Veterinary Parasitology 175 (2011) 40–46

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Prevalence and molecular typing of *Giardia* spp. in captive mammals at the zoo of Zagreb, Croatia

Relja Beck[a], Hein Sprong[b], Ingeborg Bata[c], Snjezana Lucinger[d], Edoardo Pozio[e], Simone M. Cacciò[e,*]

[a] *Department for Bacteriology and Parasitology, Croatian Veterinary Institute, Savska cesta 143, Zagreb, Croatia*
[b] *Laboratory for Zoonoses and Environmental Microbiology, National Institute for Public Health and Environment (RIVM), Bilthoven, The Netherlands*
[c] *Zagreb Zoo, Maksimirski perivoj b.b., Zagreb, Croatia*
[d] *Faculty of Veterinary Medicine, Department for Parasitology and Parasitic Diseases with Clinic, University of Zagreb, Zagreb, Croatia*
[e] *Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*

ARTICLE INFO

Article history:
Received 7 May 2010
Received in revised form 8 September 2010
Accepted 24 September 2010

Keywords:
Giardia duodenalis
Giardia microti
Captive mammals
Molecular typing
Zoonotic potential

ABSTRACT

A total of 131 faecal samples from 57 mammalian species housed at the zoo of Zagreb, Croatia, were tested for the presence of *Giardia* spp. cysts using epifluorescence microscopy. The overall prevalence (29%) was high, yet all animals were asymptomatic at the time of sampling. Positive samples were characterized by PCR and sequence analysis of both conserved and variable loci, for the identification of *Giardia* species and *G. duodenalis* assemblages and genotypes. Assemblages A and C were identified in Artiodactyla, assemblage B in Primates, Rodentia and Hyracoidea, and assemblages A, B, C and D, as well as *Giardia microti*, in Carnivora. Genotyping at the ITS1–5.8S–ITS2 region, at the triose phosphate isomerase, glutamate dehydrogenase and beta-giardin genes revealed extensive polymorphisms, particularly among assemblage B isolates. A phylogenetic analysis of concatenated sequences showed that isolates from captive mammals housed at the zoo are genetically different from isolates of human and domestic animal origin. This is the first survey in a zoological garden to include a molecular characterization of the parasite, and provides novel sequence data of *G. duodenalis* from many previously uncharacterized hosts.

1. Introduction

Giardia duodenalis (syn. *G. intestinalis*, *G. lamblia*) is the only species within the *Giardia* genus that infcets humans, pets and livestock (Cacciò and Ryan, 2008). The host range of *G. duodenalis* is wide, and includes terrestrial and marine mammals, and even fish (Lasek-Nesselquist et al., 2008; Yang et al., 2010).

It is well established that *G. duodenalis* is a species complex composed of at least seven distinct genetic groups (referred to as assemblages A to G), the taxonomy of which is still under revision (Monis et al., 2009). Assemblages A and B have been detected in a wide range of mammalian hosts, including humans, whereas assemblages C to G are to some extent host-specific, and have been very rarely, if ever, isolated from humans (Cacciò and Ryan, 2008). Genotyping data from wild animals are scarce, but some recent studies have revealed infection with assemblages A and B in wild carnivores and wild ruminants, suggesting a potential role of those animals as source of cysts infectious to other animals or to humans (van der Giessen et al., 2006; Lalle et al., 2007; Robertson et al., 2007).

In this work, mammals housed at the zoo of Zagreb, Croatia, were tested for the presence of *Giardia* sp. cysts and established PCR assays were used to characterize positive samples and to compare the identified genotypes with those already described in other hosts, including humans.

* Corresponding author. Tel.: +39 06 4990 2310; fax: +39 06 4990 3561.
E-mail addresses: simone.caccio@iss.it, caccio@iss.it (S.M. Cacciò).

0304-4017/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2010.09.026

2. Materials and methods

2.1. Study site

The Zagreb Zoo occupies 7 hectares (about 2.5 acres) and hosts 281 animal species for a total of about 2000 individuals. Samples were collected during January–February 2005 from several areas within the zoo. As fhe floor of animal cages was cleaned every evening, fresh faecal deposits were collected in the early morning. For animals that were kept in the pens during the day, faecal samples were collected from individual boxes where they spent the night. The animals tested in this work were mostly born in the zoo, or were brought into by exchange programs with other zoos.

2.2. Cyst isolation, microscopy and DNA isolation

Faecal samples were filtered through a mesh, washed with phosphate buffered saline solution (PBS) and submitted to centrifugation on 1 M sucrose gradient (specific gravity, 1.08) for 10 min at 800 × *g*. After flotation, the presence of *Giardia* spp. cysts was assessed by immunofluorescence (IF) microscopy using FITC-conjugated cyst wall-specific antibodies (Merifluor, Meridian Bioscience, Cincinnati, OH, USA), following the manufacturers' instructions.

