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Original Article

Molecular identification of *Cryptosporidium* isolates from ill exotic pet animals in Japan including a new subtype in *Cryptosporidium fayeri*

Youki Takaki[a], Yoshinori Takami[a], Takehiro Watanabe[a], Takaaki Nakaya[b], Fumi Murakoshi[b,*]

[a] *Verts Animal Hospital, 1F Kyuso Bldg 2-21-5, Naka, Hakata-ku, Fukuoka-shi, Fukuoka 812-0893, Japan*
[b] *Department of Infectious Diseases, Kyoto Prefectural University of Medicine, 465 Kajii-cho Kawaramachi-Hirokoji, Kamigyo-ku Kyoto 602-8566, Japan*

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ABSTRACT

Cryptosporidium is an obligate intracellular parasite which can cause fatal diarrheal disease in exotic animals. Sugar gliders (*Petaurus breviceps*), hedgehogs (*Atelerix albiventris*), chinchillas (*Chinchilla lanigera*), and common leopard geckos (*Eublepharis macularius*) are popular exotic animals commonly sold in pet shops in Japan. We herein investigated the species and subtypes of *Cryptosporidium* in these animals. *Cryptosporidium fayeri* was detected in a sugar glider in a Japanese animal hospital. Sequence analyses of the 60-kDa glycoprotein (*gp60*) gene revealed that *C. fayeri* belonged to subtype family IVh (IVhA13G2T1), which was proposed to be a new subtype. This is the first study to report *C. fayeri* infection in a sugar glider. In other animals, the *Cryptosporidium* horse genotype, *C. ubiquitum*, and *C. varanii* were detected in two four-toed hedgehogs (*A. albiventris*), a chinchilla (*C. lanigera*), and common leopard gecko (*E. macularius*), respectively. The *gp60* subtypes identified were VIbA13 of the horse genotype and XIId of *C. ubiquitum*. The present results revealed that potentially zoonotic *Cryptosporidium* is widespread in exotic animals in Japan.

1. Introduction

Cryptosporidium is a protozoan parasite that causes severe diarrhea and is found in mammals, reptiles, birds, fish, and various vertebrates (Fayer, 2010). Cryptosporidiosis in exotic animals is sometimes fatal in young animals and, thus, causes economic losses to pet shops and breeders (Dellarupe et al., 2016). Therefore, the impact of infections caused by *Cryptosporidium* spp. and their zoonotic potential are of importance.

Sugar gliders (*Petaurus breviceps*), hedgehogs (*Atelerix albiventris*), chinchillas (*Chinchilla lanigera*), and common leopard geckos (*Eublepharis macularius*) are exotic animals commonly sold in pet shops in Japan. The sugar glider is a mammal that is classified as a marsupial and belongs to the order Diprotodontia. They are omnivorous, live on trees, and form a flock of females and one male. *Cryptosporidium fayeri* has been detected in marsupials, including eastern grey kangaroos, yellow-footed rock wallabies, western-barred bandicoots, and koalas (Ryan et al., 2008), which generally live on the ground, except for koalas. *C. fayeri* is typically asymptomatic in adult animals (Power et al., 2005); although the pathogenicity of *C. fayeri* is currently remains unclear because of the difficulties associated with conducting wild animal surveys. *C. fayeri* infection has been reported in immunocompetent humans and causes prolonged gastrointestinal illness (Waldron et al., 2010).

In the present study, *Cryptosporidium fayeri* was detected in the sugar glider with diarrhea, and it was identified at the subtype levels using molecular techniques. *Cryptosporidium* spp. were also found in two hedgehogs, a common leopard gecko, and chinchilla that exhibited similar digestive symptoms to the sugar glider, and these isolates were also molecularly identified. The results of the present study provide epidemic information on cryptosporidiosis in exotic animals in Japan.

2. Materials and methods

Eleven exotic animals (two sugar gliders, four hedgehogs, two chinchillas, and three common leopard geckos) that were brought to the hospital between August and December 2017 were examined in the present study. Details on these animals are shown in Table 1.

