 2007). However, assemblage B has been detected in South America, both in a recent study from Brazil and in Lima, Peru, where 19 B isolates and 6 A isolates were characterized (Souza et al., 2007; Sulaiman et al., 2003).

To determine the sub-genotypes within assemblage A and B, a subset of samples from each human assemblage was sequenced at two loci, β-giardin and gdh, and aligned with the homologous sequences available in GenBank. None of the sequenced isolates showed sub-genotype A1 (Fig. 2), which is in agreement with the results of RFLP analyses. Sequencing of the β-giardin gene showed that most of the A isolates (13 of 16 isolates) was of the A3 sub-genotype and the remaining three isolates displayed the A2 sub-genotype (Caccio et al., 2002). Sequencing of the gdh gene showed no diversity at all of the assemblage A isolates, all were identical to sub-genotype AII (Wielinga and Thompson, 2007). All assemblage A sequences from both genes showed 100% homology with published sequences and no double peaks were observed in the chromatograms. In contrast, a high degree of genetic polymorphism was observed in the assemblage B samples from both genes (Tables 1 and 2, Fig. 2). Thirteen of the sequenced β-giardin assemblage B isolates appeared to originate from samples containing genetically homologous cysts, while the other half of the sequenced B isolates showed double peaks at one to seven positions, which rendered determinations of sub-genotypes of these sequences impossible. The gdh gene showed an even higher degree of polymorphism with a majority of mixed BIII and BIV patterns in RFLP and only two B sequences without double peaks. These circumstances precluded comparison of sequences from the two genes. The double peaks observed could be consistent with the presence of genetically different cysts in the same sample (Lalle et al., 2005b). One means by which different sub-genotypes in such a sample type can be properly identified is through cloning the PCR products and then sequence multiple clones. This approach was tested by cloning the PCR products from two isolates with three double peaks each. Sequencing of more than 20 clones from each sample showed that each isolate contained two main sequences and, in addition, five other variants of the β -giardin gene. Thus, seven different sequences were sub-cloned from each isolate. Whether these results represent true sub-genotypes from different cysts in the original isolates or artifacts caused by the PCR and/or cloning procedure (Beser et al., 2007), remains to be investigated further.

An alternative explanation to mixtures of different sequences in assemblage B *Giardia* is an increased allelic sequence divergence in assemblage B isolates as compared to the relatively low allelic sequence divergence identified in assemblage A *Giardia* (Baruch et al., 1996; Teodorovic et al., 2007; Yu et al., 2002). A high degree of genetic polymorphism of assemblage B has indeed been described in wastewater samples (Sulaiman et al., 2004) and in human samples from a waterborne outbreak of giardiasis (Robertson et al., 2006). Interestingly, some authors using

sequencing of the β-giardin or the gdh gene do not report any mixed B sub-genotypes at all (Souza et al., 2007; van der Giessen et al., 2006) while others do (Lalle et al., 2005b; Robertson et al., 2007). If this depends on the selected material, the PCR application used, the sequencing conditions, or other reasons is not known. Single-cyst PCR analyses of patient samples can resolve the issue of whether the mixtures of genotype B sequences are due to mixed infections or allelic sequence divergence.

No mixture of assemblage A and B was observed when RFLP and sequencing of the β-giardin was used. When one isolate, which first appeared to have changed from assemblage A at the β-giardin gene to assemblage B at the gdh gene was re-investigated, a mixture of B and A was observed in the gdh gene sequence. Another isolate, which first appeared to be a single assemblage A infection at both loci, turned out to be a mixture between A and B when the gdh sequence was carefully observed. One dog isolate showed a clear mixture of C and D in both RFLP and at the β-giardin gene sequence but a clear single assemblage C infection with both the small subunit rRNA gene and the gdh gene. This indicates that infections with mixed assemblages could be missed, especially when primers designed to amplify all different assemblages are used. PCR with assemblage specific primers has revealed a high portion of mixed assemblages compared to PCR with more universal primers (Geurden et al., 2008).

This study was mainly based on variability in the β-giardin gene, which has shown to have a rather high discriminatory power (Caccio et al., 2002; Lalle et al., 2005b; Robertson et al., 2006). To further characterize the isolates we also performed analyses of the gdh gene. This approach showed that the β-giardin gene has a higher discriminative power when assemblage A isolates are studied, which is in agreement with recently published results in a multilocus sequence evaluation (Cooper et al., 2007). However, the reverse was true when assemblage B isolates were studied, gdh showed a higher discriminatory power than the β-giardin gene but the high level of double peaks in the gdh data interfered with sub-genotype analysis. Recent data also suggest the potential for DNA recombination between isolates of different assemblages (Cooper et al., 2007; Teodorovic et al., 2007). This, together with our data, shows that it is important to combine different genotyping methods to get a clear view of the genotype of a Giardia isolate since a; different methods can group isolates into different assemblages and b; the resolution of sub-genotypes within assemblages is dependent on the selected method.

The variability of dog sub-genotypes in the area appeared to be high since no dogs seemed to share exactly the same sub-genotype pattern when results from both the β-giardin and the gdh gene were included. Among the dog isolates, no human assemblages were found, however the number of analyzed samples was limited and more data need to be obtained before dogs can be ruled out as a potential reservoir of human-specific *Giardia* in the study area.

A relatively high number of samples, 17 out of 136 (12.5%) initially regarded as positive for *Giardia* cysts by light microscopy in León, Nicaragua, were negative both with single and nested PCR of the β-giardin gene. Immunofluorescence microscopy confirmed various concentrations of *Giardia* cysts in nine of these PCR negative samples while no cysts were detected in the remaining eight samples. Whether the latter result was a reflection of initial low levels or absence of cysts or degradation of cysts during long-term storage remains unclear. Inhibition of PCR, a common problem when DNA is extracted directly from fecal samples, did not seem to occur since spiking with low levels of *Giardia* DNA resulted in proper amplification in all negative samples. As one recent report (Robertson et al., 2007) suggests the inability of the β-giardin primers to amplify certain B sub-genotypes, all 17 negative samples were also analyzed with the gdh PCR. This yielded only one additional positive sample belonging to assemblage B, thus the β-giardin primers used in this study do not seem to explain the negative outcome of our samples.

Studies from Australia, India and Turkey where both symptomatic and non-symptomatic patients were included, each found a correlation between assemblage A and diarrhea (Aydin et al., 2004; Haque et al., 2005; Read et al., 2002), while a Dutch study found that assemblage B was associated with persistent diarrhea and assemblage A with intermittent diarrhea (Homan and Mank, 2001). In this study, no correlation was observed between type of assemblage and symptoms in the 119 patients where PCR-RFLP was successful. Fecal material initially collected for other purposes was however used in the current study and most stool samples were not screened for pathogens other than parasites. A conclusion that reported diarrhea was attributed to the *Giardia* infection could therefore not be unequivocally made. Future studies designed specifically for the detection of *Giardia* and the correlation between genotype and symptoms are therefore required in the area.

Assemblage B dominated in *Giardia* isolates from humans in León, Nicaragua. The assemblage A isolates were highly homogenous genetically within the study area, while assemblage B showed very high variability on both genes studied.

Acknowledgements

Johan Ankarklev is financed by a grant from SIDA/SAREC. We thank Jadwiga Winiecka-Krusnell and Michael Storey for fruitful comments on the manuscript.

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