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Cryptosporidium spp. *infection in captive mammals from the Lisbon Zoo*

Cryptosporidium spp. em mamíferos em cativeiro do Zoo de Lisboa

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Resumo

As espécies *Cryptosporidium* estão documentadas em diferentes hospedeiros e são a causa de doença gastrointestinal em muitas espécies de mamíferos, tanto em cativeiro como na natureza. Para conhecer a epidemiologia deste protozoário parasita e identificar potenciais hospedeiros reservatórios, muitos estudos foram realizados em vários grupos de animais, mas apenas um limitado número de estudos incluiu animais de jardim zoológico. Este estudo pretendeu identificar mamíferos de diferentes famílias no Jardim Zoológico de Lisboa, como hospedeiros de *Cryptosporidium* spp.. Foram recolhidas 107 amostras de 66 espécies pertencentes a 20 famílias de 7 ordens taxonómicas diferentes. A deteção de oocistos de *Cryptosporidium* spp. foi realizada utilizando um método de sedimentação modificado seguido de coloração Ziehl-Neelsen e PCR em tempo real para o gene 18S rDNA. Os oocistos foram detetados em 28 amostras de 22 espécies de mamíferos, incluindo 13 espécies onde oocistos de *Cryptosporidium* spp. foram detetada pela primeira vez. No entanto, devido ao baixo número de oocistos em todas as amostras positivas, a identificação molecular das espécies não foi possível utilizando os métodos disponíveis. As espécies de animais em cativeiro mantidas em parques zoológicos encontram-se numa proximidade artificial que os expõe à contaminação cruzada por *Cryptosporidium* spp.. Testes regulares para a identificação deste e de outros protozoários intestinais devem ser realizados para prevenir doenças clínicas em animais suscetíveis.

Palavras-chave: *Cryptosporidium* spp., parque zoológico, mamíferos em cativeiro.

Summary

Cryptosporidium species are recognized as infectious to different hosts and the cause of gastrointestinal illness in vertebrates, such as mammals, both in captivity and in the wild. To explore the epidemiology of this protozoan parasite and to identify potential reservoirs, many studies have been performed in several animal groups but only few studies included zoo animals. Because only old data were available about the occurrence of *Cryptosporidium* spp. in Lisbon Zoo, this study was conducted to survey animals from different mammal families. A total of 107 samples were collected from 66 species belonging to 20 mammal families from 7 different taxonomic orders. Detection of *Cryptosporidium* spp. oocysts was performed using a modified diethyl ether sedimentation method followed by Ziehl-Neelsen staining and a Real-Time PCR targeting the 18S rDNA gene. Oocysts were detected in 28 samples from 22 mammal species, including 13 species where *Cryptosporidium* spp. were detected for the first time. However, due to the low number of oocysts in all positive samples, the species identification was not possible using the available methods. Captive animals species kept in zoological parks are in an artificial proximity that exposes them to cross contamination by *Cryptosporidium* spp.. Regular testing of this and other intestinal protozoan should be performed to prevent clinical disease in susceptible animals.

Keywords: Cryptosporidium spp., Zoological parks, captive mammals.

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1. Introduction

Cryptosporidium spp. Tyzzer, 1910 are protozoan parasites that infect a wide variety of vertebrate hosts, not only domestic animals but also zoo animals and wildlife. Some species of this protozoa are of worldwide concern because they represent a major public health problem due to gastrointestinal disease in humans. Currently, more than 18 species of *Cryptosporidium* are recognized in mammals and over 150 species belonging to 12 mammalian orders have been reported as hosts (Ryan et al. 2014). Different studies have been performed to detect *Cryptosporidium* spp. in captive animals with the parasite being detected in several zoos (Gomez et al. 2000; Gracenea et al. 2002; Delgado et al. 2003; Osman et al. 2017) but further work is necessary to identify potential reservoirs of cryptosporidiosis, including those responsible for human infections. The Lisbon Zoo is located in Lisbon, the capital city of Portugal, housing approximately 2000 animals belonging to more than 300 species, of which 114 are mammals. A previous study identified *C. parvum* in different species (Delgado et al. 2003 and 2005). The current study aims to identify the species of mammals infected with *Cryptosporidium* species using parasitological and molecular methods and consequently to get a better understanding of the possible reservoir hosts of cryptosporidiosis in this zoo.

2. Material and Methods

A total of 107 faecal samples from 66 animal species belonging to 20 families of Mammals from 7 different taxonomic orders were collected from the soil, after defecation, to sterile plastic containers between October of 2016 and April of 2017 (Table 1). Seventy-three samples were from individual animals and 34 were from animals of the same species within a common area (pool samples). The samples were stored at 4ºC until processing. The study was conducted under the supervision of the zoo veterinarians and since only faecal samples were collected from the facilities, approval from the Ethics Committee was not required.

