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Occurrence and molecular characterization of *Cryptosporidium* spp. in mammals and reptiles at the Lisbon Zoo

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Abstract The presence of *Cryptosporidium* parasites in mammals and reptiles kept at the Lisbon Zoo was investigated. A total of 274 stool samples were collected from 100 mammals and 29 reptiles. The species and genotype of the isolates identified by light microscopy were determined by nested PCR and sequence analysis of a fragment of the small subunit rRNA gene. *Cryptosporidium* oocysts were found in one black wildebeest (*Connochaetes gnou*), one Prairie bison (*Bison bison bison*) and in one Indian star tortoise (*Geochelone elegans*). The PCR and sequence analysis of these three isolates showed that those excreted by the Prairie bison were *Cryptosporidium* mouse genotype, those from the black wildebeest were from a new *Cryptosporidium* genotype and those infecting the Indian star tortoise were *Cryptosporidium* tortoise genotype. The present work reports a new *Cryptosporidium* genotype in a black wildebeest and the first finding of the *Cryptosporidium* mouse genotype in a ruminant.

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Introduction

Cryptosporidium spp. are widespread protozoa considered to be important causes of gastrointestinal disease in humans and vertebrates.

Cryptosporidium infections have been reported in a wide spectrum of mammals belonging to several orders – Primata, Artiodactyla, Perissodactyla, Carnivora, Lagomorpha, Rodentia, Marsupialia, Monotrema and Proboscidea – and in numerous reptilian species belonging to orders Testudines and Squamata (Graczyk et al. 1997; Majewska et al. 1997; Mtambo et al. 1997; Muriuki et al. 1997; Gómez et al. 2000; Graczyk and Cranfield 2000). Recent molecular studies have shown that there is an extensive genetic diversity in *Cryptosporidium* parasites infecting mammals, reptiles and birds and in addition to the 14 *Cryptosporidium* accepted species – *C. andersoni*, *C. hominis*, *C. parvum*, *C. canis*, *C. felis*, *C. wrairi*, *C. suis*, *C. muris*, *C. saurophilum*, *C. serpentis*, *C. baileyi*, *C. meleagridis*, *C. galli* and *C. molnari* – many host-adapted genotypes have been described such as the deer, mouse, pig, bear, cervine, mongoose, marsupial, snake and lizard genotypes (Morgan et al. 1999a; Ryan et al. 2004; Xiao and Ryan 2004; Xiao et al. 2004a, 2004b).

Studies carried out previously in artiodactyl ruminants at the Lisbon Zoo, showed a 4% prevalence of cryptosporidiosis (Delgado et al. 2003) and molecular characterization has identified the isolates as *C. parvum* (Alves et al. 2001, 2003). In the present study, we extended the range of animals from the Lisbon Zoo examined for *Cryptosporidium* to several other mammalian orders and to reptiles. We also characterized part of the small subunit (SSU) rRNA gene of *Cryptosporidium*-positive samples by PCR amplification and sequence analysis.

Materials and methods

Between April 2002 and February 2003, 274 stool samples were collected from 100 mammals (Table 1) and 28 reptiles (Table 2) kept at the Lisbon Zoo.

With the exception of two baby lions, all mammals were adults, and had no *Cryptosporidium*-related symptoms. The reptiles studied, adults and young animals, were all asymptomatic, with the exception of one Indian star tortoise, which was part of a large group of confiscated tortoises in the black market in Singapore, that was severely sick due to crowded facilities, bad nutrition status, extreme temperature changes, and transport-induced stress. This tortoise arrived at the Lisbon Zoo 3 days prior stool collection and died 2 weeks later.

