

USE OF PARASITOLOGICAL AND MOLECULAR METHODS IN *GIARDIA* SP. DETECTION IN ANIMALS HELD IN CAPTIVITY

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Protozoa of the genus *Giardia* cause the disease giardiasis in a large number of animal species, but also in humans. This disease can be either subclinical or followed by diarrhea, malabsorption, weight loss and stunted growth. The aim of this study was to examine the presence of *Giardia* sp. in different species of animals in Belgrade Zoo (asymptomatic infection) and to compare the sensitivity of parasitological and molecular-genetic methods. Examination of the presence of *Giardia* sp. protozoa was performed in 52 fecal samples, using fecal flotation and molecular-genetic PCR method. No positive samples were detected when deploying the parasitological method, while PRC analysis revealed the presence of *Giardia* sp. in one animal - ring-tailed lemur (*Lemur catta*), which indicates the advantage of the molecular-genetic method over the parasitological one. The absence of *Giardia* sp. in other samples can be explained by adequate housing conditions, successful implementation of hygienic and sanitary measures, as well as good veterinary practice. Use of PCR method for testing the presence of *Giardia* sp. showed a higher sensitivity when compared to the fecal flotation method.

Keywords: Flotation, *Giardia*, PCR, Zoo animals

INTRODUCTION

Parasites of the genus *Giardia* commonly cause diarrhea in humans, as well as in domestic and wild animals. Probably the most common intestinal protozoa infecting humans, *Giardia* sp. also appears to be common in some zoo-housed primates [1]. The clinical presentation in domestic animals infected by *Giardia* sp. is most often characterized by diarrhea, stunted growth, weight loss, decreased reproductive capacity

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and even death, which can consequently lead to significant economic losses [2,3]. In humans, these parasites can cause persistent diarrhea that can last for 3 weeks or longer [4]. *Giardia* sp. is a parasitic flagellate which morphologically has a bilaterally symmetrical body with eight flagella. They have an adhesive disk on the ventral surface of the body which enables them to attach to the epithelial cells of the intestinal mucosa. Trophozoites, vegetative forms of *Giardia* sp. multiply in the small intestine where they cause diarrhea and malabsorption, the main clinical signs of giardiasis. Cysts are responsible for the transmission of the infection. They are formed and released from the gastrointestinal system of the host, after which a new susceptible individual can be infected. Healthy animals can be infected through contaminated water, food, or direct fecal-oral route [5]. There are several pathways for the introduction of *Giardia* sp. in zoos, which include: animal food, newly acquired infected animals, staff and visitors [6]. Among the eight species within the genus *Giardia*, the most important in the field of veterinary medicine is *Giardia duodenalis*. There are eight assemblages (A – H) of this species that do not differ morphologically but show more or less host specificity [7]. Zoonotic assemblages A and B do not show host specificity and can be found in humans, as well as in a wide range of animals. Remaining assemblages (C-H) show certain host specificity: assemblages C and D are mainly found in canids, assemblages E in equines and ruminants, assemblages F in felines, assemblages G in rodents, and assemblages H in marine mammals [7]. Various studies examined the occurrence of *Giardia* sp. in animals kept in captivity [1,4,8,9], using different diagnostic methods, while centrifugal fecal flotation (using saturated aqueous zinc sulphate solution) is one of the most used methods to detect *Giardia* cysts in fecal samples. However, the disadvantage of this method can be found in the success rate of cysts detection, being 90% after consecutive examination of at least three fecal samples. This method lacks liability in differentiating species of the genus *Giardia* [10]. On the contrary, molecular genetic methods such as those based on polymerase chain reaction (PCR) are undoubtedly more reliable and can detect not only the presence or absence of parasites' DNA in the investigated sample, but can also determine the species within the genus *Giardia* [11].

Currently, PCR-based methods are generally used in the detection of *Giardia* parasites in humans and animals. The use of these methods has affected the sensitivity of determination of *Giardia* sp. [12].

Over the years, there has been an increase in the number of visitors in the zoos, which prefer interaction (direct contact) with animals. As a result, the risk of zoonosis transmission has also grown. From a public health point of view, these protozoa have a high zoonotic potential, being among the most common intestinal parasites in humans worldwide. Thus, the importance of wild animals kept in captivity must be taken into consideration since the increasing number of infectious diseases of animals poses a great health risk for humans. For this reason, research on animal diseases is one of the most important factors in maintaining human health [13].

