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Research article

Prevalence, infection intensity and genotyping of *Giardia duodenalis* in ring-tailed lemurs *Lemur catta* from European zoos and wild populations

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Abstract

Globally, *Giardia duodenalis* is probably the most common intestinal protozoan parasite infecting humans and it appears also to be common in some zoo-housed primates. Infected zoo animals present a risk for potential spill-over of zoonotic pathogens to co-residing animals, staff and visitors. Using quantitative PCR, this study compared *Giardia* spp. prevalence and infection intensity in wild and zoo-housed ring-tailed lemurs *Lemur catta*. Infection intensity of zoo-housed ring-tailed lemurs (prevalence=88.6%, median Ct value=31.1, IQR=27.1–34.5) was significantly higher (P>0.01) than in wild ring-tailed lemurs (prevalence=20.0%, median Ct value=37.7, IQR=37.5–38.7), where little or no *Giardia* was found. Comparison of the enclosure designs showed both a higher prevalence and significantly higher intensity (P>0.005) of *Giardia* infections in zoos with walk-through enclosures (prevalence=89%, median Ct value=28.6, IQR=26.5–32.3) compared to traditional enclosures (prevalence=65%, median Ct value=35.2, IQR=33.3–37.8), but there was substantial variation within groups. The potentially zoonotic *G. duodenalis* assemblage B was identified in samples from five zoos. These findings suggest that ring-tailed lemurs may be asymptomatic carriers of *G. duodenalis* and a higher parasitic load might occur in lemurs held in walk-through enclosures.

Introduction

The zoonotic potential of the intestinal parasite *Giardia duodenalis* should be a matter of concern for institutions such as zoos where a diverse selection of animal species are held in close proximity to each other and humans. Given its monoxenous life cycle (Berrilli et al. 2011), stress-tolerant cysts (Flanagan 1992), and the low infective dose required to establish infection (Cacciò and Sprong 2011), species of *Giardia* have the potential to quickly increase the infection pressure in a given environment. Giardiasis may result in severe malabsorptive diarrhoea, lactose intolerance, irritable bowel syndrome, and even death of the host (Flanagan 1992; Cacciò and Sprong 2011; Halliez and Buret 2013). So far, molecular epidemiological studies of *G. duodenalis* have revealed a species complex of at least seven assemblages, labelled A–G (Monis et al. 2009). Assemblages A and B are found in several mammals, including humans (Flanagan 1992); assemblages C and D in dogs, E in hoofed livestock, F in cats and G in rats. Hosts can be infected with a host-specific assemblage simultaneously with an assemblage of lower host specificity, such as A or B (Geurden et al. 2008; Xiao and Fayer 2008). The lack of host specificity, particularly of assemblages A and B means that zoo-housed animals are at risk of becoming infected via humans,

