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Molecular and histopathological features of *Cryptosporidium ubiquitum* infection in imported chinchillas *Chinchilla lanigera* in Japan

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ABSTRACT

Long-tailed chinchillas *Chinchilla lanigera* are popular rodent species kept both in households, where they are hand-raised as pets, and in zoological facilities. From January 2016 to February 2017, 13 juvenile chinchillas from five facilities in Japan were diagnosed with cryptosporidiosis at the animal hospital. Eight of the cases were fatal. All of the animals were imported from the Czech Republic by the same vendor. Histopathological and multilocus sequence analyses using 18S ribosomal RNA, actin, 70-kDa heat shock protein, and 60-kDa glycoprotein genes confirmed *Cryptosporidium ubiquitum* of subtype XIId as the etiological agent. Multilocus analysis demonstrated the presence of two new sequence types closely related to the *C. ubiquitum* Xlld strain isolated from a human in the USA. This study indicated that potentially zoonotic *Cryptosporidium* is widespread and may have caused a high number of deaths among imported juvenile chinchillas.

1. Introduction

The long-tailed chinchilla *Chinchilla lanigera* is a small nocturnal rodent belonging to the family Chinchillidae. The historic distribution of this species extends throughout the arid, barren, and rugged areas connecting the coastal mountain ranges and the Andes of South America. Since their population continues to decline due to the activities of humans, the international trade of wild chinchillas is prohibited [1]. Thus, all chinchillas available in the legal pet trade are bred and raised on farms, or by private breeders. Nearly every year, thousands of captively bred chinchillas are imported from other counties into Japan without quarantine. The growing international trade of these animals is contributing to the emergence and spread of infectious diseases via transmission of the pathogens carried by the imported animals.

Cryptosporidium is a genus of coccidian protozoan parasite of medical and veterinary importance and is the etiological agent for cryptosporidiosis. Currently, > 30 *Crypsotporidium* species are recognized and are capable of infecting a wide range of vertebrate animals, and causing mild to severe gastrointestinal or respiratory diseases in their host species [2–4]. One species of interest is *Cryptosporidium ubiquitum* Fayer, Santín and Macarisin, 2010 has previously been identified as the *Cryptosporidium* cervine genotype, less frequently as cervid or W4 genotype, and is considered to be an emerging zoonotic pathogen [5]. This species has been reported in various countries to infect both wild and domestic ruminants; rodents; carnivores; marsupials; and primates, including many human cases [5–7].

In the present study, we report the clinical, histopathological, and molecular features of cryptosporidiosis caused by *C. ubiquitum* in juvenile chinchillas imported into Japan. Our results serve to alert veterinarians; workers in the pet trade; animal care and service workers at zoos, animal shelters, and pet stores; and pet owners who may be exposed to imported chinchillas regarding the threat of *C. ubiquitum* infection and the development of cryptosporidiosis.

2. Materials and methods

2.1. Animals and specimens

The animals in the study included 13 juvenile chinchillas (IDs: 1604–1611, 1702–1706) taken to Banquet Animal Hospital, Tokyo, Japan from January 2016 to February 2017. The animals were all imported from the Czech Republic by the same vendor with the intent to be sold as pets and had been housed in five different facilities (Table 1). Fresh feces were collected from each animal and placed in separate vials containing 70% ethanol or 2.5% aqueous potassium dichromate ($K_2Cr_2O_7$) solution, and stored at 4 °C. In order to investigate the

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Table 1
Characteristics of thirteen *Cryptosporidium* infections in juvenile chinchillas.

Case IDs	locality	Date of diagnosis	Initial and subsequent clinical signs	Duration of illness	Outcome
1604	Saitama	Jan, 2016	Anorexia and diarrhea	several days	N/A
1605	Saitama	Jan, 2016	Rectal prolapse, diarrhea, and intussusception	several days	Death
1606	Saitama	Oct, 2016	Loss of vigorous prostration, diarrhea	several days	Death
1607	Saitama	Oct, 2016	Asymptomatic (health check)	–	N/A
1608	Tokyo A	Oct, 2016	Diarrhea	several days	Death
1609	Saitama	Oct, 2016	Loss of vigorous prostration, and diarrhea	8 days	Death
1610	Saitama	Nov, 2016	Sudden death	–	–
1611	Tokyo A	Nov, 2016	Sudden death	–	–
1702	Saitama	Nov, 2016	Anorexia, diarrhea	4 days	N/A
1703	Saitama	Dec, 2016	Asymptomatic (health check)	–	N/A
1704	Kanagawa A	Dec, 2016	Rectal prolapse and diarrhea	2 days	Death
1705	Tokyo B	Jan, 2017	Anorexia and diarrhea	10 days	N/A
1706	Kanagawa B	Feb, 2017	Anorexia, diarrhea, and rectal prolapse	2 days	Death

N/A: not available.

prevalence of *Cryptosporidium* infection among captively bred chinchillas imported from other geographical areas, 50 fecal specimens collected from 45 chinchillas (30 juvenile, 15 adult) imported from the USA, and five adult chinchillas from the Netherlands were placed in separate vials containing 70% ethanol and stored at 4 °C. No ethical approval was obtained because this study did not involve animal experiments and only used clinical specimens, obtained non-invasively from animals.