Fecal samples from sugar gliders and other animals were collected for a parasitological examination by sucrose centrifugal flotation and Kinyoun acid-fast staining, and also for DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). *Cryptosporidium*-specific fragments were amplified by a nested polymerase chain reaction (PCR) targeting an ~830-bp fragment of the small subunit ribosomal RNA (SSU rRNA) gene, as reported previously (Xiao et al., 2001). Regarding the subtyping of *C. fayeri*, the horse

* Corresponding author.
E-mail address: muraf@koto.kpu-m.ac.jp (F. Murakoshi).

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Table 1
Information of exotic animals surveyed in this study.

Animals	Age	Body condition score	Observation	Pet shop locality	Kinyoun acid-fast staining	Results of RealPCR™ Panels	PCR
Petaurus brevicep (Sugar glider)	3 month	2	Diarrhea	Kyoto	positive	*Cryptosporidium* spp.	positive
Petaurus breviceps	4 month	2.5	Diarrhea	ND	negative	not investigated	–
Atelerix albiventris (four toed hedgehog) (Aa1)	2 month	2.5	Hernia	Fukuoka	positive	*Cryptosporidium* spp.	positive
Atelerix albiventris (Aa2)	2.5 month	2	Diarrhea	Kyoto	positive	not investigated	positive
Atelerix albiventris	3 month	2.5	Diarrhea	ND	negative	not investigated	–
Atelerix albiventris	2 month	2.5	Diarrhea	Fukuoka	negative	not investigated	–
Chinchilla lanigera (chinchilla) (Cl1)	2 month	2	Loose stool	Oita	positive	not investigated	negative
Chinchilla lanigera (Cl2)	3 month	2	Loose stool	Fukuoka	positive	not investigated	positive
Eublepharis macularius (leopard gecko)	2 years	2.5	Severe diarrhea	Fukuoka	positive	not investigated	positive
Eublepharis macularius	1 years	3.5	Anorexia, diarrhea	Fukuoka	negative	not investigated	–
Eublepharis macularius	ND	3.5	Anorexia, diarrhea	Fukuoka	negative	not investigated	–

ND: No data, Body condition scores were assessed by veterinarians between 1 and 5. No clear criteria for body condition scores in these animals.

genotype, and *C. ubiquitum*, the *Cryptosporidium* 60-kDa glycoprotein gene (*gp60*) was amplified by nested PCR using primers, as reported previously (Sulaiman et al., 2005; Power et al., 2009; Li et al., 2014). PCR amplification was performed using KOD-Plus-Neo (TOYOBO, Japan), and positive fragments were purified using MonoFas DNA Purification Kit I (GL Science, Japan). DNA sequencing was conducted using the BigDye Terminator v3.1 Cycle Sequencing kit on an automated ABI3130 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA). SSU rRNA and *gp60* nucleotide sequences were sequenced using secondary primers. To detect other pathogens, fecal samples from the sugar glider and the four toed hedgehog were sent to the IDEXX company for PCR testing using the IDEXX Canine diarrhea RealPCR™ Panel (IDEXX Reference Laboratories, USA). This panel detects the following pathogens: *Clostridium perfringens*, *Salmonella* spp., *Giardia* spp., *Cryptosporidium* spp., canine enteric coronavirus, canine parvovirus 2, and canine distemper virus.

The sequences of *gp60* were aligned using Clustal X2 (Larkin et al., 2007). All gaps were eliminated. Maximum likelihood (ML) analyses were performed using MEGA 7.0.26 (Kumar et al., 2016). Substitution models and optional parameter sets were also evaluated using MEGA 7.0.26, and the most suitable sets were selected according to the Akaike information criterion (AIC). We constructed a phylogenetic tree, in which the substitution model and optional parameters used were the Tamura-Nei model (Tamura and Nei, 1993), incorporating the invariable site and Gamma distribution (five categories) options. To calculate the bootstrap value, 1000 ML trees were constructed using the same datasets. The protocol for the experiments was approved by the Committee on the Animal Experiments of the Kyoto Prefectural University of Medicine.

