IJP: Parasites and Wildlife 11 (2020) 83–87

Contents lists available at ScienceDirect

IJP: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

# Detection and molecular characterization of *Cryptosporidium* species in wild-caught pet spiny-tailed lizards

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ARTICLE INFO

*Keywords:*
Cryptosporidiosis
Reptiles
*Cryptosporidium avium*
Imported animals

ABSTRACT

*Uromastyx* is a genus of the herbivorous agamid lizards, also known as spiny-tailed lizards or mastigures, which are found in parts of Africa and the Middle East. Currently, several species of this genus are available in the international pet trade in Japan. In this study, two imported wild-caught spiny-tailed lizards (Arabian blue mastigure, *Uromastyx ornata philbyi,* and Sudan mastigure, *Uromastyx dispar flavifasciata*) were diagnosed with a *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) infection based on the presence of the oocysts in the rectal feces using sucrose flotation and light microscopy examination at a local animal hospital in Tokyo, Japan. One of the lizards had died, and histopathological examination revealed enteritis with the *Cryptosporidium* parasite. Sequence analyses using the small subunit ribosomal RNA, actin, and 70-kDa heat shock protein genes indicated that the lizards had contracted a novel variant of *C. avium* that commonly infects avian species.

## 1. Introduction

Species of the genus *Cryptosporidium* (Apicomplexa: Cryptosporidiidae), is an intracellular parasite that is associated with gastrointestinal diseases. Currently, more than 40 *Cryptosporidium* species have been described in mammals, reptiles, birds, amphibians, and fish (Zahedi et al., 2016; Feng et al., 2018).

*Cryptosporidium* infections among reptilian species are ubiquitous, and some of them cause chronic and lethal gastrointestinal diseases (Deming et al., 2008; Pasmans et al., 2008; Griffin et al., 2010; Ryan and Xiao, 2014). Two species, *C. serpentis* and *C. varanii* (syn. *C. saurophilum*) were the most frequently isolated species from the gastrointestinal tracts of snakes and lizards, respectively (Pavlásek et al., 1995; Koudela and Modrý, 1998; Plutzer and Karanis, 2007; Pavlásek and Ryan, 2008; Paiva et al., 2013; Ryan et al., 2014). In tortoises, *C. testudinis* and *C. ducismarci* have been described (Ježková et al., 2016). Furthermore, there are a few detection reports of *C. parvum*, *C. muris*, *C. tyzzeri,* and *C. andersoni* in reptiles, particularly snakes, which are thought to be a pseudoparasite due to the mechanical transmission by preying on infected rodents (Morgan et al., 1999; Xiao et al., 2004; Pedraza-Díaz et al., 2009; Díaz et al., 2013; da Silva et al., 2014; Yimming et al., 2016). There is also a single report of *C. avium* (traditionally known as avian genotype V) from two green iguanas (*Iguana iguana*) (Kik et al., 2011). In addition to these recognized species of *Cryptosporidium*, several genotypes have been reported in reptiles including; lizard (*C. serpentis*-like) genotype, snake genotype I and II, and tortoise genotype III (Xiao et al., 2004; Abe and Matsubara, 2014; Ježková et al., 2016; Zahedi et al., 2016).

The genus *Uromastyx* is in the family Agamidae and contains 15 recognized species, which are often known by the common name mastigures, spiny-tailed lizards, and dab lizards (Wilms et al., 2007). Lizards in the genus *Uromastyx* are largely herbivorous and originated from the North to Northeast Africa and the Middle East, and several species of this genus are often kept as pets. Rataj et al. (2011) reported the presence of *Cryptosporidium* spp. among *Uromastix* spp. in Slovenia, however, the species/genotype of *Cryptosporidium* infections involving *Uromastyx* has not yet been elucidated.

The present study reports *Cryptosporidium* infection in mastigures and revealed a novel variant of *C. avium* that normally infects avian species.