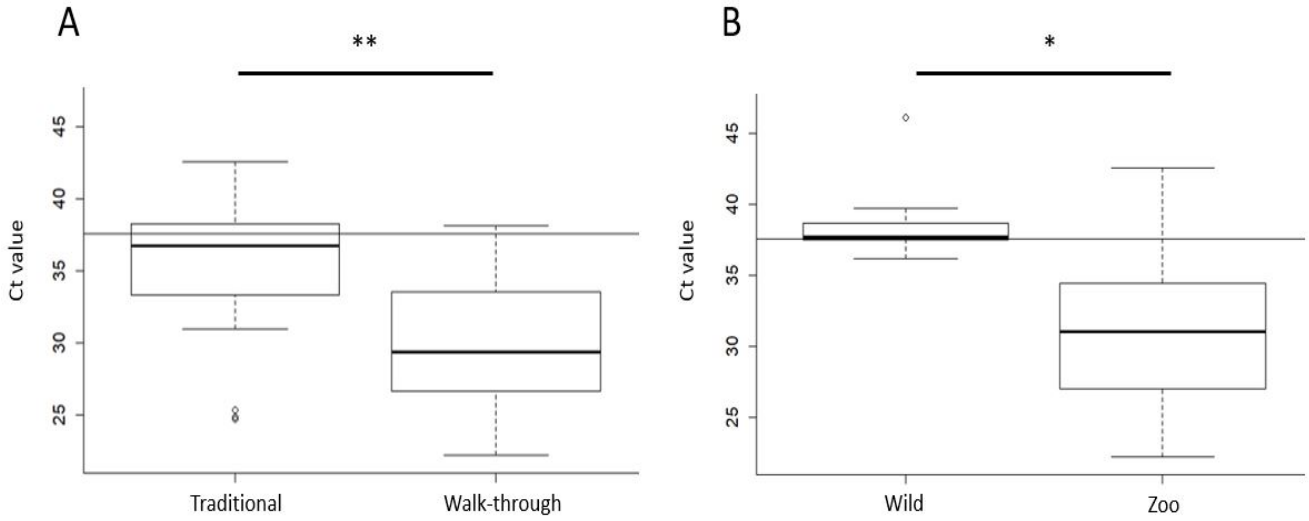

Figure 1. Infection intensity of *Giardia* by habitat of ring-tailed lemur groups. The boxplot depicts the median Ct values (bold lines), 25–75% quartiles (box), upper and lower quartiles (whiskers), and outliers (dot) for the samples collected in A) traditional enclosures versus walk-through enclosures, and B) wild versus zoos. Ct cut-off 37.6 (horizontal line) is shown, meaning that Ct values ≤37.6 denote samples infected with *G. duodenalis* while Ct value >37.6 denotes samples negative of *G. duodenalis* (*=0.01<P>0.05; **=0.001<P>0.01). Samples derived from zoos had lower Ct values than those derived from the wild (P=0.01) and samples derived from walk-through enclosures were lower than those of traditional enclosures (P=0.007).

urban wildlife and water sources. Vice versa, infected zoo-housed animals are a potential disease transmitter for other zoo-housed species, staff and visitors.

Several zoos hold some animal species such as ring-tailed lemurs *Lemur catta* in walk-through enclosures designed so that visitors can venture inside the enclosure. This enclosure design may have implications for disease spread to other zoo animals and visitors. With the modern approach to enclosure design that mimics the species' natural habitat, including a denser vegetation and sill flooring instead of concrete, stress-tolerant parasites like *Giardia* can survive in the flooring and keep being transmitted between individuals (Berrilli et al. 2011; Mir et al. 2016). Walk-through enclosures are often larger than traditional enclosures and hygiene levels more difficult to maintain, posing a risk for increased *Giardia* transmission (Mir et al. 2016).

Wild ring-tailed lemurs in Madagascar do not appear to carry *Giardia* spp. according to observations based on microscopical screening of faecal samples (Villers et al. 2008). Interestingly, several studies have identified *G. duodenalis* assemblage A and/or B in European and Chinese zoos (Levecke et al. 2007, 2009; Beck et al. 2011; Berrilli et al. 2011; Martínez-Díaz et al. 2011; Karim et al. 2015). However, it remains to be investigated whether there is a correlation between *Giardia* prevalence, infection intensity and enclosure design. The objectives of this study were to estimate and compare the prevalence and infection intensity of *Giardia* spp. in wild and zoo-housed ring-tailed lemurs and to further compare data from individuals held in walk-through and traditional enclosures, respectively, in European zoos. Moreover, *Giardia*-positive samples were characterised using Sanger and Next Generation Sequencing to report on (sub)assemblages and their zoonotic potential.

Materials and methods

Sample collection and DNA extraction

A total of 132 fresh (<12 hours old) faecal samples were collected from ring-tailed lemurs between February and July 2018 from 16 European zoos and from wild groups of ring-tailed lemurs in three locations in Madagascar (Table 1). Samples were stored in 70% ethanol during shipment. Upon arrival, samples were washed twice with phosphate-buffered saline solution (PBS) and 300±20 mg from each sample was used for further analysis. To lyse the cysts, freeze/thawing cycles were applied to the samples as described by Adamska et al. (2010). This procedure included three rounds of submersion into a 100°C water bath for 2 min and submersion in liquid nitrogen for 2 min. After the third round of exposure to liquid nitrogen, 800 µl ATL lysis buffer was added. Samples were centrifuged at 4,000 rpm for 5 min, and 500 µl supernatant was treated with 37 µl proteinase K in 500 µl AL buffer overnight at 56°C. DNA was extracted following the recommendation by Adamska et al. (2010) using the QIAamp DNA Tissue Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations with minor changes in volumes: A total of 500 µl 96% ethanol was added to cover all sample material, and the DNA was eluted in 100 µl Buffer AE. Samples were stored at −20°C until further analysis.

