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Short communication

Genotyping of *Giardia duodenalis* from Southern Brown Howler Monkeys (*Alouatta clamitans*) from Brazil

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Abstract

Giardia duodenalis is a widespread intestinal protozoan that can infect humans and animals, both domestic and wild. Independent of host, infections present with the same symptoms. However, based on host specificity, *Giardia* isolates have been grouped into genotypes A to G. Parasites of assemblage A and B are known to infect humans, in addition to primates and a wide variety of mammals. In Brazil, hitherto *Giardia* genotypes were defined only for humans and domestic animals. To evaluate the genotypes of different *Giardia* present among other animals, fecal samples from 28 Southern Brown Howler Monkeys (*Alouatta clamitans*) kept in captivity from South Brazil were screened for *G. duodenalis* using parasitological methods. All of them were asymptomatic, but positive for *Giardia*. The genotype of the *G. duodenalis* circulating among these animals was ascertained by molecular typing, performed using amplification and sequencing of the β-giardin gene. Sixteen of 28 samples were successfully amplified by PCR and sequencing of this gene s revealed that all of them were of the genotype A1. These findings suggest that *A. clamitans* represent a potential risk of environmental contamination of a *G. duodenalis* genotype that also infect humans, and therefore can be considered a potential reservoir for *G. duodenalis* of a genotype that can also infects humans. Therefore, these results highlight a potential public health problem due to the epidemiological and molecular evidence for anthropozoonotic transmission.

Keywords: *Giardia duodenalis*; *Alouatta clamitans*; Genotyping; β-Giardin; Anthropozoonosis

1. Introduction

Giardia duodenalis (syn. *G. intestinalis*, *G. lamblia*) is a protozoa that often infects vertebrates relevant to evolutionary and medical studies. Its life cycle consists of two stages: cysts that are responsible for the fecal-oral transmission and trophozoites that establish infection within animal host's duodenum (Thompson, 1994). Phylogenic analyses of different loci have ordered *G. duodenalis* on one of the earliest diverging eukaryotic lineages (Boothroyd et al., 1987; Edlind and Chakraborty, 1987; Sogin et al., 1989; Hashimoto et al., 1994; Okamoto and Hasegawa, 1995; Adam, 2001). This parasite is responsible for causing approximately 1 billion cases of diarrheal disease annually worldwide (Islam, 1990; WHO/UNICEF, 2000) and about 2.5 million annual infections in the United States (Furness et al., 2000). It also is a significant veterinary pathogen (USDA, 1994).

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Although *G. duodenalis* isolates from different host species are morphologically indistinguishable, they have been grouped into of seven genetically distinct genotypes (Monis et al., 2003). Genotypes A and B have been detected in humans and in a wide range of other mammals, while C to G appear to be restricted from humans as hosts. Genotypes A and B infect other primates than humans such mountain gorillas (*Gorilla gorilla berengei*) (Graczyk et al., 2002), Black howler Monkeys (*Alouatta pigra*) (Vitazkova and Wade, 2006) and have also been found in other domestic and wild animals, such as dogs, cats, cattle, pigs, slow loris, siamang, beavers and white tailed deer, suggesting that genotypes A and B demonstrate a zoonotic preference (Thompson, 2000; Thompson and Monis, 2004; Cacciò et al., 2005; Hunter and Thompson, 2005; Volotão et al., 2007).

The howler monkeys (genus *Alouatta* monotypic in subfamily Alouattinae) are among the largest of the New World monkeys. In Brazil, ten species are currently recognized. One of these, *Alouatta clamitans* (Gregorim, 2006), is native to South and Central American forests. The knowledge of the parasitic diseases that can infect humans carried among these monkeys and others will have an impact on human health and species conservation. To date, no genotyping studies using stocks from monkeys have been carried out in Brazil. The present study was conducted to determine the genotypes of isolates of *G. duodenalis* shed by Southern Brown Howler Monkeys (*A. clamitans*) from South of Brazil using β-giardin gene as a target.

2. Materials and methods

2.1. Sampling

Fecal samples of 28 *A. clamitans* kept in captivity ($N = 28$) were collected between April 2005 and April of 2006 in order to investigate the presence of *G. duodenalis*. The samples were collected directly from the rectum of each by their veterinarians. These animals were kept in captivity in Centro de Pesquisas Biológicas e Observatório de Primatas de Indaial, CEPESBI, Blumenau, Santa Catarina, Southern Brasil (26°54′S and 49°13′W). The research described herein was previously approved by the Ethics Committees of the Regional University of Blumenau (033/04).