Faecal samples were tested by a modified diethyl ether sedimentation method (Richie, 1948). Briefly, approximately 3 g of faeces were dissolved in tap water and filtered through gauze to a 15 mL centrifuge tube. Two milliliters of filtered feces were collected to a new 15 mL tube, 6 ml of water were added and finally 4 mL of diethyl ether were also added. The tubes were vortexed and then centrifuged at 1500 rpm for 5 min. Upper layers, with dissolved fat and debris were discarded and the supernatant was collected to a new tube. Water was added until the volume reached 12 mL and a new centrifugation was done at 4500 rpm for 10 min. The supernatant was discarded and the pellet was used to prepare faecal smears to be stained by a modified Ziehl-Neelsen method (Casemore et al., 1991). The stained faecal smear slides were examined by light microscopy using the 40x and 100x objectives (Olympus BX 40). Part of the pellet was preserved in 2.5% potassium dichromate and kept at 4ºC for molecular studies.

For the molecular studies, the samples preserved with potassium dichromate were washed three times twice with PBS and centrifuged at 12000 rpm for 10 min. After the third centrifugation, 200 □l of PBS was added to the pellet for nucleic acid extraction. The DNA extraction process started with 5 freeze-thaw cycles of 30 minutes each (- 80ºC and 56ºC). For DNA purification process the Qiagen DNA mini Kit (Qiagen, Germany) was used according to the protocol 'Isolation of DNA from Stool for Pathogen Detection'.

A Real-Time PCR targeting the 18S rRNA was performed in all samples according to the protocol previously described by Hill et al. (2007), with minor modifications. Briefly, each 25 µl reaction contained 12.5 µL of TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific, EUA), 20 pmol of primer forward (5'-ATGACGGGTAACGGGGAAT-3') and 20 pmol of primer reverse (5'-CCAATTACAAAACCAAAAAGTCC-3'), 5 pmol of probe (5'-FAM-CGCGCCTGCTGCCTTCCTTAGATG–3'-BHQ), 7.05 µL of water and 5 µL of DNA sample. A positive *C. parvum* DNA and a negative control (dH_2O) were included on each run. Real-time PCR was performed in a 7300 Real-Time PCR thermocycler (Thermo Fisher Scientific, USA) with the following conditions: 95 ºC for 15 min, followed by 45 cycles at 94 ºC for 10 seconds, 55 ºC for 30 seconds and 72°C for 20 seconds and a final extension at 72 ºC for 7 min. For *Cryptosporidium* differentiation to species level, positive samples at real-time PCR were submitted to a nested PCR followed by RFLP according to Xiao *et al*. (2001).

Table 1. Number of mammal species from different taxonomic orders and families sampled at the Lisbon Zoo, with the number of collected individual and pool samples.

Order	Family	Number of species	Individual samples (N)	Pool samples (N)
Artiodactyla	Bovidae	15	8	21
	Camelidae	2	1	2
	Cervidae	2	1	3
	Giraffidae	2	3	3
	Hippopotamidae	1	1	0
Primates	Atelidae	2	2	0
	Callitrichidae	9	9	0
	Cebidae	2	2	0
	Cercopithecidae	8	8	0
	Hominidae	3	8	2
	Hylobatidae	2	2	0
	Lemuridae	5	7	0
Carnivora	Ailuridae	1	1	0
	Felidae	6	9	0
	Procyonidae	1	1	0
	Ursidae	1	0	3
Diprotodontia	Phascolarctidae	1	1	0
Perissodactyla	Equidae	1	1	0
Xenarthra	Myrmecophagidae	1	2	0
Proboscidae	Elephantidae	1	6	0
Total		66	73	34

3. Results

A total of 28 samples (of which 14 were collected from individual animals) were positive for *Cryptosporidium* oocysts. The results distributed by families are shown in Table 2. Using the sedimentation technique and Zielh Neelsen staining, 27 samples were tested positive. Order Artiodactyla has 18 samples positive, 14 detected in pooled samples and 4 in individual animals. Order Primates had 6 positive samples, all from individual animals. One sample from each of the Carnivora, Perissodactyla and Proboscidea orders were also positive with this technique. Using real-time PCR, eight samples tested positive. Seven animals were simultaneously positive with both techniques and one animal tested positive only with real-time PCR.

Table 2. Species and number of samples positive to *Cryptosporidium* spp.

