Detection of assemblages A and B of *Giardia duodenalis* in water and sewage from São Paulo state, Brazil

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ABSTRACT

Giardia duodenalis is a protozoan that parasitizes humans and other mammals and causes giardiasis. Although its isolates have been divided into seven assemblages, named A to G, only A and B have been detected in human faeces. Assemblage A isolates are commonly divided into two genotypes, AI and AII. Even though information about the presence of this protozoan in water and sewage is available in Brazil, it is important to verify the distribution of different assemblages that might be present, which can only be done by genotyping techniques. A total of 24 raw and treated sewage, surface and spring water samples were collected, concentrated and purified. DNA was extracted, and a nested PCR was used to amplify an 890 bp fragment of the *gdh* gene of *G. duodenalis*, which codes for glutamate dehydrogenase. Positive samples were cloned and sequenced. Ten out of 24 (41.6%) samples were confirmed to be positive for *G. duodenalis* by sequencing. Phylogenetic analysis grouped most sequences with *G. duodenalis* genotype AII from GenBank. Only two raw sewage samples presented sequences assigned to assemblage B. In one of these samples genotype AII was also detected. As these assemblages/genotypes are commonly associated to human giardiasis, the contact with these matrices represents risk for public health.

Key words | genotyping, *Giardia*, glutamate dehydrogenase, sewage, water

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INTRODUCTION

Giardia duodenalis (syn. *lamblia* or *intestinalis*) is a flagellated protozoan that parasitizes the small intestine of humans and other mammals, causing giardiasis. Isolates of this species are morphologically similar, but have been divided into seven assemblages, named A to G, on the basis of genetic heterogeneity. To date only assemblages A and B have been found in humans (Thompson 2004; Monis *et al.* 2009).

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In developed countries, the prevalence of human giardiasis varies from 2 to 5%. In Brazil, studies show that it ranges from 16 to 26% (Mascarini & Donalísio 2006; Silva *et al.* 2009; Tashima *et al.* 2009).

G. duodenalis is a waterborne pathogen. Cysts of the organism can survive for long periods in water and are resistant to some of the processes used by the water industry, such as filtration and disinfection. To protect public health, therefore, it is important to search for *Giardia* cysts in water and sewage samples (Faubert 2000; Slifko *et al.* 2000; Smith *et al.* 2006; Carmena *et al.* 2007).

Only molecular characterization techniques can be used to differentiate *G. duodenalis* assemblages. These techniques have been used to characterize *Giardia* isolates from environmental sources in a number of countries (Cacciò *et al.* 2003; Sulaiman *et al.* 2004; Bertrand & Schwartzbrod 2007; Castro-Hermida *et al.* 2008; Plutzer *et al.* 2008).

In Brazil, this protozoan has been detected in water and sewage samples by immunofluorescence assay (Franco *et al.* 2001; Cantusio Neto & Franco 2004; Hachich *et al.* 2004; Heller *et al.* 2004; Cantusio Neto *et al.* 2006; Nishi *et al.* 2009; Razzolini *et al.* 2010); however, the molecular characterization of *Giardia* cysts from these sources has not yet been published. Thus, the significance of cysts in the environment for public health is not known in this country.

The objective of this study was to detect and characterize the genotypes of *Giardia* sp. in water and sewage samples from São Paulo state, Brazil, discussing the importance of findings for public health.

MATERIALS AND METHODS

Water and sewage samples

A total of 24 water and sewage samples were collected from May 2008 to March 2009 in São Paulo state, Brazil (18 in São Paulo Metropolitan Region and 6 in two small municipalities within the state: Piracicaba and São Lourenço da Serra).

Raw (6) and treated (6) sewage samples were collected at six different wastewater treatment plants that operate by stabilization ponds, activated sludge, anaerobic reactors or by a match of two of these systems. Effluent from these plants is discharged into water bodies and, in some cases, is directed to non-potable industrial uses. Two raw sewage samples were collected at different entrances of a lake that has been used as a water supply.