Giardia *spp. detection using qPCR*

Giardia was detected by qPCR with and without a TaqMan probe targeting a 62-bp fragment of the small subunit ribosomal RNA (SSU-rRNA) gene of *G. duodenalis*. Primers were *Giardia*-F: 5'-GACGGCTCAGGACAACGGTT-3', *Giardia*-R: 5'-TTGCCAGCGGTGTCCG-3', and the TaqMan probe *Giardia*-T: FAM-5'-CCCGCGGCGGTCCCTGCTAG-3'-TAMRA designed by

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Table 1. Prevalence of *Giardia duodenalis* (proportion of infected samples) and median Ct values with Interquartile range (IQR) at each location. Zoo I, Zoo II, Zoo III, and Zoo IV denotes four anonymous zoos. Madagascar coordinates: Beza Mahafaly: 23°39'S 44°37'E, Amoron'l Onilahy: 23°31'S 44°05'E, Ivohiboro forest: 22°34'S 46°42'E.

Exhibit type	Location	No. of faecal samples tested	% samples positive for *Giardia*	Median Ct value across positive samples
Walk-through enclosures	Givskud Zoo (DK)	7	100.0%	26.5 (24.2–28.4)
	Zoo I	2	100.0%	27.3 (26.8–27.8)
	Jesperhus Feriepark (DK)	9	88.9%	27.2 (26.3–28.3)
	Zoo II	27	100.0%	28.0 (25.9–29.3)
	Planckendael Zoo (BE)	10	100.0%	29.1 (26.6–31.9)
	Zoo III	3	100.0%	32.0 (30.5–32.8)
	Ree Park Safari (DK)	11	100.0%	32.2 (31.6–33.5)
	Apenheul Primate Park (NL)	5	80.0%	32.3 (28.6–33.0)
	Whipsnade Zoo (UK)	14	78.5%	34.0 (30.3–35.9)
	Zoo IV	4	0.0%	-
Traditional enclosures	Copenhagen Zoo (DK)	5	100.0%	25.3 (24.9–33.0)
	Kristiansand Zoo and Amusement Park (NO)	12	83.3%	34.0 (32.7–35.3)
	Royal Burger's Zoo (NL)	5	60%	34.6 (34.3–38.4)
	Odense Zoo (DK	7	85.7%	36.1 (34.2–36.8)
	Antwerp Zoo (BE)	2	50.0%	37.5 (37.3–37.7)
	Aalborg Zoo (DK)	9	11.1%	38.5 (37.8–39.4)
Madagascar	Beza Mahafaly	15	33.3%	37.7 (37.2–38.5)
	Amoron'l Onilahy	9	22.2%	38.7 (37.6–38.1)
	Ivohiboro forest	11	0.0%	39.5 (39.5–39.5)

Verweij et al. (2003). The TaqMan assay was performed in duplicates in a qPCR in a volume of 50 µl as described by Verweij et al. (2004). H_2O was used as a negative control.

A 1:10 serial dilution was performed of DNA isolated from 5.0x106 purified non-viable *G. duodenalis* cysts in PBS (plus penicillin, streptomycin, gentamicin, 0.01% Tween-20) from experimentally infected gerbils *Meriones unguiculatus* (WaterborneTM, INC). The logarithmic regression line derived from qPCR showed, that at a cycle threshold (Ct) at 37.6, the faecal sample contained less than one cyst per mg faeces (1.256ln (number of cysts)+37.546=thresholds, R2=0.995). This Ct value was set as the cut-off between *Giardia* positive and negative faecal samples. The infection intensity was defined as the median level of parasitic load in a lemur group for each zoo and reported as the median Ct value of the group with interquartile ranges. The infection intensity was compared among lemur groups in walk-through enclosures, traditional enclosures and the Madagascar locations. A Kruskal-Wallis test was applied using the software R 3.3.2 (R Core Team 2003) to identify statistically significant differences ($P<0.05$) of parasite infection intensity between samples.

Molecular characterisation of* Giardia *spp.

Giardia spp. were molecularly characterised via nested and semi-nested PCR targeting the triose-phosphate isomerase (TPI) gene and the glutamate dehydrogenase (GDH), respectively, as described in previous studies (Sulaiman et al. 2003; Read et al. 2004; Geurden et al. 2008; Levecke et al. 2009). After visualising the primary and secondary PCR products on 1.0–1.2% agarose gels stained with SYBR Safe (Invitrogen), the PCR products were sequenced using Sanger sequencing and Next Generation Sequencing (NGS). The PCR products for Sanger sequencing were purified using the MSB Spin PCRapace kit (Invitek GmbH, Berlin, Germany) according to the manufacturer's protocol. For a subset of the samples, paired-end DNA libraries were generated using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA) and the Nextera XT Index Kit (Illumina, San Diego, CA) according to the manufacturer's protocol and sequenced on an Illumina MiSeq sequencer.