Family	Species	Individual	Pool
Bovidae	Black-faced Impala (*Aepyceros melampus petersi* Bocage, 1879)	0	1
	Roan Antelope (*Hippotragus equinus* Desmarest, 1804)	0	1
	Southern Sable Antelope (*Hippotragus niger niger* Harris, 1838)	0	2
	Scimitar-horned Oryx (*Oryx dammah* Cretzschmar, 1827)	1	0
	Arabian Oryx (*Oryx leucoryx* Pallas, 1777)	1	1
	African Buffalo (*Syncerus caffer caffer* Sparrman, 1779)	0	1
	African forest buffalo (*Syncerus caffer nanus* Boddaert, 1785)	0	1
	Nyala (*Tragelaphus angasii* Gray, 1849)	0	1
	Common Eland (*Tragelaphus oryx* Pallas, 1766)	0	2
	Sitatunga (*Tragelaphus spekii* Sclater, 1863)	0	1
Camelidae	Bactrian Camel (*Camelus ferus* Przewalski, 1878)	0	1
	Dromedary (*Camelus dromedarius* Linnaeus, 1758)	1	0
Giraffidae	Angolan giraffe (*Giraffa camelopardalis angolensis* Lydekker, 1903)	0	2
	Okapi (*Okapia johnstoni* Sclater, 1901)	1	0
Cercopithecidae	Javan Lutung (*Trachypithecus auratus* Geoffroy, 1812)	1	0
Hominidae	Chimpanzee (*Pan troglodytes* Blumenbach, 1775)	3	0
Lemuridae	White-fronted Lemur (*Eulemur albifrons* Geoffroy, 1796)	1	0
	Black-and-white Ruffed Lemur (*Varecia variegata* Kerr, 1792)	1	0
Felidae	Persian Leopard (*Panthera pardus saxicolor* Pocock, 1927)	1	0
	Eurasian Lynx (*Lynx lynx* Linnaeus, 1758)	1	0
Equidae	Grevy's Zebra (*Equus grevyi* Oustalet, 1882)	1	0
Elephantidae	African Elephant (*Loxodonta africana* Blumenbach, 1797)	1	0
Total		14	14

4. Discussion and Conclusion

Cryptosporidium spp. has been detected in a wide variety of animals. In this study, several mammal species belong to the following families were detected as infected: Artiodactyla, Camelidae, Giraffidae, Cercopithecidae, Hominidae, Lemuridae, Felidae, Equidae and Elephantidae. Most of those positive samples had low numbers of oocysts and were not identified as positive using the real-time PCR possibly due to loss of oocysts during DNA extraction.

Artiodactyla included the higher number of animals infected with *Cryptosporidium* spp.. From family Bovidae, the *Cape buffalo* (*Syncerus caffer caffer* Sparrman, 1779) with one positive in three pool samples was previously identified as a host not only in the wild but also in captivity in zoos (Gómez et al. 1996; Mtambo et al. 1997). African forest buffalo (*Syncerus caffer nanus* Boddaert, 1785) with also one positive pool sample was previously detected as a wild host in Rwanda (Hogan et al. 2014). One positive Scimitar-horned oryx (*Oryx dammah* Cretzschmar, 1827) was found but it had been previously detected in this species in an animal with diarrhoea (Van Winkle 1985). *Cryptosporidium* spp. positive pool samples were detected in Common eland (*Tragelaphus oryx* Pallas, 1766), Nyala (*Tragelaphus angasii* Gray, 1849) and Southern sable antilope (*Hippotragus niger niger* Harris, 1838). Those species had also been detected infected in Belgian and the Czech Republic respectively (Heuschele et al. 1986; Geurden et al. 2009; Ryan et al. 2003), with *C. ubiquitum* being identified in the captive Nyala. In two species, Sitatunga (*Tragelaphus spekii* Sclater, 1863) and the Roan antilope (*Hippotragus equinus* Desmarest, 1804) oocysts were detected in the present study but no references were found on the occurrence in wild captive animals from those animals. Considering the molecular approaches, two species were confirmed infected by real-time PCR, namely the Black-faced Impala (*Aepyceros melampus petersi* Bocage, 1879), with only one pool collected, tested and found positive. The regular Impala was already tested as positive, from the Kruger National Park, South Africa (Abu Samra et al. 2011) and further studies identified the species as *C. ubiquitum* Fayer et al. 2010 (Abu Samra et al. 2013). In a pool sample from the Arabian Oryx (*Oryx leucoryx* Pallas, 1777) oocysts were detected and with a second test it was possible to confirm the infection in one specific animal. This animal species was previously detected as infected in 2005 at Lisbon Zoo (Alves et al. 2005). Also, an earlier study on gastrointestinal parasites in Arabian Oryx showed positive animals for *Cryptosporidium* spp. (Mohammed et al. 2012). Other species previously detected at the Lisbon Zoo in 2003-2005 and not detected as infected in this study were: Black wildebeest (*Connochaetes gnou* Zimmermann, 1780), Blesbok (*Damaliscus pygargus phillipsi* Harper, 1939), Kudo (*Tragelaphus strepsiceros strepsicerus* Pallas, 1766), Barbary sheep (*Ammotragus lervia* Pallas, 1777) and South African oryx (*Oryx gazella* Linnaeus, 1758) and Eld's deer (*Panolia eldi* M'Clelland, 1842) (Delgado et al. 2003; Alves et al. 2005).