Direct and concentrated faecal smears were prepared and *Cryptosporidium* oocysts identified by light microscopy at a ×400 magnification after modified

Table 1 List of mammals studied

Mammals
Red-necked wallaby (*Macropus rufogriseus*)
Ring-tailed lemur (*Lemur catta*)
White-fronted brown lemur (*Lemur macaco albifrons*)
Black lemur (*Lemur macaco macaco*)
Mongoose lemur (*Lemur mongoz*)
Red ruffed lemur (*Varecia variegata rubra*)
Black and white ruffed lemur (*Lemur variegatus variegatus*)
Black howler monkey (*Alouatta caraya*)
Brown capuchin (*Cebus apella*)
White-faced saki (*Pithecia pithecia*)
Goeldi's monkey (*Callimico goeldii*)
Black-tailed marmoset (*Callithrix argentata*)
White-fronted marmoset (*Callithrix geoffroyi*)
Golden-headed lion tamarin (*Leontopithecus rosalia chrysomelas*)
Golden lion tamarin (*Leontopithecus rosalia rosalia*)
Saddleback tamarin (*Saguinus fuscicollis*)
Emperor tamarin (*Saguinus imperator subgrisescens*)
White-lipped tamarin (*Saguinus labiatus labiatus*)
Red handed tamarin (*Saguinus midas midas*)
Black-handed tamarin (*Saguinus midas niger*)
Cotton-top tamarin (*Saguinus oedipus oedipus*)
Green monkey (*Cercopithecus aethiops sabeus*)
Red-tail monkey (*Cercopithecus ascanius ascanius*)
Moustached monkey (*Cercopithecus cephus cephus*)
Diana monkey (*Cercopithecus diana diana*)
Mona monkey (*Cercopithecus mona*)
deBrazza's monkey (*Cercopithecus neglectus*)
Lesser white-nosed monkey (*Cercopithecus petaurista petaurista*)
Colobus monkey (*Colobus guereza kikuyuensis*)
Japanese macaque (*Macaca fuscata fuscata*)
Lion-tailed macaque (*Macaca silenus*)
Guinea baboon (*Papio cynocephalus papio*)
Hamadryas baboon (*Papio hamadryas hamadryas)*
Mandrill (*Papio sphinx*)
Red-shanked douc langur (*Pygathrix nemaeus nemaeus*)
Western lowland gorilla (*Gorilla gorilla gorilla*)
White-handed gibbon (*Hylobates lar*)
Siamang (*Hylobates syndactylus*)
Chimpanzee (*Pan troglodytes*)
Two-toed sloth (*Choloepus didactylus*)
Iberian wolf (*Canis lupus signatus*)
Fennec fox (*Fennecus zerda*)
Red fox (*Vulpes vulpes silacea*)
Asiatic black bear (*Selenarctos thibetanus*)
Brown bear (*Ursus arctos*)
Cheetah (*Acinonyx jubatus jubatus*)
Iberian lynx (*Felis lynx lynx*)
Ocelot (*Felis pardalis*)
Serval (*Felis serval*)
Clouded leopard (*Neofelis nebulosa*)
Angolan lion (*Panthera leo bleyenberghi*)
Jaguar (*Panthera onca*)
Leopard (*Panthera pardus*)
Siberian tiger (*Panthera tigris altaica*)
Sumatran tiger (*Panthera tigris sumatrae*)
Snow leopard (*Pantera uncia*)
California sea lion (*Zalophus californianus*)
Harbour seal (*Phoca vitulina*)
Indian elephant (*Elephas maximus indicus*)
African bush elephant (*Loxodonta africana africana*)
Grevy's zebra (*Equus grevyi*)
Domestic donkey (*Equus asinus*)
Horse (*Equus caballus*)
Southern white rhinoceros (*Ceratotherium simum simum*)
Great Indian rhinoceros (*Rhinoceros unicornis*)
Wild boar (*Sus scrofa*)
Hippopotamus (*Hippopotamus amphibius)*
Pygmy hippopotamus (*Choeropsis liberiensis liberiensis*)
Bactrian camel (*Camelus bactrianus bactrianus*)
Dromedarian camel (*Camelus dromedarius*)
Llama (*Lama glama glama*)
Axis deer (*Cervus axis axis*)
American elk (*Cervus elaphus canadensis*)
Eld's deer (*Cervus eldi thamin*)
Angolan giraffe (*Giraffa camelopardalis angolensis*)
Domestic goat (*Capra hircus*)
Addax (*Addax nasomaculatus*)
Black faced impala (*Aepyceros melampus petersi*)
Red hartebeest (*Alcelaphus buselaphus caama*)
Barbary sheep (*Ammotragus lervia*)
Blackbuck (*Antilope cervicapra*)
Prairie bison (*Bison bison bison*)
Domestic yak (*Bos mutus grunniens*)
Black wildebeest (*Connochaetes gnou*)
Blue wildebeest (*Connochaetes taurinus taurinus*)
Bontebok (*Damaliscus dorcas dorcas*)
Roan antelope (*Hippotragus equinus*)
Sable antelope (*Hippotragus niger niger*)
Waterbuck (*Kobus ellipsiprymnus ellipsiprymnus*)
Red lechwe (*Kobus leche leche*)
Scimitar-horned oryx (*Oryx dammah*)
Gemsbok (*Oryx gazella gazella*)
Arabian oryx (*Oryx leucoryx*)
African buffalo (*Syncerus caffer caffer*)
Forest buffalo (*Syncerus caffer nanus*)
Eland (*Taurotragus oryx oryx*)
Nyala (*Tragelaphus angasi*)
East African bongo (*Tragelaphus eurycerus isaaci*)
Sitatunga (*Tragelaphus spekei gratus*)
Greater kudu (*Tragelaphus strepsiceros strepsiceros*