3. Results

Table 1 shows information on the exotic animals surveyed in the present study. Of the 11 animals examined, 6 tested positive for *Cryptosporidium* on Kinyoun acid-fast staining. Only *Cryptosporidium* spp. were positive in the samples tested with the RealPCR™ Panel. A PCR analysis using SSU rRNA showed that all samples with Kinyoun acid-fast staining were positive for *Cryptosporidium*, except for one chinchilla (Cl1) sample. The SSU rRNA and *gp60* genes were deposited in GenBank under the accession numbers LC483882-LC483888 (Table 2).

An NCBI blast (Basic Local Alignment Search Tool) search (blastn optimized for highly similar sequences) was used to search for the sequence with the highest homology to the detected sequence. The SSU rRNA gene sequences of *Cryptosporidium* from the sugar glider showed the highest homology with *C. fayeri* (AF112570) (100%, 758/758 bp homologous), and the *gp60* sequence showed the highest homology with that of subtype family IVa in *C. fayeri* (FJ490092, isolate from a kangaroo). The *gp60* sequence of *C. fayeri* had 90% homology (1036/1151 bp) with FJ490092, and Gap was present at 4% (47/1151 bp). Table 3 shows nucleotide sequence similarities (%) among the subtype families of *C. fayeri* (XIVa – IVh (detected in the present study)) at the *gp60* gene. Fig. 1 shows the results of the phylogenetic analysis of the *gp60* subtype of *C. fayeri*, including the sequence isolated from the sugar glider. Based on these results, the *gp60* subtype family detected in this study, IVh, was proposed to be a new subtype family, and the *gp60* serine-coding trinucleotide repeat of this isolate was IVhA13G2T1.

Cryptosporidium isolated from the hedgehog was 99% (780/784 bp) homologous in the SSU rRNA gene to the *Cryptosporidium* horse genotype (FJ435962) and the *gp60* gene sequence was 100% (750/750 bp) homologous to FJ435961 (VIbA13). The SSU rRNA and *gp60* sequences detected from Aa1 and Aa2 were identical (Table 2). *Cryptosporidium* isolated from the chinchilla was 100% (825/825 bp) homologous in the SSU rRNA gene to *C. ubiquitum* (KT922236) and the *gp60* gene sequence was 100% (844/844 bp) homologous to that of LC334004 (XIId). *Cryptosporidium* isolated from the common leopard gecko was 99% (614/617 bp) homologous in the SSU rRNA gene to *C. varanii* (EU553556).

Table 2
Information of exotic animals and *Cryptosporidium* detected in this study.

Host	species	subtype	accession number	
			SSU rRNA	*GP60*
Petaurus breviceps	*C. fayeri*	IVaA13G2T1	LC483882	LC483886
Atelerix albiventris (Aa1, Aa2)	*Cryptosporidium* horse genotype	VIbA13	LC483885	LC483888
Chinchilla lanigera (Cl2)	*C. ubiquitum*	XIId	LC483883	LC483887
Eublepharis macularius	*C. varanii*	–	LC483884	–

The SSU rRNA and *GP60* sequences detected form Aa1 and Aa2 were identical.

Table 3
Nucleotide sequence similarity (%) among subtype families of *C. fayeri* (XIVa–IVh) at the *gp60* locus.

	IVa	IVb	IVc	IVd	IVe	IVf	IVg	IVh
IVa (FJ490092)								
IVb (FJ490087)	88							
IVc (FJ490069)	87	90						
IVd (FJ490059)	88	90	94					
IVe (FJ490071)	78	80	85	91				
IVf (FJ490070)	79	80	76	80	81			
IVg (MG516790)	87	95	86	87	79	79		
IVh (LC483886)	**90**	**80**	**79**	**79**	**84**	**77**	**80**	