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https://doi.org/10.1016/j.ijppaw.2020.01.002
Received 28 November 2019; Received in revised form 7 January 2020; Accepted 7 January 2020
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## 2. Materials and methods

### 2.1. History

The first case was a male imported wild-caught Arabian blue mastigure (*Uromastyx ornata philbyi*) and was purchased from a pet shop in Tokyo, Japan, in December 2017. Just a few months later, on March 19, 2018, this lizard was admitted to an animal hospital with emaciation, diarrhea, and vomiting. The parasitological examination could not be performed since the specimen was not available at the hospital. On day 0, the lizard was administrated enrofloxacin, metronidazole, mosapride, and a lactobacillus preparation. On day 3, the animal was found dead and diagnosed with cryptosporidiosis based on the presence of the oocysts in the rectal feces using sucrose flotation and light microscopy examination.

The second case was a Sudan mastigure (*Uromastyx dispar flavifasciata*), which was an imported wild-captured individual purchased by the same owner on November 23, 2017, at a pet shop in Saitama, Japan. On January 22, 2018, the lizard was admitted to the animal hospital with constipation. An abdominal operation revealed intestinal obstruction with flooring materials, feces, and numerous pinworms. On March 26, 2018, *Cryptosporidium* oocysts were detected from a new fecal specimen using sucrose flotation during a health check in the hospital.

As pets in their new environment, the lizards lived together in an indoor cage and were fed with commercial pellets for tortoises, commercial seeds for birds, waxworms, and plants (leaves of kale and turnips and chrysanthemum flowers).

### 2.2. Histopathological examination

The dead Arabian blue mastigure was necropsied and subjected to histopathological analysis at Rakuno Gakuen University, Hokkaido, Japan. The intestines were fixed in 10% neutral-buffered formalin, embedded in paraffin, and cut into 4 μm-thick sections using a microtome and stained with hematoxylin and eosin (HE), giemza, Zeel-Neelsen (ZN), and Periodic acid-Schiff (PAS).

### 2.3. Molecular analyses of the Cryptosporidium

Fecal specimens from both lizards were placed in separate vials containing 2.5% aqueous potassium dichromate ($K_2Cr_2O_7$) solution and were submitted to molecular analyses at Nippon Veterinary and Life Science University, Tokyo, Japan.

Total genomic DNA was extracted using PowerSoil DNA Isolation Kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions and used as a template for PCR analysis. For genotyping of the *Cryptosporidium* species, a nested PCR assay that targeted the partial fragment of the nuclear small subunit ribosomal RNA (SSU) gene was amplified as previously described by Sevá Ada et al. (2011). The nested PCRs targeting the partial fragment of actin and 70-kDa heat shock protein (HSP70) genes were amplified and sequenced using specific primers for subtyping analysis (Sulaiman et al., 2000, 2002). Primary and secondary PCR reactions were carried out in a volume of 25 μl and 50 μl respectively, containing 10 × Ex Taq buffer, 2.5 mM of each dNTP mixture, 400 nM of forward and reverse primers, 5 units of TaKaRa ExTaq (TaKaRa Shuzo Co. Ltd., Otsu, Japan), and 1 μl of DNA template or primary PCR product. Amplification of the SSU gene involved the following, the templates were subjected to an initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 45 s, 60 °C for 45 s (for the primary amplification) or 56 °C for 90 s (for the second amplification), and 72 °C for 60 s, a final extension at 72 °C for 5 min. Furthermore, to determine the primary reaction of actin and the HSP70 genes, the reaction conditions were an initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 60 s, a final extension at 72 °C for 10 min. The second amplification was altered slightly and the annealing temperature was 45 °C for actin and 55 °C for HSP70. The PCR products from the second amplification were analyzed using 1.5% agarose gel electrophoresis and sequenced with Applied Biosystems 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA) at Macrogen Japan (Kyoto, Japan) using the secondary primers.

Sequence similarity was determined using BLAST analysis from the National Center for Biotechnology Information website. In order to construct a phylogenetic tree, the present sequences were aligned with reference sequences obtained from DDBJ/ENA/GenBank using MAFFT version 7 online service with the option Q-INS-I setting (Katoh and Standley, 2013), followed by a manual edit. The concatenated dataset of SSU, actin, and HSP70 genes sequences were then aligned with the reference (Holubová et al., 2016). Selection of the optimum DNA/Protein models using the MEGA version 7.0 software was the first step of phylogenetic analyses (Kumar et al., 2016). Then the phylogenetic tree was inferred by neighbor-joining (NJ) and the maximum likelihood (ML) methods. All positions contained gaps and the missing data were eliminated. Bootstrap support for branching was based on 1000 replicates.