2.2. Pathological examination

For histopathological analyses, two dead animals (animal IDs: 1605 and 1608) were autopsied. The brain, esophagus, intestines, kidney, heart, lung, liver, spleen, and adrenal glands were dissected from each animal, fixed in 10% neutral-buffered formalin, embedded in paraffin, cut at 4 μm-thick sections using a microtome, and stained with hematoxylin and eosin (HE). Specimens were analyzed by light microscopy.

Intestinal tissues were also analyzed by immunofluorescence assay (IFA). For IFA, *Cryptosporidium* oocysts in the formalin-fixed paraffin-embedded sections of intestines were stained with EasyStain (BTF, North Ryde, Australia), which contained fluorescein isothiocyanate (FITC)-labeled antibodies specific for *Cryptosporidium* and *Giardia.* Conventional methods were used for staining. Fluorescence signals in the sections were visualized using a BZ-X700 fluorescence microscope (Keyence, Osaka, Japan).

2.3. DNA extraction, PCR, and sequencing

The fecal specimens preserved in ethanol or $K_2Cr_2O_7$ were used for molecular analyses. Oocysts collected from the feces were washed with double-distilled water, purified using sucrose centrifugal flotation, and subjected to three freeze-thaw cycles in transfers between a − 70 °C freezer and a 56 °C heat block in order to ensure efficient lysis. Total genomic DNA was extracted using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions. The isolated DNA was used as template for PCR analysis.

PCR analysis included amplification of the 18S ribosomal RNA (18S), actin, and the 70-kDa heat shock protein (HSP70) genes using nested PCR as previously described for identification of *Cryptosporidium* species [8–11]. In general, PCR amplification was performed in a 25 μl volume containing 1 μl of each forward and reverse primers (10 μM), 12 μl of PCR mixture (OnePCR, GeneDirex, USA), 10 μl of double distilled water, and 1 μl of template. For the nested amplification, 1 μl of the first PCR product was added to 49 μl of a second PCR containing 25 μl of OnePCR mixture, 22 μl of double distilled water, and 1 μl of each of the internally nested forward and reverse primers. Cycling for both rounds of amplification was performed at 94 °C for 5 min for initial template denaturation, followed by 30 or 35 cycles of 94 °C for 45 s, the specific annealing temperature for each primer set, and 72 °C for 1 min for product extension. A final extension was performed at the end of the cycling profile at 72 °C for 7 min. Detailed information regarding the primers, amplification cycling conditions, and the appropriate references are shown in Supplemental table 1. The PCR products from the second amplification were purified and sequenced at Macrogen Japan (Kyoto, Japan) using the same primers that were used during the secondary PCR amplification.

The DNA samples were also subjected to multilocus sequence typing (MLST) of the 60-kDa glycoprotein (GP60) gene, and the four genetic loci of cgd6_1590, cgd6_60, cgd2_3690, and cgd4_370 [12–14]. The sequencing results were used for detecting intra-subtype variability of *Cryptosporidium* species.

2.4. Sequence analyses

Sequence data obtained for the 18S, actin, HSP70, and GP60 genes were aligned using the ClustalW with initial fixed parameter values in Molecular Evolutionary Genetics Analysis (MEGA) (https://megasoftware.net/home), version 7.0 software [15]. Sequence similarity was determined using BLAST analysis from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/Blast.cgi).

Naming of the four genetic loci cgd6_1590, cgd6_60, cgd2_3690, and cgd4_370 for MLST analysis was done in accordance with the nomenclature used for the allelic profile of *C. ubiquitum* [14] and the MLST sequence type was then determined by the combination of alleles identified. The gene sequences obtained for the four loci were concatenated and aligned using MEGA. Phylogenetic trees were constructed using neighbor-joining (NJ) [16] and maximum likelihood (ML) methods [17] by MEGA. A substitution model and optional parameter sets were determined using the Tamura-Nei model [18], and selected according to the Akaike information criterion (AIC), respectively. Since there was no sequence for use as an out-group sequence for the four genetic loci, midpoint rooting was used to estimate phylogenetic relationships. The phylogenetic tree was evaluated using bootstrap methodology based on 1000 replicates for both trees. Representative nucleotide sequences generated in the study have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC3340000 (18S), LC3340001 (actin), LC3340002 (HSP70), LC3340004 (GP60), LC3340005 (cgd2_3690), LC3340006- LC3340007 (cgd4_370), LC3340008 (cgd6_60), and LC3340009 (cgd6_1590).

3. Results

3.1. Diagnosis

Following delivery to the animal hospital, the chinchillas were

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