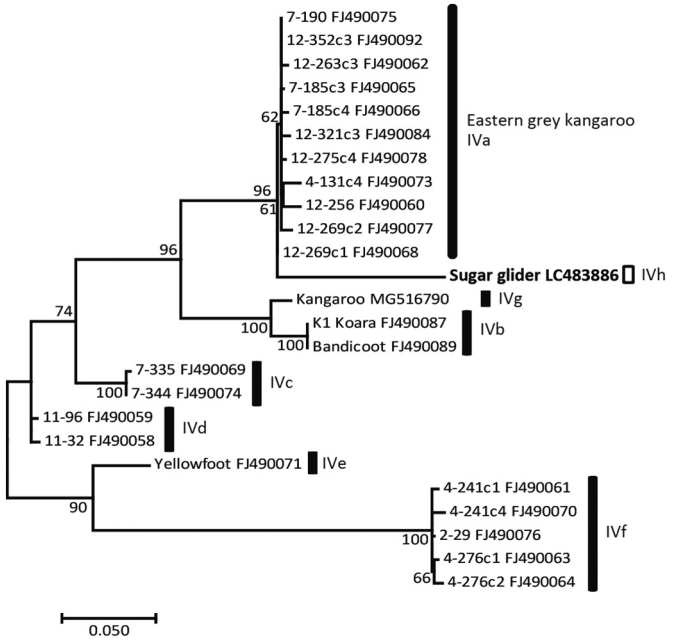

Fig. 1. Phylogenetic tree based on partial sequences of the *gp60* gene in *Cryptosporidium fayeri.*

A phylogenetic tree based on partial sequences of the *gp60* gene constructed by maximum likelihood *(*ML) analyses for *C. fayeri* using 911 nucleotides without gaps (substitution model and optional parameters = TN93 + Γ + I). Only bootstrap values > 50% from 1000 pseudo-replicates are shown.

4. Discussion

The present study is the first to have detected *C. fayeri* from a sugar glider, a captive animal in Japan, and a novel *C. fayeri* subtype family (IVhA13G2T1) was identified. *C. fayeri* was previously identified as marsupial genotype I, with this pathogen being observed in Australian marsupials, such as the red kangaroo, koala, eastern grey kangaroo, yellow-footed rock wallaby, western-barred bandicoot, western grey kangaroo, and Tasmanian devils (Ryan and Power, 2012; Wait et al., 2017). These animals generally live on the ground, except for the koala. The *gp60* sequence detected from the sugar glider in the present study formed a clade with the IVa subtype family isolated from eastern grey kangaroos in Australia (Power et al., 2009). The reported homology of the *gp60* subtype family (IVa - IVg) is 76–95%. The sequence detected in the present study was 90% identical to the closest IVa, suggesting a new subtype family, IVh (Table 3).

Since the sugar glider in this survey was bred and sold at the same pet shop, we assumed that the pathogen was brought in with the infected animal (it may have originated from other sugar gliders) and spread among animals in the pet shop. In Japan, sugar gliders are mainly imported from the Republic of Indonesia and Thailand; however, there was no information on the origin of the sugar glider examined in the present study. The clinical symptoms of *C. fayeri* currently remain unclear. A previous study reported that adult eastern grey kangaroos shed oocysts, but remained asymptomatic (Power et al., 2005). In the present study, the 3-month-old sugar glider presented with diarrhea and weight loss. It tested positive for *C. fayeri* and negative for *C. perfringens*, *Salmonella* spp., and *Giardia* spp. The symptoms of diarrhea in this animal may have been caused by other pathogens, but suggest that *C. fayeri* causes digestive dysfunction symptoms in young sugar gliders. In a previous study, immunocompetent humans who had contact with marsupials were infected by *C. fayeri* (Waldron et al., 2010). In 2006, 8490 sugar gliders were imported into Japan (Ozone, 2006). In total, 8816 sugar gliders in 2014 and 7286 marsupials, including sugar gliders, in 2017 were imported into Japan (https://www.mhlw.go.jp/). The captive breeds of sugar gliders in Japan are a type of companion animal that make close contact with the owner. Furthermore, some tearooms in Japan allow humans to make contact with sugar gliders. Therefore, it is important to consider the risk of human infection with *C. fayeri* from sugar gliders and veterinarians need to adequately inform owners about this risk.