DNA was extracted directly from faecal samples using the Fast Prep (Qbiogene, Illkirch Cedex, France) procedure as described by Da Silva et al. (1999). Briefly, an aliquot of faecal sample (0.4 ml) was homogenized using the FP120 Fast Prep Cell disruptor (Savant, Thermo Electro Corporation, Woburn MA, USA). The DNA released after the lysis step was purified using the Fast DNA extraction kit (Qbiogene, Illkirch Cedex, France).

2.3. Molecular methods

Protocols for the amplification of a 292-bp fragment of the small subunit ribosomal DNA (SSU-rDNA) gene, of a ~315-bp fragment encompassing the ITS1–5.8S–ITS2 region in the ribosomal unit, of a 511-bp fragment of the beta-giardin gene (*bg*), and of a 530-bp fragment of the glutamate dehydrogenase (*gdh*) gene were as described previously (Hopkins et al., 1997; Cacciò et al., 2008, 2010; Lalle et al., 2005). For the amplification of the triose phosphate isomerase (*tpi*) gene, two protocols were used, one with broad specificity (Sulaiman et al., 2003) and the other that specifically amplifies *G. duodenalis* assemblage D (Lebbad et al., 2010). In all cases, the primary PCR reaction consisted of 25 μl of 2× PCR master mix (Promega, Milan, Italy), 10 pmoles of each primer, and 1–3 μl of DNA in a total reaction volume of 50 μl. For the nested PCR, 2.5–5 μl of the first PCR was used as template. PCR products were separated by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

PCR products were purified using the Qiaquick purification kit (Qiagen, Milan, Italy) and sequenced on both strands using the ABI Prism BIGDYE Terminator Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The sequencing reactions were analysed using the ABI 3100 automatic sequencer (Applied Biosystems), and sequences were assembled using the software program SeqMan II (DNASTAR, Madison WI, USA).

2.4. Phylogenetic analysis

Reference sequences from axenic strains and closely related field isolates were retrieved from the ZoopNet database (Sprong et al., 2009). All molecular epidemiological data were stored and analysed in Bionumerics (Version 6.01; Applied Math, Belgium). Sequences were aligned using Clustal X (Thompson et al., 1997), and distance-based analyses were conducted using Kimura 2-parameters distance estimates and trees were constructed using the Neighbour-Joining (NJ) algorithm, implemented in the MEGA program version 4.0 (Tamura et al., 2007). Bootstrap proportions were calculated by the analysis of 1000 replicates for NJ trees.

3. Results

3.1. Prevalence of Giardia sp. in zoo mammals

The presence of *Giardia* sp. cysts in faecal samples was assessed using cyst wall-specific monoclonal antibodies and microscopic analysis. The parasite was detected in 38 of the 131 (29%) animals tested (Table 1); the highest prevalence was observed in species of Artiodactyla (5 isolates of 10 tested), Carnivora (12 of 21), Primates (6 of 12), and Rodentia (3 of 5). Albeit not systematically recorded, the number of cysts was generally low (less than 5 cysts per field), and only a few faecal samples contained a high number of cysts (data not shown). All animals were asymptomatic at the time of sample collection.

3.2. Molecular characterization

3.2.1. Identification of Giardia species

Of the 38 positive isolates, sufficient faecal material for DNA extraction was available only for 27 samples, which were submitted to a nested PCR for the amplification of a fragment of the *SSU-rDNA* gene. Positive results were obtained for 23 of the 27 (85%) samples. Sequence analysis identified *G. duodenalis* assemblage A in isolates from a serval (*Leptailurus serval*), a lynx (*Lynx lynx*), a wolf (*Canis lupus*), a coatimundi (*Nasua nasua*), a peccary (*Pecari tajacu*) and a oryx (*Oryx dammah*); *G. duodenalis* assemblage B was found in isolates from a Malayan sun bear (*Ursus malayanus*), a Prevost' squirrel (*Callosciurus prevosti*), a Patagonian cavy (*Docilchotis patagonum*), a rock hyrax (*Procavia capensis*), 3 ring-tailed lemurs (*Lemur catta*), a mantled guereza (*Colobus guereza*), a white-handed gibbon (*Hylobates lar*) and a chimp (*Pan troglodytes*); *G. duodenalis* assemblage C was detected in an isolate from a peccary; *G. duodenalis* assemblage D was detected in isolates from a cheetah (*Acinonyx jubatus*) and an African buffalo (*Synceros caffer nanus*); finally, *Giardia microti* was detected in isolates from a leopard (*Panthera pardus japonensis*) and a cheetah (Table 2).

Table 1
List of the zoo mammals examined in the present study. Scientific and common names, number of faecal samples tested and number of samples positive for *Giardia* are indicated.