The partial nucleotide sequence of the SSU, actin, and HSP70 gene sequences for *Cryptosporidium* sp. detected from the Arabian blue mastigure have been deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers, LC416466 (401bp), LC416467 (1,831bp) and LC416468 (918bp).

## 3. Results

### 3.1. Histopathological examination

Histopathological examination of the gastrointestinal tract of the Arabian blue mastigure revealed catarrhal enteritis with villous atrophy and edema of the muscle layer of the small intestine (Fig. 1A). There were *Cryptosporidium*-like structures on the intestinal mucosal surface (Fig. 1B). These structures were approximately 6 μm in diameter, spherical to subspherical in shape, and were positive in ZN and PAS

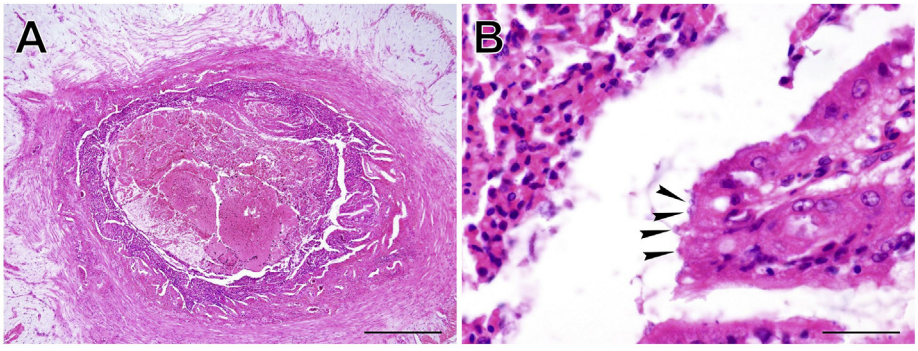


**Fig. 1.** Histological findings of the deceased Arabian blue mastigure. (A) jejunoileum showing villous atrophy, muscle layer edema, and degenerated epithelial cells in the lumen. Bar = 500 μm. (B) *Cryptosporidium* appear as rounded purple structures (arrowheads) on the microvilli of the epithelial cells. HE staining. Bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