Giardia sp. has been detected in zoos across Europe [1,8,14,15]. There are reports of their high prevalence in wild ruminants kept in captivity which can serve as reservoirs for zoonosis transmission [16]. *Giardia* sp. was detected in the Zagreb Zoo in Artiodactyla, Carnivora, Hyracoidea, Perisodactyla, Primates and Rodentia, [8], while in two zoos in Spain (Madrid and Valencia) their presence was confirmed in 16 different species of primates [14].

Having in mind the zoonotic potential of *Giardia* sp. and the fact that animals often do not show clinical signs, as well as the limitations of parasitological methods in *Giardia* sp. detection, the aim of this study was to investigate the occurrence of *Giardia* sp. in various mammal species in Belgrade Zoo, using centrifugal fecal flotation and PCR methods and to compare the sensitivity rate of those methods.

MATERIAL AND METHODS

Fecal samples of 52 animals belonging to 19 species were collected in Belgrade Zoo (Table 1). All animals were clinically healthy. Samples were collected in sterile plastic bags during morning hours and were transported to the Laboratory for Animal Genetics, Department of Biology and Laboratory for parasitology, Department of Parasitology, Faculty of Veterinary Medicine, University of Belgrade, where they were stored at 4°C until further processing. Positive control was obtained from the laboratory of the Department for Parasitology in the same institution.

Table 1. The number of samples and the taxonomic position of sampled animals

Order	Family	Species	Number of animals whose feces was sampled
Sphenisciformes	Spheniscidae	Humboldt penguin (<i>Spheniscus humboldti</i>)	2
Diprotodontia	Macropodidae	Parma wallaby (<i>Macropus parma</i>)	10
		Red kangaroo (<i>Macropus rufus</i>)	5
Primates	Hominidae	Bornean orangutan (<i>Pongo pygmaeus</i>)	1
	Callitrichidae	Silvery marmoset (<i>Mico argentatus</i>)	1
	Hominidae	Chimpanzee (<i>Pan troglodytes</i>)	2
	Lemuridae	Ring-tailed lemur (<i>Lemur catta</i>)	1
	Lemuridae	Common brown lemur (<i>Eulemur fulvus</i>)	1
Rodentia	Caviidae	Patagonian mara (<i>Dolichotis patagonum</i>)	2
		Capybara (<i>Hydrochoerus hydrochaeris</i>)	2
Cingulata	Chamyoheridae	Southern three-banded armadillo (<i>Tolypentes matacus</i>)	1
Artiodactyla	Bovidae	Domestic yak (<i>Bos grunniens</i>)	5
		American bison (<i>Bison bison</i>)	4
	Camelidae	Bactrian camel (<i>Camelus bactrianus</i>)	2
	Giraffidae	Giraffe (<i>Giraffa camelopardalis</i>)	1
Perisodactyla	Equidae	Zebra (<i>Equus zebra</i>)	4
	Felidae	Cheetah (<i>Acinonyx jubatus</i>)	2
Carnivora	Canidae	Arctic wolf (<i>Canis lupus arctos</i>)	4
		Fennec fox (<i>Vulpes zerda</i>)	2

Centrifugal fecal flotation with zinc sulphate solution

Fecal samples were analyzed by using centrifugal fecal flotation method with saturated aqueous zinc sulfate solution ($ZnSO_4$, specific gravity 1.18 at $20^\circ C$), following the procedure described by Bogunović et al. [17]. Each fecal sample was examined in duplicate by two experienced specialists under light microscope (Olympus CX 23, Japan) using $100\times$ and $400\times$ magnifications.