Scientific name (common name)	Samples tested/positive
Order Carnivora	
Acinonyx jubatus (Cheetah)	2/1
Lynx lynx (Lynx)	2/1
Leptailurus serval (Serval)	4/1
Panthera leo (Lion)	2/1
Panthera pardus japonensis (Leopard)	2/1
Panthera tigris altaica (Siberian tiger)	2/0
Paradoxurus hermaphrodites (Palm civet)	3/1
Uncia uncial (Snow leopard)	2/1
Ailurus fulgens (Red panda)	2/0
Amblonyx cinerea (Small-clawed otter)	1/0
Suricata suricatta (Meerkat)	Pooled faeces/0
Ursus americanus (American black bear)	2/0
Ursus malayanus (Malayan sun bear)	2/1
Ursus arctos (Brown bear)	2/0
Cynictis penicillata (Yellow mongoose)	Pooled faeces/0
Zalophus californianus (Californian sea lion)	4/0
Halichoerus grypus (Gray seal)	2/1
Nasua nasua (Coatimundi)	4/1
Vulpes zerda (Fennec)	1/0
Canis lupus (Wolf)	9/4
Chrysocyon brachyurus (Maned wolf)	2/1
Order Artiodactyla	
Addax nasomaculatus (Addax)	4/1
Oryx dammah (Scimitar-horned oryx)	5/2
Camelus bactrianus (Bactrian camel)	3/0
Synceros caffer nanus (African buffalo)	4/1
Bison bonasus (Wisent)	3/1
Pecari tajacu (Peccary)	2/2
Connochaetes taurinus (Blue gnu)	1/0
Boselaphus tragocamelus (Nilgai)	1/0
Hexaprotodon liberiensis (Pygmy hippopotamus)	1/0
Axis axis (Chital)	2/0
Order Perissodactyla	
Equus przewalski (Przewalski's horse)	3/0
Equus hemonius kulan (Asian wild ass)	2/0
Order Rodentia	
Callosciurus prevosti (Prevost's squirrel)	1/1
Pedetes capensis (Spring hare)	2/0
Hydrochoerus hydrochaeris (Capibara)	2/1
Myocastor coypus (Nutria)	Pooled faeces/0
Docilchotis patagonum (Patagonian cavy)	1/1
Order Insectivora	
Echinops telfairi (Tennec)	1/0
Order Xenarthra	
Choloepus didactylus (Two-toed sloth)	1/0
Order Hyracoidea	
Procavia capensis (Rock hyrax)	2/2
Order Lagomorpha	
Oryctolagus cuniculus (Rabbit)	2/0
Order Chiroptera	
Rhinolophus hipposideros (Horseshoe bat)	Pooled faeces/0
Order Marsupialia	
Trichosurus vulpecula (Brush tail possum)	2/1
Order Primates	
Pan troglodytes (chimp)	5/2
Cercopithecus diana (Diana monkey)	4/0
Colobus guereza (mantled guereza)	4/1
Cebus paella (Tufted capuchin)	5/0
Semnopithecus entellus (Hanuman langur)	3/2
Lemur catta (Ring-tailed lemur)	4/3
Nycticebus coucang (Slow loris)	1/0
Hylobates lar (white-handed gibbon)	2/1
Perodicticus potto (Potto)	1/0
Callithrix pygmaea (Pygmy marmoset)	1/0
Cercocebus torquatus (Mangabey)	1/0
Galago crassicaudatus (Greater galago)	1/0
	131/38

Table 2
Genotyping results obtained at four different loci. Genotypes having zoonotic potential are shown as bold, underline characters.

Code	Species	ssu-rDNA	ITS1–ITS2	*tpi*/*tpi*-D	*bg*	*gdh*
GDA282	Snow leopard	neg	A1	C	neg	neg
GDA292	Leopard	*G. microti*	A1	neg	neg	neg
GDA293	Cheetah	*G. microti*	A1	neg	neg	neg
GDA691	Cheetah	D	A1	neg	neg	neg
GDA838	Serval	**A**	neg	neg	neg	neg
GDA689	Lynx	**A**	A1	A1/D	neg	neg
GDA690	Wolf	pos	A1	A1/D	neg	neg
GDA697	Wolf	**A**	A+C	A1/D	neg	neg
GDA281	Maned wolf	neg	A1	BIV	neg	neg
GDA696	Coatimundi	**A**	A	**A**	A	A
GDA303	Malayan sun bear	**B**	B	B	**B**	B
GDA315	African buffalo	D	neg	neg	neg	neg
GDA317	Wisent	neg	neg	C	neg	neg
GDA331	Collared pecari	C	neg	C	neg	neg
GDA332	Collared pecari	**A**	A	**A**	**A**	**A1**
GDA334	Scimitar-horned oryx	**A**	A	**A**	**A**	**A1**
GDA307	Prevost's squirrel	**B**	B	**B**	**B**	neg
GDA748	Patagonian cavy	**B**	B	**BIV**	**B**	B
GDA311	Rock hyrax	**B**	B	**BIV**	**B**	neg
GDA693	Ring-tailed lemur	**B**	B	B	**B**	neg
GDA808	Ring-tailed lemur	**B**	B	B	neg	neg
GDA811	Ring-tailed lemur	**B**	neg	BIV	neg	neg
GDA809	Guereza colobus	**B**	B	**B**	**B**	**B**
GDA810	White-handed gibbon	**B**	B	B	B	**B**
GDA297	Chimp	**B**	neg	B	**B**	B