Surface water grab samples (11) were taken from a reservoir, close to the abstraction point supplying a drinking water treatment plant. The reservoir is also used as a recreational area. A spring water sample (1) was also collected. All samples were processed within 24 hours after collection.

Sample concentration

Samples (10 L) were concentrated by the modified membrane filtration technique (Araújo *et al.* 2010), which consists of filtration and centrifugation steps. Membranes with pores of 1 m and diameters of 14 cm (Millipore®, Brazil) were employed. Pre-filters were also required for treated sewage filtration.

Raw sewage samples (100 ml) were concentrated by centrifugation. Sewage was distributed into 50 ml conical tubes and centrifuged at $7428 \times g$ for 15 minutes at 24°C. The sediments were resuspended and pooled into one tube and then washed with ultra-pure water (Milli Q®). The suspension was centrifuged ($7.428 \times g/15'/24°C$) and supernatant was discharged.

Sample purification

Sediment obtained from the concentration step was purified by fluctuation in zinc sulfate solution (33%) according to Faust *et al.* (1938), with some modifications. Briefly, sediment was washed with ultra-pure water (Milli Q®). Supernatant was discharged after centrifugation ($7428 \times g/15'/24°C$). Ten millilitres of zinc sulfate solution was added. After homogenization, tubes were centrifuged ($3714 \times g/3'/24°C$). The pellet was removed with a 10 mL pipette. The supernatant left in the tube (2 ml) was washed twice with ultra-pure water (Milli Q®). The pellet was resuspended in ultra-pure water (Milli Q®).

DNA extraction and amplification

Genomic DNA was extracted using the phenol-chloroform-isoamilic alcohol protocol (Araújo *et al.* 2010), with small

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modifications as follows: all concentrated sediment was submitted to DNA extraction and the incubation period at 65°C was reduced to 80 minutes.

The PCR mix consisted of 25 ng of DNA, 10× buffer, 2 mM of $MgCL_2$, 1% of PVP, 300 μM of deoxynucleoside triphosphate mix, 0.5 μM of each primer, 1.25 U of *Tth* DNA polymerase (Biotools®, Madrid, Spain) and ultra-pure water (Milli Q®) to make up to a final volume of 25 μl. The same PCR mix, but without the PVP, was used for the second amplification. Amplification primers and conditions are described in Table 1.

The nested PCR product was resolved by electrophoresis using a 1.8% agarose gel prepared with 1× TAE buffer and stained with ethidium bromide. A 100 bp DNA Ladder Plus (Fermentas) was included as a size marker. Gel images were obtained by UV transillumination and captured by a gel documentation system (Epi Chemi II Darkroom and Software Labworks – UVP).

Following successful PCR, the products were purified (Ilustratm GFXtm PCR DNA and Gel Band Purification Kit, GE® Healthcare, UK). Molecular cloning and sequencing were done according to the methods of Ivanova *et al.* (2001). Analyses of 825 positions of the *gdh* gene sequences of the clones were compared with their closest relatives in the GenBank database by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Further phylogenetic and molecular evolutionary analyses were performed with the MEGA 4.1 program (Tamura *et al.* 2007).