DNA extraction

The extraction was performed using a Quick-DNA™ Faecal/Soil Microbe Miniprep kit (Zymo Research, USA). From each collected sample, 200 mg of feces was added to ZR BashingBead™ Lysis Tube and mixed with 800 μl of Lysis solution. The Lysis Tube was placed in TissueLyser II (Qiagen, USA) and processed at maximum speed for 5 minutes. The Lysis Tube was then centrifuged at $10000 \times g$ for 1 minute. Acquired supernatant in the volume of 400 μl was transferred to a Zymo-Spin™ IV Spin Filter placed in a collection tube and centrifuged at $7000 \times g$ for 1 minute. Further on, 1200 μl of Faecal DNA Binding Buffer was added to the filtrate in the collection tube. The mixture acquired in the previous step in the volume of 800 μl was transferred to a Zymo-Spin™ IIC Column, which was placed in a collection tube and centrifuged at $10000 \times g$ for 1 minute. The flow-through was discarded and the step was repeated. Zymo-Spin™ IIC Column was placed in a new collection tube, 200 μl of DNA Pre-Wash Buffer was added in the column and centrifuged at $10000 \times g$ for 1 minute, while the flow-through was discarded. Further on, 500 μl of Faecal DNA Wash Buffer was added to the Zymo-Spin™ IIC Column and the flow through was discarded. The column was transferred to a sterile 1.5 ml tube and 100 μl of DNA Elution Buffer was added directly to the column matrix and centrifuged for 30 seconds at $10000 \times g$ in order to elute the DNA. In order to purify the DNA sample, a Zymo-Spin™ IV-HRC Spin Filter was placed in a sterile 1.5 ml tube and the eluted DNA from the previous step was added inside and centrifuged at $80000 \times g$ for 1 minute. The quality and quantity of extracted DNA were determined using BioSpec-nano Micro-volume UV-Vis Spectrophotometer (Shimadzu, Japan).

PCR amplification

In order to amplify a 292-base pair (bp) long *ssrRNA* fragment, the following primers were used: RH11: 5'-CATCCGGTCGATC-CTGCC-3' and RH4: 5'-AGTCGAAC-CCTGATTCCTCCGCCAGG-3' [18]. The PCR mixture was prepared using a commercial set of KAPA2G Robust HotStart ReadyMix PCR mixture (Kapa Biosystems, USA) according to the manufacturer's instructions. PCR was performed in a final volume of 25 μl containing: 10 μl KAPA2G Robust HotStart ReadyMix, 1.25 μl of each primer, 10 ng of a DNA sample and 2.5 μl of ddH₂O. PCR amplification was performed in a T100 Thermal Cycler device (Bio-Rad, USA), while the PCR parameters were as follows: activation at $95^\circ C$ for 5 minutes, followed by 40 cycles of denaturation at $95^\circ C$ for 30 seconds, primer annealing at $65^\circ C$ for 30 seconds, extension at $72^\circ C$ for 60 seconds and final extension at $72^\circ C$ for 7 minutes.

Visualization of the PCR products

The amplification products (5 μ l) were mixed with 1 μ l 6X DNA Loading Dye (Thermo Scientific, USA), subjected to electrophoresis in 2% agarose gel, stained with ethidium bromide, visualized under UV light, examined for different band patterns and documented.

RESULTS

When the centrifugal fecal flotation method was deployed, no *Giardia* cysts were found in any of the analyzed samples (Table 2). Unlike this classical parasitological procedure, molecular analysis using PCR has shown different results. Namely, PCR analysis revealed one positive sample. An electrophoretic band of 292 bp shown in Figure 1 indicates a PCR product amplified from the fecal sample no. 5, originating from ring-tailed lemur (*L. catta*).

Table 2. Findings of endoparasites in the investigated fecal samples

Species \ Endoparasite	Three-banded armadillo <i>n</i>	Red kangaroo <i>n</i>	Parma wallaby <i>n</i>	Arctic wolf <i>n</i>	Ring-tailed lemur <i>n</i>
G	0	0	0	0	1*
S1	1	4	0	0	0
O	1	0	0	0	0
C	0	4	1	0	0
S2	0	0	0	1	0

n – number of positive samples; G – *Giardia* sp; S¹ – strongylid eggs; O – oxyurid eggs; C – coccidian oocysts; S² - *Sarcocystis* spp. oocysts; *positive result obtained using PCR