3.2.2. Genotyping at the ITS1–5.8S–ITS2 region

All isolates were further analysed at the ITS1–5.8S–ITS2 region for the identification of genotypes within assemblages. Clear amplification products were obtained from 19 of 27 (70%) isolates (Table 2). Assemblage A, genotype A1, was identified in isolates from cheetah (two isolates), snow leopard (*Uncia uncia*), leopard, lynx, maned wolf (*Chrysocyon brachyurus*) and wolf. A novel sequence, differing from genotype A1 by three single nucleotide substitutions (SNPs), was found in isolates from a peccary, a oryx and a coatimundi (Table 2).

Assemblage B was identified in 8 isolates: the isolates from a mantled guereza, two ring-tailed lemurs, a white-handed gibbon and Malayan sun bear had a sequence identical to that previously found in a chimp (GU126438), whereas the isolates from a rock hyrax and a Prevost' squirrel had a sequence with a 37-bp deletion in the ITS2 region, representing a novel variant. The isolate from a Patagonian cavy had a sequence with a 36-bp deletion in the ITS2 region (this sequence has been previously deposited in GenBank under the accession number GU126441). Finally, an isolate from a wolf had a sequence compatible with a mixed A+C infection (Table 2).

3.2.3. Genotyping at the triose phosphate isomerase locus

At the *tpi* locus, clear amplification products using generic primers were obtained from 20 of 27 (75%) isolates (Table 2). Assemblage A was identified in 5 isolates: the isolates from a lynx and two wolves had a sequence identical to genotype A1, whereas the isolate from a coatimundi had a sequence that differs from genotype A1 by a single SNP. Finally, the isolate from a peccary had a sequence identical to that described in isolates from humans (GQ329678, GQ329677 and EU041756) and a cat (EU781027).

Assemblage B was identified in 11 isolates: the isolates from a ring-tailed lemur, a rock hyrax, a Prevost' squirrel, and a maned wolf had a sequence identical to genotype BIV, whereas the isolates from a white-handed gibbon and a mantled guereza had a sequence which differs from genotype BIV at two undetermined positions. The isolate from a Patagonian cavy had a sequence identical to that reported from a human isolate (EF688023) and from a wastewater sample (AY368171). The isolates from two ring-tailed lemurs had a sequence which differs by a single SNP from that found in a Brazilian human isolate (EU272153). Finally, the isolates from a chimp and a Malayan sun bear had a sequence which differ by three SNPs from that found in both a chimp and a mandrill (EU637590).

Assemblage C was detected in three isolates: the isolate from a peccary had a sequence identical to that reported from a dog (AY228641), whereas the isolate from a snow leopard had a sequence which differs from AY228641 by four SNPs, and the isolate from a wisent (*Bison bonasus*) had a sequence which differs from AY228641 at five undetermined positions.

Interestingly, when the *tpi* primers specific for assemblage D were used, only isolates from a lynx and two wolves gave a clear amplification product. Sequencing revealed assemblage D in these 3 isolates, which were typed as assemblage A1 using the generic *tpi* primers (Table 2).

3.2.4. Genotyping at the beta-giardin locus

At the *bg* locus, only 11 of 27 (41%) isolates could be successfully analysed by sequencing (Table 2). The isolate from a peccary had a sequence identical to genotype A1, the isolate from a oryx had a sequence identical to genotype A2, whereas the isolate from a coatimundi had a sequence that differs from genotype A1 by two SNPs.

The other eight isolates were identified as assemblage B: the isolates from a Patagonian cavy, a Prevost' squirrel and a rock hyrax had a sequence identical to that found in humans (e.g., EU594668, EU274393, EU881697, EF455593), in a rabbit from Sweden (EU769210) and in a monkey from Sweden (EU769208). The isolates from a mantled guereza, a white-handed gibbon and ring-tailed lemur had a sequence identical to that found in humans (EU274389 and EU637579) and several captive animals, e.g., a Thomson gazelle (EU626199) a Barbary macaque (EU637581), and an ant-eater (FJ009209). The isolates from a chimp and a Malayan sun bear had a sequence identical to that found in humans in New Zealand (FJ560593 and EU274393) and in the USA (DQ116605), and in a Barbary macaque (EU637580).

3.2.5. Genotyping at the glutamate dehydrogenase locus

At the *gdh* locus, only 8 of 27 (30%) isolates could be successfully analysed by sequencing (Table 2). The isolates from a peccary and a oryx had a sequence identical to genotype A1, whereas the isolate from a coatimundi showed a very distinct sequence, which differs from genotype A1 by ten SNPs.