Geneious Prime (Biomatters Ltd, Auckland, New Zealand) was used for extraction and removal of low quality reads (length <10 bp and/or Pred-score <20), trimming (using the BBduk-plugin), and mapped to reference using Bowtie2 (Langmead and Salzberg 2012) with a 65% similarity cut-off. GenBank reference sequence

Table 2. Genotyping of samples using the two loci TPI and GDH. NEG denotes negative amplification. NA: Not Available. Samples isolates are marked; Givskud Zoo: G, Zoo II: X, Planckendael Zoo: PL, Whipsnade Zoo: W, Copenhagen Zoo: CPH.

Location	Isolate	TPI	GDH	TPI Accession no	GDH Accession no
Givskud Zoo	G1	NEG	BIV	NA	MN616939
	G2	NEG	BIV	NA	MN616940
	G3	NEG	BIV	NA	MN63005
	G7	NEG	BIV	NA	MN63004
Zoo II	X6	NEG	BIII/BIV	NA	MN616928/MN616929
	X7	BIII	BIII/BIV	MN625239	MN616930/MN616931
	X9	BIII/BIV	BIII/BIV	MN625243	MN616937/MN616938
	X11	BIII/BIV	BIII/BIV	MN625240	MN616932/MN616933
	X16	BIII/BIV	BIII/BIV	MN625241	MN616934/MN616935
	X18	BIII	BIV	MN625242	MN616936
Planckendael Zoo	PL8	BIII	BIII/BIV	MN625244	MN623000/ MN622999
Whipsnade Zoo	W1	NEG	BIII/BIV	NA	MN622998/MN622997
Copenhagen Zoo	CPH2	BIII	BIII/BIV	MN625245	MN63003/ MN63002

Accessions L02120, U57879, DQ650648, AF06956, AF069560, AY228641, DQ246216, AY228645, AF069558, EU781013, M84604, AY178737, EU637582, AF069059, AY178738, U60983, U60986, AY178741, AF069057, AY178746 (Sprong et al. 2009). Samples with a consensus sequence with coverage greater than 100 reads were extracted and aligned to the reference library using ClustalW with default settings.

Results

All 16 participating zoos had *Giardia*-infected lemurs except Zoo IV (Table 1). The overall infection intensity as represented by the median Ct value of positive samples was 31.1 (IQR=27.1–34.5). Between enclosure designs, there was a significantly higher prevalence and intensity of *Giardia* infections in zoos with walk-through enclosures compared with traditional enclosures (Figure 1A). Of the 92 faecal samples collected from walk-through enclosures, 89% were positive with a median Ct value 28.6 (IQR=26.5–32.3) whereas only 65% from traditional enclosures were positive (median Ct value=35.2, IQR=33.3–37.8) (P>0.005, Figure 1B). Of the 35 samples collected from wild ring-tailed lemurs in Madagascar, 20% were positive (Table 1) but the overall infection intensity (median Ct value=37.7, IQR=37.5–38.7; Figure 1B) was significantly lower (P>0.01) than in zoo-held ring-tailed lemurs.

Samples from five zoos were successfully amplified for the GDH gene and three zoos for the TPI gene (Table 2). Some samples mapped to a single subtype and some to both recognised subtypes, BIII and BIV, of assemblage B. Sanger sequences always mapped to only one of the two subtypes for a given sample (Table 2).

Discussion

This study observed a high prevalence of *G. duodenalis* in ring-tailed lemurs in European zoos, a result consistent with findings from similar studies where most tested ring-tailed lemurs were found to be infected with *G. duodenalis* (Levecke et al. 2007; Beck et al. 2011; Berrilli et al. 2011; Martínez-Díaz et al. 2011). *Giardia* in ring-tailed lemurs have also been reported in American and Chinese zoos, suggesting that *Giardia* is common in ring-tailed lemurs in zoos across the globe (Villers et al. 2008; Karim et al. 2015).

Looking at the different enclosure designs in the zoos, ring-tailed lemurs held in walk-through enclosures were more frequently infected and carried a larger load than ring-tailed lemurs held in traditional enclosures (P>0.005). Walk-through enclosures are often larger than traditional enclosures, leaving cleaning more challenging. This situation may result in re-infection of treated animals. Although traditional enclosures are often smaller than walk-through enclosures with fewer square metres available per individual, this type of enclosure might allow for increased infection rate due to crowding (Daly et al. 2013). It is important to note, that these findings document a very large variation in infection intensity between samples from the same zoo and even within the enclosure design (Table 1). Factors that likely contribute to variation in infection prevalence and intensity include group density within enclosures, cleaning frequency, inadequate manure containment and disposal (Daly et al. 2013) but also stress levels and age and gender distribution across the lemur groups, which have been found to affect shedding of cysts in other species (Flanagan 1992; Charles-Smith et al. 2010). For instance, *Giardia* is significantly more prevalent in younger individuals in several other mammalian species, including humans (Flanagan 1992), and young males of the lemur species Coquerel's sifaka *Propithecus coquereli* are more susceptible to the epidemiologically similar protozoan, *Cryptosporidium* spp., than females (Charles-Smith et al. 2010).