2.2. Diagnosis and isolation

Fecal samples were examined for *G. duodenalis* at Laboratory of Parasitology, Regional University of Blumenau. The samples were analyzed by microscopy (Faust et al., 1939) and kept under refrigeration until genotyping. One positive sample from each animal was stored in new vials and transported under refrigeration to the Laboratory of Molecular Epidemiology and Infectious Diseases in Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, RJ. Cysts were purified approximately 6 months after collection by sucrose density gradient centrifugation as previously described (Maddox-Hyttel et al., 2006).

2.3. Molecular characterization

Total DNA from *G. duodenalis* cysts was extracted using a commercial kit (QIAmp DNA Stool Mini Kit, Qiagen GmbH. Germany) according to the manufacture's instructions with minor modifications. Lysis temperature was increased to 95 °C, and the DNA was eluated with 100 μL of elution buffer and stored at 4 °C. Polymerase chain reaction (PCR) to amplify fragment of the β-giardin gene was performed as described (Cacciò et al., 2002). Amplified products were purified using the Wizard® SV Gel and Pcr Clean-Up System (Promega, Madison, WI, USA). DNA fragments were sequenced in both directions using the ABI Prism™ BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3730 automatic DNA sequencer (Applied Biosystems, Foster City, CA).

Sequences were analyzed using Chromas (http://www.technelysium.com.au/chromas.html) and compared with known *Giardia* spp. sequences obtained from GenBank using ClustalW algorithm (Thompson, 1994) in the MEGA version 4.0 (http://www.megasoftware.net). Phylogenetic analyses of 328 nucleotides length β-giardin sequences were performed through MEGA 4.0, on alignments obtained from ClustalW. The distance estimations were carried out using Jin and Nei (1990) equation (Kimura two-parameter model). The phylogenetic trees were constructed using a neighbor-joining algorithm (Saitou and Nei, 1987). For each calculation, branch reliability was assessed using bootstrap analysis (1000 replicates). A reference β-giardin gene sequence representing each major *G. duodenalis* genotypes (GenBank accession numbers listed in Fig. 1) was aligned against the sequences obtained from the new isolates. The nucleotide sequence of β-giardin from *G. muris* (GenBank accession number AY258618) was used as an outgroup.

2.4. Nucleotide sequence accession numbers

The new nucleotide sequences were deposited in the GenBank database under accession numbers

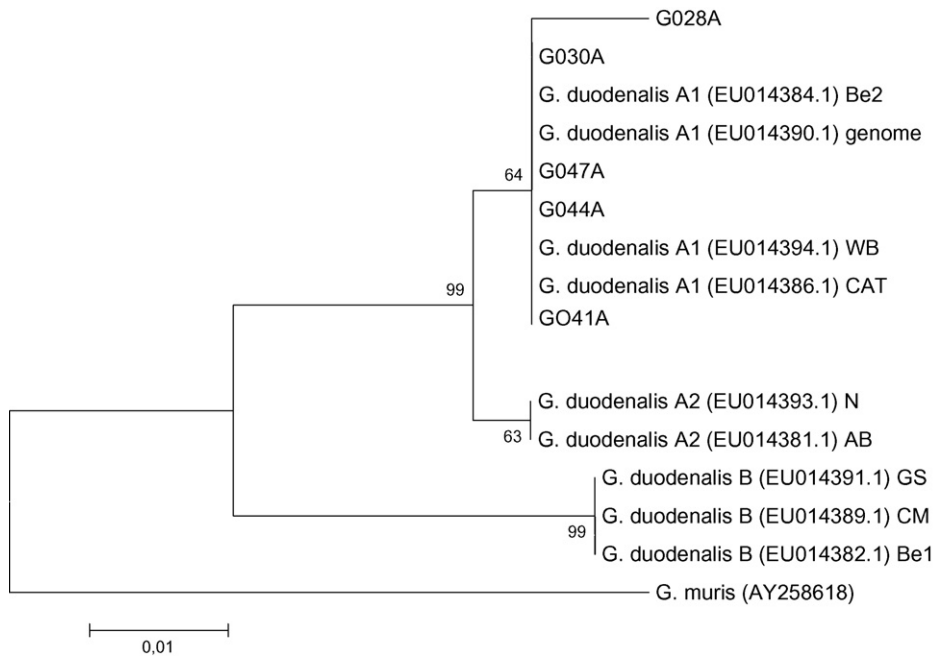

Fig. 1. Neighbor-joining phylogenetic analyses of *G. duodenalis* isolates from *A. clamitans* at beta giardin loci, carried out using the Kimura two-parameter model.