The other five isolates were identified as assemblage B: the isolate from a mantled guerza had a sequence identical to that found in a captive Barbary macaque in Italy (EU637586), whereas the isolate from a white-handed gibbon had a sequence identical to that found in a Brazilian human isolate (EF507682). The isolate from a Malayan sun bear had a sequence showing a single SNP from that found in a marmoset (AY178753). The isolate from a Patagonian cavy had a sequence that differs from that of reference strains Ad28 (AY178738) and Ad45 (AY178739) at three undetermined positions. Finally, the isolate from a chimp had a sequence that differs from that found in a human isolate (EU834844) at five undetermined positions.

3.2.6. Phylogenetic analysis

A phylogenetic analysis of the ITS-1-5.8S-ITS2 sequences was performed on a multiple alignment that included the 19 zoo isolates (Table 2) as well as representatives of all *G. duodenalis* assemblages and *G. microti* (Fig. 1). The global topology of the tree is comparable to that obtained using concatenated *tpi-bg-gdh* sequences (see insert of Fig. 2): assemblages A, E and F cluster together, as do assemblages B, C, D and G. However, the position of assemblages F and G is markedly different in the two trees, most likely due to specific indels in the ITS-1-5.8S-ITS2 sequences from these assemblages.

To further explore the relationship between the zoo isolates and other human and animal isolates, we performed a phylogenetic analysis on concatenated *tpi-bg-gdh* sequences. As shown in Fig. 2, the eight zoo isolates for which multi-locus sequence information was available (Table 2), cluster with isolates belonging to either assemblage A or B. In the case of assemblage A, the isolates from a coatimundi, peccary and oryx are closer to the group of genotypes from subassemblage AI, and differ from genotypes of both subassemblages AII and AIII. In the case of assemblage B, no obvious clusters can be identified (Fig. 2); indeed, human, domestic and captive animal isolates, including the zoo isolates from a Malayan sun bear, a Patagonian cavy, a chimp, a mantled guereza and a white-handed gibbon, are interspersed in a less resolved tree compared to that obtained with assemblage A isolates.

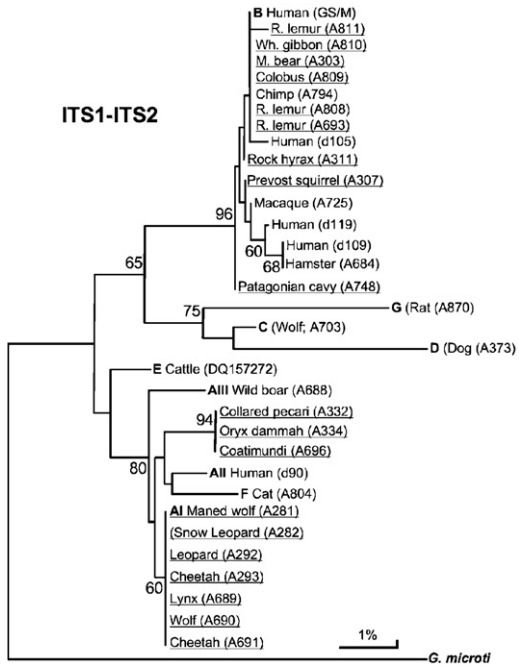

Fig. 1. Neighbour-joining tree based on the ITS1, 5.8S and ITS2 sequences of isolates from *G. duodenalis*. The homologous *G. microti* sequence was used as outgroup. Only bootstrap values >60 are indicated. Sequences from this study are underlined, whereas others were described in and retrieved from Cacciò et al. (2010).

4. Discussion

Based on the direct observation of cysts or trophozoites in faecal samples, the presence of *Giardia* spp. has been reported in many mammalian species, including both wild and captive animals, with prevalence that varied from very low to 100% (reviewed by Olson and Buret, 2001). In the last two decades, interest in *Giardia* infection of wild mammals has been further motivated by the possible involvement of some species, such as beavers, in the zoonotic transmission of giardiasis (Appelbee et al., 2005).

In the present study, a variety of wild mammal species kept in captivity at the zoological garden of Zagreb, Croatia, was tested for the presence of *Giardia* cysts, and an overall prevalence of 29% was estimated. To the best of our knowledge, this is the first report of the parasite in faecal samples of addax (*Addax nasomaculatus*), oryx, peccary, Prevost' squirrel, Patagonian cavy, cheetah, lynx, palm civet (*Paradoxurus hermaphroditus*), coatimundi and maned wolf (Table 1). We attempted to characterize positive isolates by amplification and sequencing of fragments of both conserved and variable loci. As shown in Table 2, only eight isolates could be analysed at all tested loci, whereas the remaining isolates showed amplification at some loci only. This can be ascribed to mismatches in the binding region