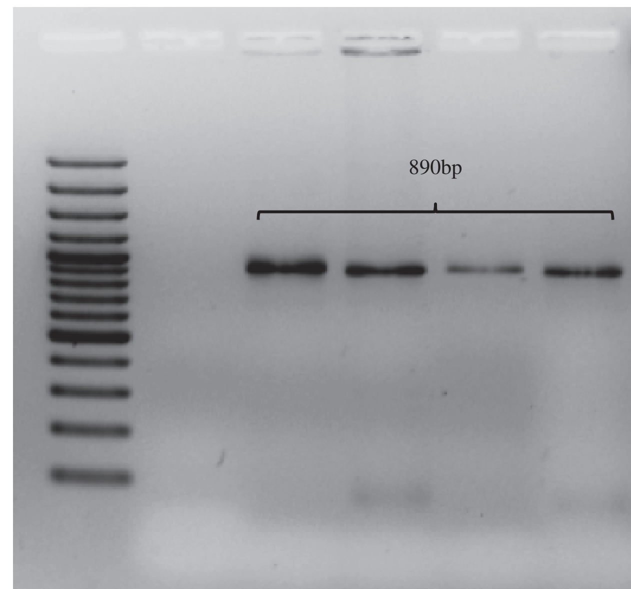

Figure 1 | Ethidium bromide stained agarose gel electrophoresis of the 890 bp fragment amplified from *gdh* gene of *Giardia duodenalis*. Lane 1: 100 bp DNA Ladder Plus (Fermentas); Lane 2: negative control; Lane 3: positive control; Lane 4: raw sewage positive sample; Lane 5: treated sewage positive sample; Lane 6: surface water positive sample.

RESULTS AND DISCUSSION

PCR amplification of the 890 bp *gdh* gene fragment was detected in 14 (58.3%) out of 24 water and sewage samples analysed (Figure 1). Eleven of the positive samples were cloned and sequenced; ten (41.6%) were confirmed to be

Table 1 | Primers sequence and PCR cycles used for amplification of *Giardia gdh* genes from environmental samples

			PCR conditions						
PCR reaction	Primer ID	Sequence*	ID	Cycles	D	A	E	FE	Band size
1st	GdhR (F)	5′CGAGCGCGAGCCGAAGT ATATCC3′	95°C, 7 min	40	95°C, 2 min	50°C 1 min	72°C 1.5 min	72°C 5 min	1190 bp
	#579II (R)	5′GATGTTYGCRCCCATCTG RTAGTTC3′							
2nd	GDH-FI (F)	5′AAYGAGGTYATGCGCTT CTGCCA3′	94°C, 2 min	30	94°C, 35 s	56°C 35 s	72°C 1 min		890 bp
	#579II (R)	5′GATGTTYGCRCCCATCT GRTAGTTC3′							

ID = Initial denaturation, D = Denaturation, A = Annealing, E = Extension, FE = Final Extension and * = position 659–1485 from the start of the gene.

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positive by sequencing (Table 1). One to ten clones of each sample were sequenced, resulting in a total of 71 nucleotide sequences. Some samples had clones with identical sequences, therefore only 64 nucleotide sequences were submitted to GenBank (GenBank accession numbers: GQ503101–GQ503141, GQ503152–GQ503157 and GQ503159–GQ503174). Phylogenetic analyses grouped most of the 64 sequences with *G. duodenalis* genotype AII sequences from GenBank. Only two raw sewage samples contained sequences assigned to assemblage B. In one of these samples, both genotype AII and assemblage B were detected (Table 2, Figure 2).

The presence of *Giardia* in water and sewage is well documented worldwide (Karanis *et al.* 2006; Robertson *et al.* 2006; Anceno *et al.* 2007; Bertrand & Schwartzbrod 2007; Castro-Hermida *et al.* 2008; Plutzer *et al.* 2008). In Brazil, the detection of this protozoan has also been documented in some studies (Franco *et al.* 2001; Cantusio Neto & Franco 2004; Hachich *et al.* 2004; Heller *et al.* 2004; Cantusio Neto *et al.* 2006; Nishi *et al.* 2009; Razzolini *et al.* 2010).

Table 2 | PCR, sequencing and genotyping results of *Giardia* isolates detected in water and sewage samples from São Paulo, Brazil