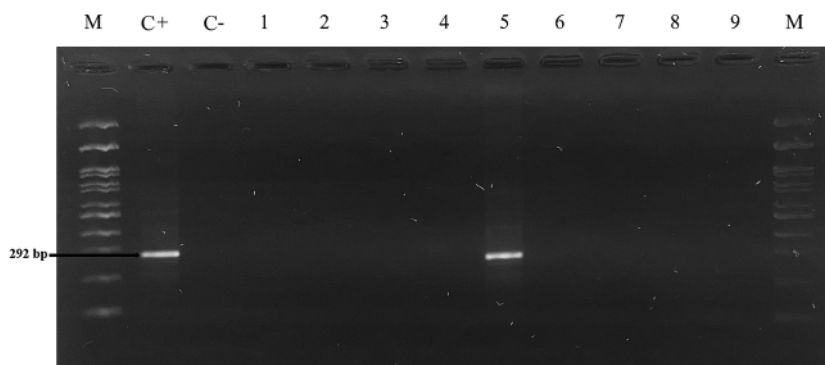


Figure 1. Visual presentation of PCR products on agarose gel after electrophoresis. M-DNA marker (100 bp), C+ positive control; C- negative control; 1-9 - tested samples: 1) Parma wallaby (*Macropus parma*); 2) Red kangaroo (*Macropus rufus*); 3) Bornean orangutan (*Pongo pygmaeus*); 4) Silvery marmoset (*Mico argentatus*); 5) Ring-tailed lemur (*Lemur catta*); 6) Chimpanzee (*Pan troglodytes*); 7) Southern three-banded armadillo (*Tolypeutes matacus*); 8) Domestic yak (*Bos grunniens*); 9) American bison (*Bison bison*).

Further on, centrifugal fecal flotation revealed other parasitic forms as a side finding in seven out of 52 inspected fecal samples. These were identified as: trichostrongyle and oxyurid eggs in a southern three-banded armadillo (*Tolypeutes matacus*); trichostrongyle eggs and coccidian oocysts in four red kangaroos (*Macropus rufus*); coccidian oocysts in one parma wallaby (*Macropus parma*); *Sarcocystis* spp. oocysts in one arctic wolf (*Canis lupus arctos*) (Table 2, Figure 2).



Figure 2. Visual presentation of various parasitic forms revealed with centrifugal faecal flotation method. **A**-trichostrongyle eggs; **B**-coccidian oocysts; **C**- *Sarcocystis* spp. oocysts.

DISCUSSION

The absence of *Giardia* sp. DNA in most of analyzed animals can be explained by good hygiene measures, as well as good practice of animal health control applied by the staff in the Belgrade Zoo. Additionally, the absence of mutual contact between individuals of different mammal species and communication of animals with humans (excluding workers that are in direct or indirect contact during cleaning, feeding and other technical work) are also factors that explain the low prevalence of infection. As previously described, infection occurs after the consumption of contaminated water/food or by the fecal-oral route. The finding of *Giardia* sp. in only one sample, which originated from the ring-tailed lemur, indicates that animals, although housed close to each other, have not come into contact with contaminated water or food, suggesting very successful hygienic and sanitary measures and good veterinary practice implemented by the employees of the Belgrade Zoo. Furthermore, we should have in mind that in our study samples were collected only once from each animal, which can affect the acquired results, having in mind that *Giardia* sp. is excreted intermittently in the feces. In the study conducted by Chagas et al. [19] in São Paulo Zoo (Brazil), the prevalence of *Giardia* sp. was evaluated using passive flotation and was 1.5% while it was highest (2%) within the class of Mammals. This finding is in accordance with our results, in which the prevalence was 3.1%. Research conducted by Matsubayashi et al. [4] in Osaka Zoo (Japan), similarly to our results, showed a low prevalence of *Giardia* sp. Namely, out of 284 examined samples, *Giardia* sp. was detected only in one mandarin duck (*Aix galericulata*) and two ruddy shelducks (*Tadorna ferruginea*). The authors suggest that the low prevalence may be due to the good quarantine system conducted by veterinary surgeons in the Zoo. Beck et al. [8] conducted an investigation on 131

asymptomatic animals in Zagreb Zoo (Croatia) and revealed 38 positive findings. The highest *Giardia* sp. prevalence among the investigated animals in the research of Beck *et al.* [8] was found within Artiodactyla, while in our research there were no positive samples within this order. Further on, in the same study high prevalence of *Giardia* sp. was found in animals belonging to orders of Carnivora, Primates and Rodentia, while in our research there was only 1 positive sample originating from a primate.