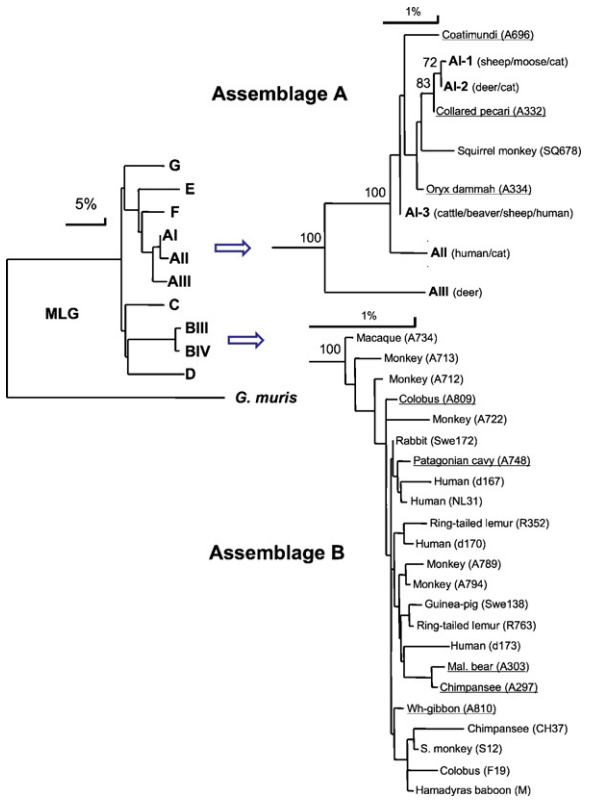

Fig. 2. Bottom-left image: neighbour-joining tree based on the concatenated sequences of beta-giardin (*bg*), glutamate dehydrogenase (*gdh*), and triose phosphate isomerase (*tpi*) loci of representative isolates of each *G. duodenalis* assemblage and sub-assemblage (see Section 2). The concatenated sequences of *bg* and *tpi*-loci of *G. muris* (isolate Ad-120) were used as outgroup. Main image: neighbour-joining tree showing the genetic relationship of isolates from *G. duodenalis* assemblages A and B. The tree included all zoo isolates (underlined; see Table 2) and isolates having the highest homology (see Section 2). Only bootstrap values >60 are indicated.

of the primers, which might prevent an effective amplification or to lower sensitivity of some assays (i.e., the *gdh* assay). Similar results have been reported by other authors (e.g., Lasek-Nesselquist et al., 2009), and clearly indicate that the current genotyping scheme must be improved, particularly when isolates from new hosts are analysed.

The occurrence of assemblage B in different species of Primates (Table 2) is in agreement with isoenzyme data from a marmoset and a siamang (Monis et al., 2003) and with DNA sequence data from Prosimians, Old World and New World monkeys, and apes (Itagaki et al., 2005; Cacciò et al., 2008; Levecke et al., 2009). Recalling that assemblage A has been identified in brown howler monkeys from Brazil (Volotão et al., 2008), gorillas from Uganda (Graczyk et al., 2002), captive squirrel monkeys and chimps from Europe (Levecke et al., 2009), non-human primates appear to be infected exclusively with *G. duodenalis* assemblages A and B, like humans.

Similarly, the occurrence of assemblage B in Prevost' squirrel and Patagonian cavy supports previous data from other rodent species (beavers and muskrats) that were characterized at the *tpi* and *bg* loci (Sulaiman et al., 2003; Fayer et al., 2006). The presence of assemblage A in isolates from peccary and oryx corroborates previous genotyping results from livestock and wild ruminants (Trout et al., 2004; Geurden et al., 2009). Isolates from Carnivora were more variable and characterized by the presence of both *G. microti* and different *G. duodenalis* assemblages (A, B, C and D). Moreover, the use of *tpi* primers specific for the assemblage D (Lebbad et al., 2010) revealed the presence of this assemblage in isolates from wolf and lynx (Table 2), which were genotyped as assemblage A using generic *tpi* primers.

With respect to zoonotic potential, when data from the ITS1–5.8S–ITS2 region are considered, seven isolates from Carnivores harboured genotype A1, which is zoonotic, albeit its prevalence in humans is low (Sprong et al., 2009); for assemblage B, no zoonotic genotypes were observed, in agreement with recent data obtained by sequence analysis of this locus (Cacciò et al., 2010). At the *tpi* locus, zoonotic genotypes included the BIV genotype, and another B genotype found in a Patagonian cavy that has been reported in an axenic strain of human origin (Lasek-Nesselquist et al., 2009). Finally, at the *gdh* locus, the zoonotic genotype A1 was found in peccary and oryx, while the B genotype found in a white-handed gibbon has been previously reported in a human from Brazil (Souza et al., 2007).