In the present study, the *Cryptosporidium* horse genotype was detected in a hedgehog. While *C. parvum* and *C. erinacei* (*Cryptosporidium* hedgehog genotypes) are common in hedgehogs (Kváč et al., 2014), only one previous study reported the detection of the *Cryptosporidium* horse genotype in an imported hedgehog in Japan (Abe and Matsubara, 2015). Isolates from the hedgehog were placed into the subtype VIb family (VIbA13), which is the same subtype reported in the present study. However, the clinical symptoms of the hedgehog in that study were not described. In the present study, the hedgehog had a hernia, and we found inflammation of the cranial intestine from the serosa during proctopexy. Hence, this pathogen may cause severe digestive symptoms in hedgehogs. While this species of pathogen is mainly detected in foals and calves (Thompson et al., 2007; Caffara et al., 2013), it has also been identified in immunocompetent humans (Robinson et al., 2008; Xiao et al., 2009). Since the subtype of the horse genotype isolated from the hedgehog was similar to the genotype isolated from humans in a previous study (Xiao et al., 2009), infections in humans need to be considered. In total, 11,950 hedgehogs in 2014 and 14,479 in 2017 were imported into Japan (https://www.mhlw.go.jp/). Due to the large number of breeders in Japan, the prevention of infection and mitigation of potential epidemics are crucial.

C. ubiquitum was isolated from the chinchilla (Cl2). Cl1 was positive for cryptosporidium on acid-fast staining, but negative on PCR. The lack of amplification of SSU rDNA in Cl1 may have been due to the low intensity of oocysts in the fecal sample. This pathogen is isolated from many animals worldwide, such as ruminants, rodents, and primates, including humans (Fayer et al., 2010). In a previous study that examined 140 chinchillas in China, 1 chinchilla tested positive for *C. parvum* and 13 for *C. ubiquitum*, and the subtype was XIId (Qi et al., 2015). In the Czech Republic and Poland, *C. ubiquitum* was isolated from 2 out of 50 chinchillas, and the subtype was XIIa (Kellnerová et al., 2017). In an animal hospital in Japan, *C. ubiquitum* was isolated from 13 chinchillas, 11 of which had diarrhea and 8 of which died (Kubota et al., 2019). Similarly, the chinchilla in the present study had severe diarrhea. In Japan, *C. ubiquitum* has also been isolated from wild large Japanese field mice (Murakoshi et al., 2013); however, there is no information on the subtype. *C. ubiquitum* has recently been attracting increasing attention due to infections in humans, causing cryptosporidiosis (Fayer et al., 2010; Li et al., 2014). Since the chinchilla is a popular pet for humans, the zoonotic capability of *C. ubiquitum* needs to be considered.

C. varanii was isolated from the common leopard gecko. *C. varanii* is a common *Cryptosporidium* species isolated from numerous reptiles (Pavlasek and Ryan, 2008). In Japan, it has been isolated from a Baron's green racer, veiled chameleon, Chinese wonder geckos, and a common leopard gecko (Abe and Matsubara, 2015). This pathogen causes epidemics among reptiles. The main symptoms observed include anorexia, weight loss, and emaciation. Young reptiles are more prone to lethality with cryptosporidiosis (Deming et al., 2008; Dellarupe et al., 2016). In the present study, we found similar symptoms to those in previously reported cases of *C. varanii* infection.

These pathogens may have been introduced by infected animals imported from foreign countries or through infections contracted in pet shops or Japanese homes. Therefore, in order to prevent infection and epidemics in the future, investigations into the routes of infection of cryptosporidiosis are needed. Furthermore, the occurrence of these pathogens needs to be clarified by elucidating the rate of infection in captive animals in pet shops.

Ethical statement

Animal studies

The protocol for the experiments was approved by the Committee on the Animal Experiments of the Kyoto Prefectural University of Medicine, Kyoto, Japan.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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