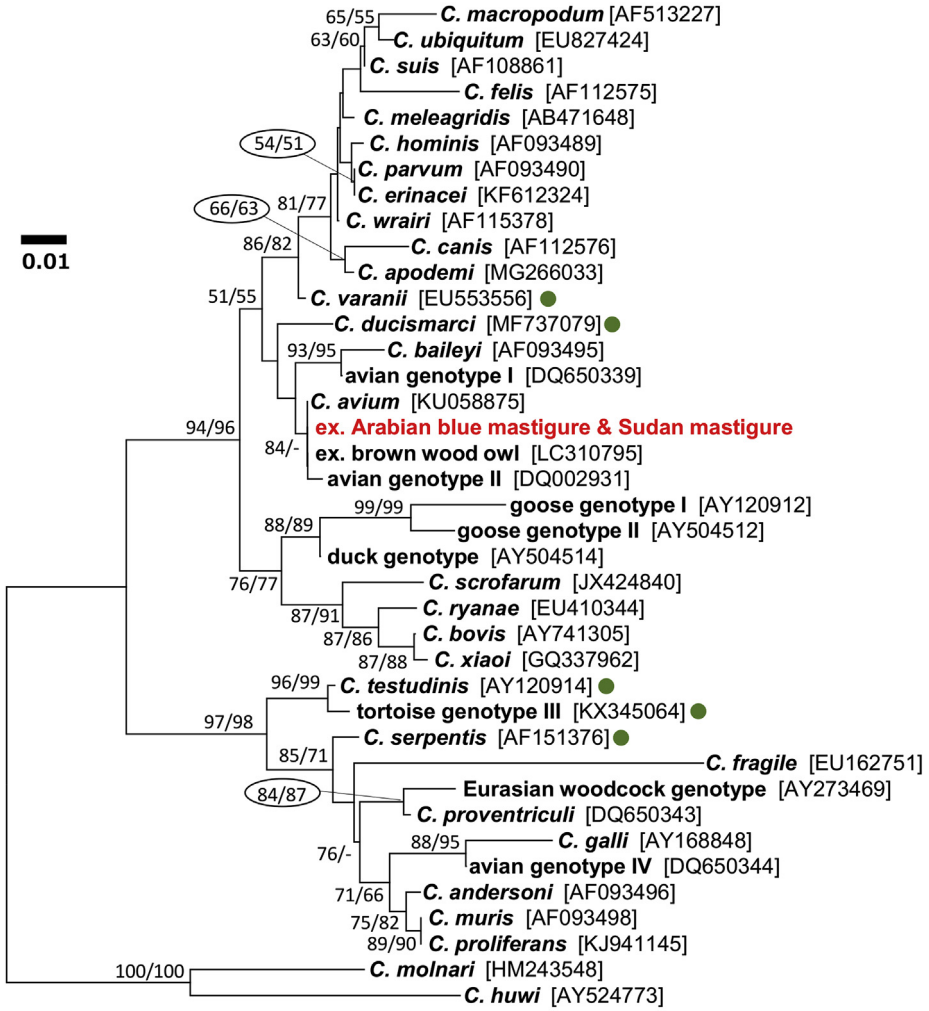


**Fig. 2.** Phylogenetic relationships between *Cryptosporidium* sp. from the mastigures and other *Cryptosporidium* spp. The mid-point tree was generated with the neighbor-joining method using Tamura 3-parameter plus Gamma distribution. Nodal values represent boot strap values (> 50%) for neighbor-joining (left) and ML (right). Circle represents the major reptile-associated species. Bar represents the number of nucleotide substitutions per sites.

staining.

### 3.2. Sequence analysis of Cryptosporidium spp. from reptiles

The partial fragment of the SSU gene (401 bp) was successfully amplified and sequenced in both of the lizards. SSU sequences from two lizards were identical to each other and showed 100% identity (401/401 bp) with the *Cryptosporidium* sp. (accession no. LC310795) from a brown wood owl (*Strix leptogrammica*) in Japan (Makino et al., 2018). Identities of 99.8% (400/401 bp, derived from a single adenine insertion) for *C. avium* (accession nos. KU058875 and JX548299) and avian genotype II (accession nos. KU058875 and KJ487974) were also noted. Phylogenetic analyses using SSU sequences showed that the *Cryptosporidium* from the mastigure and brown wood owl and *C. avium* were monophyletic and placed within a clade of avian-associated *Cryptosporidium* clade (Fig. 2).

Partial nucleotide sequences of the actin (1831 bp) and HSP70 (918 bp) genes were successfully amplified and sequenced from the Arabian blue mastigure. The sequence at the actin gene was 99% identity (906/918bp) with *Cryptosporidium* sp. from the brown wood owl (accession no. LC310796) and *C. avium* from the common ostrich (*Struthio camelus*) (accession no. AB696815) and red-crowned parakeet (*Cyanoramphus novaezelandiae*) (accession no. KU058882). The sequence of the HSP70 gene showed 98.4% (1801/1831 bp) to 98.3% (1798/1829bp) identities with *C. avium* from several pet birds (accession nos. JQ798893, AB471665, MK311173). Moreover, an identity of 97.6% (1787/1831 bp) was found between this isolate and the brown wood owl (accession no. LC310797). The phylogenetic tree using concatenating data across multiple sequences of SSU, actin, and HSP70 genes revealed that the present species clustered with the *Cryptosporidium* species recorded from the brown wood owl, and this clade appeared to branch near the monophyletic *C. avium* clade (Fig. 3).

## 4. Discussion

In this study, the deceased Arabian blue mastigure displayed typical symptoms (diarrhea, vomiting, and oocyst excretion) of intestinal cryptosporidiosis. Although no viral or bacterial examinations were performed, histopathological findings supported this diagnosis. The prognosis of the Sudan mastigure that discharged *Cryptosporidium* oocysts is not currently known.