Two aspects emerged when data from multiple loci are considered. On the one hand, isolates from Carnivora, with the exception of those from a Malayan sun bear and coatimundi, could not be assigned unequivocally to one assemblage (or even to one species in the case of leopard, snow leopard and cheetah; Table 2). This can be due either to mixed infections, with preferential amplification of one assemblage/species over the other, or to the occurrence of recombinants carrying genetic information from different assemblages/species (Cacciò and Sprong, 2010). On the other hand, even when data from different loci are congruent at the level of assemblages, the combination of genotypes (i.e., the resulting haplotype) is still difficult to interpret. For example, the isolates typed as BIV at the *tpi* locus are not typed as BIV at the *gdh* locus (Table 2), and a number of sequences, all from assemblage B, show heterogeneous positions that complicate their assignment to specific genotypes. A phylogenetic analysis, using concatenated sequences from the *tpi*, *bg* and *gdh* genes, was performed to compare multi-locus genotypes (MLGs) of zoo mammals with MLGs from humans and other animals present in a dedicated database (Sprong et al., 2009). This showed that MLGs from zoo mammals are genetically distinct from all other previously characterized MLGs (Fig. 2). Thus, using MLG analysis, a higher resolution is obtained, and the zoonotic potential of most isolates is no longer supported, at least based on available data.

The high genetic heterogeneity of *G. duodenalis* isolates in a closed environment like a zoo makes identification of transmission routes difficult. Clearly, life in zoological gardens not only impose biological and spatial restrictions to the animals, preventing them from performing their species-specific behaviour, but can also alter the transmission of infective diseases, due to increased contacts among individuals, and exposure to humans (caretakers and visitors). The mammals tested in this work were all born in the zoo, or were brought into the Zagreb zoo by exchange

programs with other zoological gardens. This may have allowed the introduction of asymptomatically infected animals from beyond the boundaries of the zoo (Verweij et al., 2003). The occurrence of *G. microti* in a few carnivores may be explained by the presence of synanthropic rodents in the zoo, which may also serve, along with cats, as reservoir of infectious *G. duodenalis* cysts. A better understanding of the transmission routes requires the genetic characterization of *Giardia* isolates from humans and synanthropic animals having a direct interaction with the zoo animals, as well as from the water used in this closed environment.

Conflict of interest statement

The authors declare the absence of any conflict of interest.

Acknowledgments

This work has been partially supported by a grant (FOOD-CT-2004-506122) from the 6th European Union Framework Programme (Med-Vet-Net, a Network of Excellence for the Integrated Research on the Prevention and Control of Zoonoses). We thank Daniele Tonanzi for his excellent technical support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2010.09.026.

References

Appelbee, A.J., Thompson, R.C., Olson, M.E., 2005. *Giardia* and *Cryptosporidium* in mammalian wildlife-current status and future needs. Trends Parasitol. 21, 370–376.

Cacciò, S.M., Ryan, U., 2008. Molecular epidemiology of giardiasis. Mol. Biochem. Parasitol. 160, 75–80.

Cacciò, S.M., Beck, R., Lalle, M., Marinculic, A., Pozio, E., 2008. Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. Int. J. Parasitol. 38, 1523–1531.

Cacciò, S.M., Beck, R., Almeida, A., Bajer, A., Pozio, E., 2010. Identification of *Giardia* species and *Giardia duodenalis* assemblages by sequence analysis of the 5.8S rDNA gene and internal transcribed spacers. Parasitology 17, 1–7.

Cacciò, S.M., Sprong, H., 2010. *Giardia duodenalis*: genetic recombination and its implications for taxonomy and molecular epidemiology. Exp. Parasitol. 124, 107–112.

Da Silva, A.J., Bornay-Llinares, F.J., Moura, I.N.S., Slemenda, S.B., Tuttle, J.L., Pieniazek, N.J., 1999. Fast and reliable extraction of protozoan parasite DNA from faecal specimens. Mol. Diag. 4, 57–64.

Fayer, R., Santín, M., Trout, J.M., DeStefano, S., Koenen, K., Kaur, T., 2006. Prevalence of Microsporidia, *Cryptosporidium* spp., and *Giardia* spp. in beavers (*Castor canadensis*) in Massachusetts. J. Zoo Wildl. Med. 37, 492–497.

Geurden, T., Goossens, E., Levecke, B., Vercammen, F., Vercruysse, J., Claerebout, E., 2009. Occurrence and molecular characterization of *Cryptosporidium* and *Giardia* in captive wild ruminants in Belgium. J. Zoo Wildl. Med. 40, 126–130.

Graczyk, T.K., Bosco-Nizeyi, J., Ssebide, B., Thompson, R.C., Read, C., Cranfield, M.R., 2002. Anthropozoonotic *Giardia duodenalis* genotype (assemblage) A infections in habitats of free-ranging human-habituated gorillas, Uganda. J. Parasitol. 88, 905–909.

Hopkins, R.M., Meloni, B.P., Groth, D.M., Wetherall, J.D., Reynoldson, J.A., Thompson, R.C., 1997. Ribosomal RNA sequencing reveals differences between the genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. J. Parasitol. 83, 44–51.