Positive results were found in both species of the Camelidae family. One individual sample of a Dromedary (*Camelus dromedarius* Linnaeus, 1758) was positive and a pool of from three female Wild Bactrian Camel (*Camelus ferus* Przewalski, 1878) tested positive both showing the presence of *Cryptosporidium* spp. oocysts and DNA. There are references in dromedaries in Iran and China and the occurrence of *C. andersoni* (Lindsay et al. 2000) in this species (Razawi et al. 2009; Wang et al., 2008). Although there is no reference on the occurrence of *Cryptosporidium*

spp. in Wild Bactrian Camel (wild or captive), there is a report of cryptosporidiosis in a Bactrian camel (*Camelus bactrianus* Linnaeus, 1758) in China, with the *C. andersoni* isolate being biologically similar to most bovine *C. andersoni* isolates characterized so far (Wang et al 2008). First report of *Cryptosporidium* spp. in a Bactrian camel was in 1991, in a female with chronic cryptosporidiosis; at that time, the species was morphologically similar to *C. muris* Tyzzer 1907 or to a species infecting cattle abomasum, currently known as *C. andersoni* (Fayer et al. 1991).

There were several samples tested from the family Giraffidae, with positive results in two pools of Angolan giraffe (*Giraffa camelopardalis angolensis* Lydekker, 1903) and one individual sample from an Okapi (*Okapia johnstoni* Sclater, 1901). *Cryptosporidium muris* and *Cryptosporidium* spp. have been detected in giraffes (*Giraffa camelopardalis* Linnaeus, 1758) from zoos in the Czech Republic and Spain, respectively (Kodádkova et al. 2010; Gómez et al. 1996). No references of infection (wild or captive) were found on Okapi.

From the 38 individual samples from Primates, three chimpanzees (*Pan troglodytes* Blumenbach, 1775) were positive, one Javan langur (*Trachypithecus auratus* Geoffroy, 1812), one White-fronted Lemur (*Eulemur albifrons* Geoffroy, 1796) and one Black-and-white Ruffed Lemur (*Varecia variegata* Kerr, 1792). Several studies have detected *Cryptosporidium* spp. in chimpanzees (Gonzalez-Moreno et al. 2013; Mbaya et al. 2011; Parsons et al. 2015; Debenham et al. 2015) and in the Black-and-white Ruffed Lemur (Gómez et al. 2000). In the present work, the Javan langur was detected as positive although other *Trachypithecus* species such as the Purple-faced langur (*Trachypithecus vetulus* Erxleben, 1777) has been positive for *Cryptosporidium parvum* in Sri Lanka (Ekanayake et al. 2007). No older reference of infection of the White-fronted Lemur were found.

From the order Carnivora, only two species were shedding *Cryptosporidium* oocysts, the Persian Leopard (*Panthera pardus saxicolor* Pocock, 1927) and the Eurasian lynx (*Lynx lynx* Linnaeus, 1758). The presence of *C. parvum* rat genotype was detected in a Black Leopard during a study carried out in 2007, with molecular characterization of samples from Qinghai Province, China (Karanis et al. 2007).

Our study also detected *Cryptosporidium* spp. oocysts by Ziehl-Nelseen staining and real-time PCR in one individual sample of Grevy's zebra (*Equus grevyi* Oustalet, 1882). No references were found on the occurrence in this species but *Cryptosporidium* spp. were already detected in zebras (*Equus zebra* Linnaeus, 1758) at Mikumi National Park in Tanzania (Mtambo et al. 1997). From the order Proboscidea, *Cryptosporidium* spp. have been detected in African elephants (*Loxodonta africana* Blumenbach, 1797) in the wild at Kruger National Park, South Africa and captive at the Barcelona Zoo (Abu Samra et al. 2011; Gracenea et al. 2002).

In conclusion, this study presents the new epidemiological data regarding *Cryptosporidium* spp. at the Lisbon Zoo. The results indicate that *Cryptosporidium* spp. are still occurring in captive wild mammals at the Lisbon Zoo with new host being recorded. Those animals should be considered as potential reservoirs for this zoonotic parasite. Despite de number of positive animals, clinical data of juvenile diarrhoea due to the parasite is scarce, possible due to strict sanitary and hygiene rules in the animal facilities and the constant improvements in the quality and care of the safety of housed animals.

Ethical statement: This study did not require Animal Ethics Committee approval since faecal samples were collected from the animal facilities after the spontaneous defecation of the animals.

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