Fomsgaard *et al.* [1] conducted a comparative study of the prevalence and intensity of *G. duodenalis* infection in samples of ring-tailed lemurs from five zoos in Denmark and those living freely in nature. Quantitative PCR results revealed that the prevalence (88.6%) and the degree of infection (median Ct value 31.1) in lemurs from zoos that have an area for contact with visitors (without a fence) is significantly ($P>0.01$) higher than in lemurs living freely in nature (prevalence 20.0%, median Ct value 37.7). Within the same study, a comparison was made between different types of accommodation spaces within zoos. It was determined that both the prevalence (89.0%) and the degree of *G. duodenalis* infection (median Ct value 28.6) in zoo accommodation spaces that have an area for contact with visitors (without fence) were statistically significantly ($P>0.005$) higher than in those with traditional accommodation (cages), in which the prevalence of 65% was determined, while the intensity of infection expressed over a median Ct value was 35.2 [1]. Analysis of the results of previously mentioned studies, as well as the results of our study indicate that low occurrence of *Giardia* sp. is mainly due to the absence of contact of animals with visitors, along with good hygiene and veterinary practice applied by the Belgrade Zoo. In our study, no positive samples were revealed when the centrifugal fecal flotation method was deployed for the detection of *Giardia* sp. cysts, while one positive sample was detected by the PCR method, which indicates a higher sensitivity of molecular genetics over fecal flotation method. On the contrary, in a study conducted by Solarczyk and Majewska [15], who analyzed 242 samples originating from 131 species from Poznan Zoo (Poland), parasitological analysis of feces revealed the presence of *Giardia* sp. cysts in five samples, while PCR detected only one positive sample originating from anteater (*Tamandua tetradactyla*). The low sensitivity of PCR in the study of Solarczyk and Majewska [15] can be explained by inadequate DNA extraction method and primers that amplify long fragments of DNA, {*i.e.*, 753 bp [20]; 530 bp [21]; 430 bp [22]}. Unlike Solarczyk and Majewska [15] we used Quick-DNATM Faecal/Soil Microbe Miniprep set (Zymo research, USA) for DNA extraction and primers designed by Hopkins *et al.* [18] that provide a relatively short amplification product (292 bp). Side finding of trichostrongyle eggs and coccidian oocysts in red kangaroos in our study is not surprising, having in mind they are commonly found parasites in marsupials [23,24]. *Sarcocystis* spp. oocysts found in one arctic wolf are probably caused by infection through raw feed diet which occurs in captivity. Having in mind that the feces was collected from a three-banded armadillo which has just arrived in the Belgrade Zoo and was under anthelmintic therapy, the finding of oxyurid eggs, a common parasite of this animals [25], does not surprise.

CONCLUSIONS

Our study aims to contribute to the prevention of giardiasis and other parasitic diseases that poses a risk to public health. A low level of infection with *Giardia* sp. and other parasitic forms important for animal and human health were determined in the Belgrade Zoo, probably due to the implementation of hygiene measures, as well as good practice of animal health control along with the absence of contact of animals with visitors. Furthermore, PCR method exerted higher sensitivity in detection of *Giardia* sp. over classical parasitological methods.

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Authors' contributions

MR and JÖ designed the study and drafted the manuscript. JV, ND and JÖ were responsible for the sampling. MR, JV, ND and MV performed laboratory molecular analyses. MŽR and DB performed centrifugal fecal flotation. MR, JÖ, MV and DB have contributed to the interpretation of data, revision of the manuscript and supervision of the experiment, as well.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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PRIMENA PARAZITOLOŠKIH I MOLEKULARNIH METODA U DETEKCIJI *GIARDIA* SP. KOD ŽIVOTINJA DRŽANIH U ZATOČENIŠTVU

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Protozoe iz roda *Giardia* uzrokuju đardijazu kod velikog broja životinjskih vrsta, ali i ljudi. Klinička slika može biti asimptomatska ili se mogu javiti dijareja, malapsorpcija, gubitak težine i usporen rast. Cilj rada bio je ispitivanje prisustva *Giardia* sp. kod životinja iz Zoološkog vrta grada Beograda (Beo Zoo Vrt), koje nisu ispoljavale kliničke znakove, uz poređenje osetljivosti parazitoloških i molekularno-genetičkih metoda. Ispitivanje prisustva protozoa izvedeno je u 52 uzorka fecesa prikupljenom od različitih vrsta životinja u Beo Zoo Vrtu, upotrebom parazitoloških metoda tj. flotacije sa mikroskopiranjem i molekularno-genetičke PCR metode. Parazitološkim metodama nije ustanovljen nijedan pozitivan nalaz, dok je PCR analizom ustanovljeno prisustvo protozoa *Giardia* sp. kod prstenorepog lemura (*Lemur catta*). Odsustvo protozoa kod ostalih uzorkovanih životinja može se objasniti kontrolisanim uslovima u smeštajnim prostorima, uspešno sprovedenim higijensko-sanitarnim merama i primenom dobre veterinarske prakse. PCR analiza je pokazala višu senzitivnost u otkrivanju *Giardia* sp. u odnosu na parazitološku metodu flotacije.