Table 2 List of reptiles studied

Reptiles
Aldabra giant tortoise (*Geochelone gigantea*)
Indian star tortoise (*Geochelone elegans*)
Egyptian tortoise (*Testudo kleinmanni*)
African pancake tortoise (*Malacochersus tornieri*)
Common snapping turtle (*Chelydra serpentina*)
Alligator snapping turtle (*Macroclemys temminckii*)
Bearded dragon (*Acanthodraco vitticeps*)
Panther chameleon (*Chamaeleo pardalis*)
Rhinoceros iguana (*Cyclura cornuta*)
Green iguana (*Iguana iguana*)
Cuban iguana (*Cyclura nubila nubila*)
Komodo dragon (*Varanus komodoensis*)
Yellow anaconda (*Eunectes notaeus*)
Jamaican boa (*Epicrates subflavus*)
Cuban boa (*Epicrates angulifer*)
Madagascar tree boa (*Sanzinia madagascariensis*)
Burmese python (*Python molurus bivittatus*)
Gray-banded kingsnake (*Lampropeltis alterna*)
Pueblan milksnake (*Lampropeltis triangulum campbelli*)
Forest cobra (*Naja melanoleuca*)
Monocellate cobra (*Naja naja kaouthia*)
Cape cobra (*Naja nivea*)
Eastern diamondback rattlesnake (*Crotalus adamanteus*)
Western diamondback rattlesnake (*Crotalus atrox*)
South american rattlesnake (*Crotalus durissus terrificus*)
Uracoan rattlesnake (*Crotalus vegrandis*)
West african gaboon viper (*Bitis gabonica rhinoceros*)
Boelen's python (*Morelia boeleni*)

Ziehl-Neelsen staining. In samples that were positive by microscopy, oocysts' DNA was extracted by a KOH/QIAamp DNA stool mini kit protocol (QIAGEN GmbH) (Alves et al. 2003). The species and genotype of the isolates identified were determined by nested PCR of a fragment of the small subunit (SSU) rRNA gene (Xiao et al. 2001) and sequencing of the secondary PCR products in both directions on an ABI Prism 3100 analyser (Applied Biosystems). The nucleotide sequences obtained were aligned with additional *Cryptosporidium* SSU rRNA sequences obtained from GenBank using the ClustalX program. A neighbour-joining tree was constructed from the aligned sequences with the TreeconW program using a sequence of *Eimeria tenella* (AF026388) as an outgroup. Genetic distance was calculated based on the Kimura 2-parameter model. Bootstrap analyses were conducted using 1,000 pseudo replicates.