				Sequencing results Number of clones successfully sequenced/GenBank accession numbers	
Sample origin	**Sample number**	**PCR results**	**Number of sequenced clones**	**AII**	**B**
Raw sewage	1	P	5	5/GQ503101–GQ503104[a]	0
	2	P	10	0	10/GQ503106–GQ503115
	3	P	9	9/GQ503116–503120[b]	0
	4	P	4	4/GQ503121–GQ503124	0
	5	N	–	–	–
	6	P	7	6/GQ503125–GQ503130	1/GQ503131
Treated sewage	7	N	–	–	–
	8	P	–	–	–
	9	P	–	–	–
	10	P	9	9/GQ503152–GQ503158[c]	0
	11	P	1	–	–
	12	N	–	–	–
Surface water	13	P	8	1/GQ503105	0
	14	N	–	–	–
	15	N	–	–	–
	16	N	–	–	–
	17	N	–	–	–
	18	N	–	–	–
	19	P	10	10/GQ503132–GQ503141	0
	20	P	10	10/GQ503159–GQ503168	0
	21	P	6	6/GQ503169–GQ503174	0
	22	N	–	–	–
	23	N	–	–	–
Spring water	24	N	–	–	–

P = positive sample
N = negative sample
[a] = 2 identical sequences (GQ503102)
[b] = 5 identical sequences (GQ503116)
[c] = 3 identical sequences (GQ503154)

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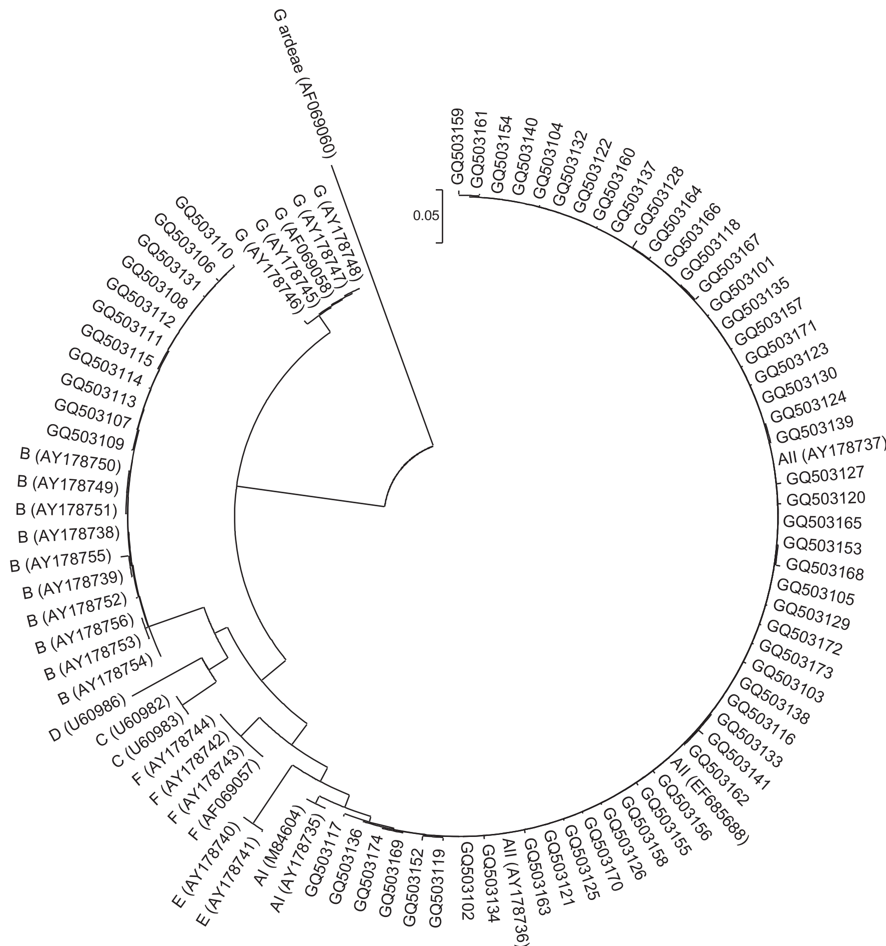

Figure 2 | Phylogenetic tree based on the alignment of 64 nucleotide sequences of *gdh* gene fragment. Tree was constructed by Neighbour-Joining method in MEGA 4.1 Beta program using maximum composite likelihood model and bootstrap of 1000 repetitions. Gene Bank reference sequences were: AII (EF685688, AY178736, AY178737), AI (AY178735, M846004), B (AY178738, AY178739, AY178749, AY178750, AY178751, AY178752, AY178753, AY178754, AY178755, AY178756), C (U60982, U60983), D (U60986), E (AY178740, AY178741), F (AY178742, AY178743, AY178744, AF069057), G (AY178745, AY178746, AY178747, AY178748, AF069058) and *G. ardeae* (AF069060).