Itagaki, T., Kinoshita, S., Aoki, M., Itoh, N., Saeki, H., Sato, N., Uetsuki, J., Izumiyama, S., Yagita, K., Endo, T., 2005. Genotyping of *Giardia intestinalis* from domestic and wild animals in Japan using glutamate dehydrogenase gene sequencing. Vet. Parasitol. 133, 283–287.

Lalle, M., Pozio, E., Capelli, G., Bruschi, F., Crotti, D., Cacciò, S.M., 2005. Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. Int. J. Parasitol. 35, 207–213.

Lalle, M., Frangipane di Regalbono, A., Poppi, L., Nobili, G., Tonanzi, D., Pozio, E., Cacciò, S.M., 2007. A novel *Giardia duodenalis* assemblage A subtype in fallow deer. J. Parasitol. 93, 426–428.

Lasek-Nesselquist, E., Bogomolni, A.L., Gast, R.J., Welch, D.M., Ellis, J.C., Sogin, M.L., Moore, M.J., 2008. Molecular characterization of *Giardia intestinalis* haplotypes in marine animals: variation and zoonotic potential. Dis. Aquat. Organ. 81, 39–51.

Lasek-Nesselquist, E., Welch, D.M., Thompson, R.C., Steuart, R.F., Sogin, M.L., 2009. Genetic exchange within and between assemblages of *Giardia duodenalis*. J. Eukaryot. Microbiol. 56, 504–518.

Lebbad, M., Mattsson, J.G., Christensson, B., Ljungström, B., Backhans, A., Andersson, J.O., Svärd, S.G., 2010. From mouse to moose: Multilocus genotyping of *Giardia* isolates from various animal species. Vet. Parasitol. 168, 231–239.

Levecke, B., Geldhof, P., Claerebout, E., Dorny, P., Vercammen, F., Cacciò, S.M., Vercruysse, J., Geurden, T., 2009. Molecular characterisation of *Giardia duodenalis* in captive non-human primates reveals mixed assemblage A and B infections and novel polymorphisms. Int. J. Parasitol. 39, 1595–1601.

Monis, P.T., Andrews, R.H., Mayrhofer, G., Ey, P.L., 2003. Genetic diversity within the morphological species *Giardia intestinalis* and its relationship to host origin. Infect. Genet. Evol. 3, 29–38.

Monis, P.T., Cacciò, S.M., Thompson, R.C., 2009. Variation in *Giardia*: towards a taxonomic revision of the genus. Trends Parasitol. 25, 93–100.

Olson, M.E., Buret, A.G., 2001. *Giardia* and giardiasis. In: Samuel, W.M., Pybus, M.J., Kocan, A.A. (Eds.), Parasitic Diseases of Wild Mammals, pp. 399–416.

Robertson, L.J., Forberg, T., Hermansen, L., Hamnes, I.S., Gjerde, B., 2007. *Giardia duodenalis* cysts isolated from wild moose and reindeer in Norway: genetic characterization by PCR-RFLP and sequence analysis at two genes. J. Wildl. Dis. 43, 576–585.

Souza, S.L., Gennari, S.M., Richtzenhain, L.J., Pena, H.F., Funada, M.R., Cortez, A., Gregori, F., Soares, R.M., 2007. Molecular identification of *Giardia duodenalis* isolates from humans, dogs, cats and cattle from the state of Sao Paulo, Brazil, by sequence analysis of fragments of glutamate dehydrogenase (gdh) coding gene. Vet. Parasitol. 149, 258–264.

Sprong, H., Cacciò, S.M., van der Giessen, J., 2009. Identification of zoonotic genotypes of *Giatrdia duodenalis*. PLOS Neg. Trop. Dis. 3, e558.

Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H., Trout, J.M., Schantz, P.M., Das, P., Lal, A.A., Xiao, L., 2003. Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. Emerg. Infect. Dis. 9, 1444–1452.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Trout, J.M., Santin, M., Greiner, E., Fayer, R., 2004. Prevalence of *Giardia duodenalis* genotypes in pre-weaned dairy calves. Vet. Parasitol. 124, 179–186.

van der Giessen, J.W., de Vries, A., Roos, M., Wielinga, P., Kortbeek, L.M., Mank, T.G., 2006. Genotyping of *Giardia* in Dutch patients and animals: a phylogenetic analysis of human and animal isolates. Int. J. Parasitol. 36, 849–858.

Verweij, J.J., Vermeer, J., Brienen, E.A., Blotkamp, C., Laeijendecker, D., van Lieshout, L., Polderman, A.M., 2003. *Entamoeba histolytica* infections in captive primates. Parasitol. Res. 90, 100–103.

Volotão, A.C., Júnior, J.C., Grassini, C., Peralta, J.M., Fernandes, O., 2008. Genotyping of *Giardia duodenalis* from Southern Brown Howler Monkeys (*Alouatta clamitans*) from Brazil. Vet. Parasitol. 158, 133–137.

Yang, R., Reid, A., Lymbery, A., Ryan, U., 2010. Identification of zoonotic Giardia genotypes in fish. Int. J. Parasitol. 40, 779–785.