Molecular analyses showed one nucleotide insertion/deletion in the SSU gene between the sequences of the mastigures and the reference sequence for *C. avium*. However, one or two nucleotide substitutions or insertions/deletions were found in the other *Cryptosporidium* species or genotypes (Ryan and Xiao, 2014); thus, we have not proposed the present species as a novel *Cryptosporidium* genotype and have designed this species as *Cryptosporidium* cf. *avium*.

*Cryptosporidium avium* has been reported from psittacine birds such as budgerigars, cockatiels, red-crowned parakeets, and galliform birds

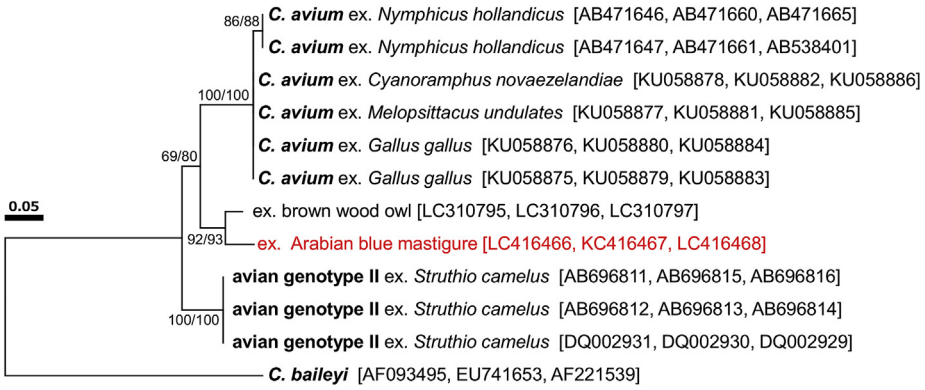


**Fig. 3.** Phylogenetic relationships of *Cryptosporidium* detected from the Arabian blue mastigure and closely-related species/subtypes as inferred by a maximum likelihood analysis of concatenated sequences were constructed from the partial DNA sequences of SSU (left), actin (middle), and HSP70 (right) loci. Numbers represent the boot strap values for NJ (left) and ML (Right). Bar represents the number of nucleotide substitutions per sites. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

such as chickens (Abe and Makino, 2010; Qi et al., 2011; Curtiss et al., 2015; Nakamura and Meireles, 2015; Li et al., 2016). Recently, a novel *Cryptosporidium* variant which is closely related to *C. avium* was reported in a brown wood owl (Aves: Strigiformes) (Makino et al., 2018). However, it has not been explicated whether *C. avium* can infect humans and animals other than birds and reptiles. *Cryptosporidium* species reported from the green iguanas (Reptilia: Squamata) appeared to be *C. avium* because the 124 bp of the SSU gene determined from the iguana was identical to *C. avium,* and differentiated from other species including reptile-associated *Cryptosporidium* species/genotypes such as *C. serpentis* and *C. varanii* (Kik et al., 2011). In addition to this, our finding was the first detection of avian-associated *Cryptosporidium* species in the family Agamidae (Reptilia: Squamata) and suggested that host specificity has a wider spectrum than previously thought. While substantial sequence divergences of actin and the HSP70 genes were observed between the present species from mastigure and that of birds, the monophyletic group implied the presence of several *C. avium* variants. Interestingly, although most birds infected with *C. avium* showed no clinical signs of cryptosporidiosis (Ng et al., 2006; Holubová et al., 2016), green iguanas showed colitis and cystitis (Kik et al., 2011). Genetic diversity of *C. avium* and the potential for pathogenesis to reptilian hosts will need to be examined in the future.

*Cryptosporidium* species was transmitted by ingestion or inhalation of oocysts excreted in the feces of infected animals. The infection can; therefore, be transmitted from animal to animal by contact with or in an environment contaminated with feces or via ingestion of contaminated water or food. Furthermore, the owner stated that the two lizards kept indoors without pet birds and not fed with birds (quail and chick) as food; and thus the transmission routes cannot be identified.

In summary, this report represents the novel variant of *C. avium* from imported mastigures. Further studies focusing on experimental cross-transmission are needed to define the host preference, host range, and pathogenic potential of this novel *C. avium* variant.

## Declaration of competing interest

None.

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