Results

Oocysts were found in faeces of two artiodactyl bovids, a black wildebeest (*Connochaetes gnou*) and a Prairie bison (*Bison bison bison*), and in one Testudinidae, the Indian star tortoise (*Geochelone elegans*) that came from Singapore. In both mammals, the level of oocyst shedding was low and none had apparent symptoms of cryptosporidiosis. Subsequent stool collection was conducted on these two animals, but no oocysts were found. The level of oocyst shedding in the Indian star tortoise was low.

A fragment of the SSU rRNA gene was successfully amplified and sequenced for these three isolates. BLASTn GenBank searches performed on the sequences obtained from the Prairie bison and the Indian star tortoise, showed 100% similarity with the sequences of *Cryptosporidium* mouse genotype (AF112571) and *Cryptosporidium* tortoise genotype (AY120914), respectively (Xiao et al. 1999, 2004b).

The black wildebeest isolate revealed a unique sequence, different from all other animal *Cryptosporidium* sequences, and thus constituted a new *Cryptosporidium* genotype in animals. The SSU rRNA partial nucleotide sequence of the black wildebeest *Cryptosporidium* isolate was deposited in the GenBank database under accession number AY883022. The new genotype from black wildebeest had the highest sequence homology to the W5 genotype (AF262332) previously found in a storm water sample collected in the Eastern Catskill Mountains, NY, with an A to G mutation, a TA insertion and a T deletion (Xiao et al. 2000). A neighbour-joining analysis showed that this genotype from wildebeest formed a cluster with several intestinal *Cryptosporidium* species (*C. parvum*, *C. hominis*, *C. wrairi*, *C. suis*, *C. canis*, *C. felis*, *C. meleagridis* and *C. saurophilum*) and genotypes (rabbit, horse, ferret, mouse, skunk, cervine, fox, squirrel, deer mouse, bear, opossum I and II, marsupial I and II, and muskrat I and II), with 87% of bootstrap support (Fig. 1).

Discussion

The present finding of *Cryptosporidium* mouse genotype in a Prairie bison constitutes the first report of this genotype in a ruminant. Gómez et al. (2000) also identified *Cryptosporidium* oocysts in a Prairie bison at the Barcelona Zoo, but the molecular identification was not performed. The mouse genotype is very common in mice and small rodents (Morgan et al. 1999b; Bajer et al. 2003). Whether the bison was infected with mouse genotype parasites or merely passing oocysts through the gastrointestinal tract after ingestion of food/water in facilities contaminated with rodents' faeces remains to be determined. Phylogenetically, the mouse genotype has the closest relatedness to *C parvum*, which infects mainly ruminants (Xiao et al. 2004a). Thus, it is possible that the bison was actually infected by the