In the present study, 10 out of 24 (41.6%) water and sewage samples analysed were found to be positive for *G. duodenalis*, corroborating previous reports that *G. duodenalis* is the only species of *Giardia* found in water sources.

Data on the genotypes of *Giardia* isolates found in Brazil are scarce and mainly derived from analysis of human and animal stool specimens (Rocha *et al.* 2003; Souza *et al.* 2007; Volotão *et al.* 2007, 2008). Although Paulino (2005) employed the technique of PCR to detect *G. duodenalis* in surface water samples, genotyping of the isolates was not performed.

The present study is the first to genotype *G. duodenalis* from environmental samples in Brazil and to demonstrate that assemblages A (genotype AII) and B of *G. duodenalis* are present in different water sources including surface water and raw and treated sewage. These findings are similar to those reported worldwide demonstrating the presence of assemblages A and B in environmental samples (Cacciò *et al.* 2003; Sulaiman *et al.* 2004; Robertson *et al.* 2006; Anceno *et al.* 2007; Bertrand & Schwartzbrod 2007; Castro-Hermida *et al.* 2008; Plutzer *et al.* 2008).

As observed in other studies, assemblage A of *G. duodenalis* was more frequently observed than assemblage B, which was detected in only two raw sewage samples (Cacciò *et al.* 2003; Sulaiman *et al.* 2004; Bertrand & Schwartzbrod 2007). In a study carried out in São Paulo, in 2007, Souza *et al.* (2007) demonstrated that genotype AII was observed in 29 out of 32 clinical samples analysed, and assemblage B in only eight samples. This finding indicates that, in the present study, genotype AII observed in sewage samples may represent the genotype circulating in the population.

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An unexpected number of Single Nucleotide Polymorphisms (SNPs) was detected in the majority of sequences obtained in this work. Most sequences had from one to five SNPs. None of these SNPs was observed in sequences recovered from GenBank. Besides, most SNPs resulted in non-synonym substitutions. Once sequencing presented high-quality and very well-defined peaks, these substitutions were not considered to be real, but were assumed to have occurred due to nucleotide incorporation problems during PCR, possibly because *Tth* DNA polymerase, a low-fidelity enzyme, was used.

The presence of *G. duodenalis* genotypes associated to human giardiasis in raw sewage indicates that there is risk in the reuse of sub-products of the sewage treatment process as observed in Table 2. Detection of genotype AII and assemblage B in treated sewage reveals that more attention should be given to sewage treatment processes, since effluents of sewage treatment plants can be used for domestic, agricultural or industrial purposes. Furthermore, this matrix can be released in surface waters that are subjected to indirect reuse including drinking water treatment. Sewage sludge may contain 10^5 to 10^6 *Giardia* cysts/kg^{-1} (Straub *et al.* 1993; Thiriat *et al.* 1997), and even though this matrix was not analysed in the present work it will be important to characterize *G. duodenalis* isolates recovered from this kind of sample, especially because of the lack of data (Rimhanen-Finne *et al.* 2001).

The detection of genotype AII in surface waters also represents risk for public health because this matrix is commonly used as recreation areas or as a source of water supply for humans.

CONCLUSIONS

The presence of genotype AII and assemblage B, commonly associated to human giardiasis, in water and sewage samples from São Paulo state indicates that contact with these matrices is a risk to public health. Therefore, more attention should be given to water and sewage treatment processes and to the uses of treated sewage and its discharges in watersheds, which should be protected against sources of contamination.

Studies of detection, quantification, infectivity and genotyping of *G. duodenalis* cysts from environmental samples should be encouraged in order to provide enough data for the decisions that need to be taken by public health authorities.

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