EU200933–EU200937 corresponding to partial *G. duodenalis* β-giardin sequences from five *Alouatta clamitans* isolates.

3. Results

This group of monkeys all tested positive for Giardia infection at least once during the timeframe of this project (April 2005–April 2006). One positive sample from each Southern Brown Howler Monkeys (*Alouatta clamitans*) was used for cyst purification, DNA extraction and genotyping of *G. duodenalis* isolates. Sixteen out of the 28 samples collected yielded amplified products using the β-giardin primers. Sequence analysis of a 328 bp β-giardin fragment of these isolates suggested that all of them were *G. duodenalis* genotype A1 (Fig. 1). Fifteen out of the sixteen sequences were homologous and one amplified product (G028A sample – accession number EU200933) revealed three single nucleotide polymorphisms (SNPs) that are not known to be present in the prototype A1 (reference sequence – EU014394.1) (Fig. 1). All of the SNPs are transitions and two of them cause a conversion of the amino acid (A53V and R221H). Our results showed that the SNPs in β-giardin sequence from sample G028A (accession number EU200933) are unique and have not been described so far in samples belonging to genotypes A.

4. Discussion

Little information is available regarding *G. duodenalis* genotypes in wildlife, although genotype A is the most prevalent. Several sylvan hosts have been identified bearing this genotype on different continents: gorillas (Africa), deers and moose (North America and Europe), kangaroos and fox (Australia) (Graczyk et al., 2002; Trout et al., 2003; van der Giessen et al., 2006; Lalle et al., 2007; Robertson et al., 2007; McCarthy et al., 2008). This is the first description of this genotype in Southern Brown Howler Monkeys (*A. clamitans*). This genotype is widespread worldwide (Cacciò et al., 2005) and has been previously detected in Brazil in humans and domestic animals (Volotão et al., 2007).

The amplification of the β-giardin gene by PCR directly from cysts has been used previously to genotype *G. duodenalis* (Cacciò et al., 2002; Eligio-Garcia et al., 2005; Lalle et al., 2005a,b; Volotão et al., 2007). Among the 28 samples that tested positive for *G. duodenalis* according to microscope examination, 16 yielded PCR products for the β-giardin gene. For the other 12, it is possible that the load of *G. duodenalis* in the sample was insufficient for PCR amplification since cysts are shed intermittently.

The *A. clamitans* screened in this study were kept in captivity in an area surrounded by human dwellings. Indeed, the monkeys were in direct contact with

domestic cats, which eventually began circulating through the cages. This continual contact could create a link between monkeys and humans within the peri-domestic setting developed by the cats. Considering that monkeys and humans are both infected by the same genotypes of *G. duodenalis*, there is a potential public health risk for the traffic of *G. duodenalis* cysts to humans (Macpherson, 2005; Papini et al., 2007; Volotão et al., 2007).

Other hypothesis for the presence of *G. duodenlais* in these monkeys could be (i) the contact of the monkeys with other wildlife animals that live nearly in a fragment of the forest and circulate near human dwellings, such as opossums. This hypothesis is strengthened by the fact that Southern Brown Howler Monkeys in the wild environment have been found infected with *Giardia* within the same geographical area (Müller et al., 2000) and (ii) the transmission of these genotypes by humans who have contact with these monkeys, such as Biologists and Veterinarians.

One of the 16 sequenced samples showed a distinct β-giardin sequence from genotype A1 (EU014394.1) with three specific SNPs that have not been described previously. All of them are transitions, but two of these are in the second codon position altering the primary structure of the codified protein, changing an alanin to valine and an arginine to a histidine. It is important to note that both have the same biochemical features. The genetic similarities between *G. duodenalis* isolates from humans and other primates as *A. clamitans* suggest epidemiological and molecular evidence of occurrence of anthropozoonotic transmission. The high prevalence of *G. duodenalis* and its asymptomatic feature demonstrates that *A. clamitans* represent a potential risk for environmental contamination and can be considered a potential public health problem.

Increasing the typing resolution and further molecular epidemiological studies of giardiasis in humans using case–control study design and subtyping analysis of specimens from both humans and animals are needed to provide more evident conclusions whether the human A1 infections are results of anthroponotic or zoonotic transmission.

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