Giardia has previously not been found in wild ring-tailed lemurs and other wild lemur species, such as greater bamboo lemurs *Prolemur simus*, brown mouse lemurs *Microcebus rufus*, and the white-fronted brown lemur *Eulemur fulvus albifrons* with the use of microscopical analysis (Junge et al. 2008; Villers et al. 2008; Rasambainarivo et al. 2013). The current study re-evaluated the presence/absence of *Giardia* in wild ring-tailed lemurs using molecular detection methods which are more sensitive than microscopy (Gotfred-Rasmussen et al. 2016). The

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TaqMan-based qPCR revealed 20% of the 35 Madagascar samples to be positive for *Giardia*. However, in all samples, except for one from Beza Mahafaly (Ct value=36.2), had detectable *Giardia*-specific DNA only at the limit of detection (median Ct value=37.7, IQR=37.5–38.7). This study shows tentatively that wild ring-tailed lemurs are positive for *Giardia*, however with an infection intensity significantly lower than zoo-held ring-tailed lemurs. It is recommended that wild lemurs should continuously be screened for *Giardia* using qPCR rather than microscopy considering that *Giardia* is found in humans in Madagascar (Randremanana et al. 2012) posing a potential risk of a spill-over to the wildlife.

PCR amplification and genotyping were only possible in 14 samples from five out of the 15 zoos that were tested *Giardia* positive according to qPCR assay. This partial low genotyping success is a recurrent challenge, where previous studies report a 0–70% successful genotyping of qPCR-amplified samples and mixed results being dependent on the markers used (Pelayo et al. 2008; Beck et al. 2011; Berrilli et al. 2011; Karim et al. 2015). This highlights the need for improved genotyping practices; specifically finding genes that amplify better and using NGS and whole-genome sequencing approaches. The use of NGS in this study enabled the identification of both sub-assemblage BIII and BIV in most successfully amplified samples, suggesting mixed infections. Identifying assemblage B is consistent with previous findings that report assemblage B as the dominating strain in non-human primates (Feng and Xiao 2011; Ryan and Cacciò 2013; Karim et al. 2015). The zoonotic potential of assemblage B is still being discussed, but it is agreed upon that assemblage B (and A) carry the highest zoonotic risk (Flanagan 1992; Lalle et al. 2005; Savioli et al. 2006; Xiao and Fayer 2008; Sprong et al. 2009; Plutzer et al. 2010; Thompson and Ash 2016; Brynildsrud et al. 2018).

The lemurs held in European zoos appear not to be clinically affected by *Giardia* infections, and asymptomatic carriage has also been reported in other studies (Villers et al. 2008; Berrilli et al. 2011). The primary focus should be, therefore, to prevent transmission of this zoonotic assemblage to other zoo-held species and humans where numerous incidences of severe diarrhoea have been documented (Flanagan 1992; Hamlen and Lawrence 1994). Some studies also report asymptomatic carriers among other zoo-held species but most studies fail to report whether the samples collected were from individuals with or without diarrhoea. Nevertheless, it is important not to neglect the not fully understood subclinical effects of *Giardia* infections such as fail-to-thrive and malabsorption, especially in young individuals (Flanagan 1992). Efficient hygiene and awareness have been the primary management strategies because complete eradication of *Giardia* in ring-tailed lemurs have failed (Copenhagen Zoo, unpublished). Hygienic measures include the use of disinfectants, disposable shoe covers and gloves, while awareness has centred around knowing the risks of transfer through day-to-day tasks to other locations within zoos (Daly et al. 2013). Guests should therefore also be advised not to consume anything while in a walk-through enclosure and wash hands when leaving the enclosure as a precaution. For future zoo management, it is important to recognise that ring-tailed lemurs can carry *Giardia*. Thus, ring-tailed lemurs should always be screened upon arrival since it is difficult to exterminate for *Giardia* later once a group or the environment in the enclosure is contaminated.

Data accessibility

Sanger sequences are deposited in GenBank (accession numbers MN625239-MN625246). NGS data has been submitted to the Genbank archive at NCBI (accession numbers MN616929-MN616940 and MN622997-MN623005)

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