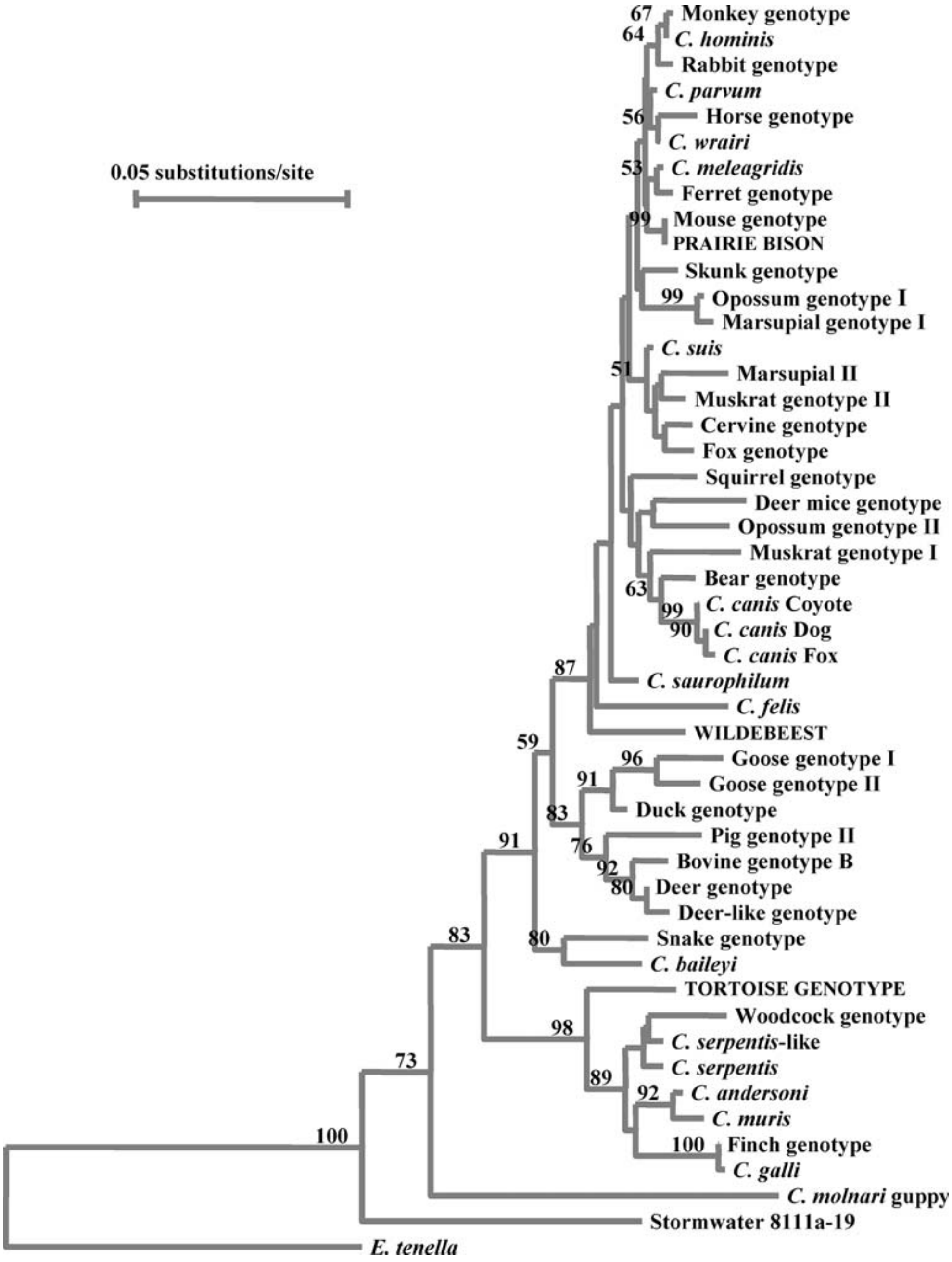

Fig. 1 Phylogenetic relationships of *Cryptosporidium* parasites inferred by neighbour-joining analysis of the SSU rRNA gene based on genetic distances calculated by Kimura 2-parameter model. The tree was rooted with an SSU rRNA sequence from *Eimeria tenella* (AF026388)

mouse genotype. However, the absence of clinical signs, the low level of oocyst shedding and its detection only once in only one individual animal cannot exclude the possibility of the mere passage of oocysts in the animal. The turtle genotype found in the Indian star tortoise has already been found in three Indian star tortoises from the Saint Louis Zoo and thus should represent a true parasite of these reptiles (Xiao et al. 2004b).

Cryptosporidium infection is apparently common in wildebeests, as an earlier study identified *Cryptosporidium* spp. in 27% of black wildebeests from the Mikumi National Park, Morogoro, Tanzania (Mtambo et al. 1997). Because no molecular characterization of the isolates was done, the identity of *Cryptosporidium* spp. in these animals was not clear. *Cryptosporidium* was also previously found in faeces of a blue wildebeest (*Connochaetes taurinus taurinus*) at the Barcelona Zoo (Gómez et al. 2000). Molecular characterization of the parasite identified it as *C parvum* (Morgan et al. 1999b). The black wildebeest in this study was apparently passing oocysts of a new *Cryptosporidium* genotype. Due to its similarity with the W5 genotype from storm water, this new genotype may represent a new *Cryptosporidium* parasite that the black wildebeest acquired from other animals in the Zoo, rather than a native parasite from Africa. Thus, like many species or groups of vertebrates (Xiao et al. 2004a), wildebeests may be infected with at least two *Cryptosporidium* parasites, *C. parvum* and this new *Cryptosporidium* genotype.

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