ORIGINAL ARTICLE

First Characterization in China of Encephalitozoon cuniculi in the Blue Fox (Alopex lagopus)

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ABSTRACT

Encephalitozoon cuniculi is a microsporidian parasite that infects a wide range of vertebrates, including primates. It has recently emerged as an opportunistic parasite of patients infected with the human immunodeficiency virus. The blue fox (Alopex lagopus; also known as the arctic fox) is one of the most susceptible species for encephalitozoonosis. Here, we report an outbreak of encephalitozoonosis at a fox farm in China. The isolated parasites displayed the typical morphology of E. cuniculi as assessed by Masson’s trichrome staining. Analysis of the internal transcribed spacer sequence indicated that the isolated parasite is a genotype III strain of E. cuniculi. Furthermore, phylogenetic analysis of the PTP1 gene verifies classification of this new strain (termed LN-1) with other genotype III E. cuniculi strains, though the PTP3 and SWP1 sequences diverge from the reference strain. This is the first report of encephalitozoonosis in farmed blue foxes in China.

ENCEPHALITOZOON Cuniculi belongs to the phylum Microspora, a group of obligate intracellular parasites. This parasite has been found to infect a wide host distribution that includes rabbits, mice, rats, guinea pigs, ground shrews, goats, sheep, pigs, horses, domestic dogs, wild and captive foxes, domestic cats, a variety of exotic carnivores, and primates (Canning and Lorn 1986). The damage caused by this parasite is minimal for healthy humans, but infection with the parasite can cause severe complications in immunosuppressed individuals such as AIDS patients (Didier et al. 1996; Weber et al. 1994). Many E. cuniculi surveillance studies have described samples isolated from animals and humans in South and North America, Europe, Africa, Korean, and Japan (Akerstedt et al. 2002; Ashmawy et al. 2011; Cray and Rivas 2013; Didier et al. 1995; Igarashi et al. 2008; Lallo et al. 2012; Lee et al. 2011; Mathis et al. 1996; Murphy et al. 2007; Reetz et al. 2009). At present, three genotypes of E. cuniculi, which can be discriminated based on the number of GTTT repeats in the internal transcribed spacer (ITS) region of the rDNA gene have been identified: genotype I with three repeats, genotype II with two repeats, and genotype III with four repeats (Xiao et al. 2001).

Foxes are very susceptible to E. cuniculi infection (Mohn et al. 1974). Generally only fox pups display symptoms, such as stunted growth, blindness and occasionally, sud-
den premature death. *Encephalitozoon cuniculi* spores are able to persist for a long time in the environment, particularly in water sources. This pathogen can cause serious economic loss and is very difficult to clear from a farm once it affects a fox population (Akerstedt 2002). In China, *E. cuniculi* has been isolated from rabbits. However, no report on the outbreak caused by this pathogen in the blue fox has been issued, and little is known about the prevalence of the disease in blue foxes. In this article, we report an outbreak of *E. cuniculi* at a blue fox farm in northeast China. To our knowledge, this report is the first study to describe the identification of *E. cuniculi* (genotype III) in China.

**MATERIAL AND METHODS**

**Clinical and pathological examination**

In the autumn of 2012, an outbreak of an infectious disease occurred in a fox farm located in Liaoning province of northeast China. This farm maintained about 1,000 foxes and 80% of them are pups. In this outbreak, around 50% of the pups were affected by this disease as described by the farmer. Approximately 50% of the infected pups died, and others had stunted growth.

Dead foxes were dissected to observe the pathological changes. A fox with clinical symptoms was sacrificed for parasite preparation. The blood was collected to prepare serum for the indirect fluorescence antibody test (IFAT). All the experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute (permit number: 20122054).

**Parasite purification and cultivation**

The *E. cuniculi* spores (designated as strain LN-1) were extracted as described previously with some modification (Mathis et al. 1996). Briefly, the fox kidneys were cut into small pieces, mechanically homogenized with a mortar and pestle, and passed through a 70-µm cell strainer (BD Falcon™; Corning Incorporated, Durham, NC). The cell suspensions were washed once with phosphate-buffered saline (PBS), and the sediment was resuspended in 10 ml HCl (6 mM). After incubation for 10 min at room temperature (RT), the sediment was washed twice with minimum essential medium (MEM; Gibco, Basel, Switzerland). The pellet was resuspended and inoculated into a monolayer of Madin–Darby canine kidney (MDCK) cells in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 100 U/ml penicillin, 100 mg/ml streptomycin, and 100 U/ml L-glutamine. The cultures were grown in a humidified incubator (5% CO₂ at 37 °C). The remaining suspension was centrifuged again to harvest the pellets for DNA extraction. The culture medium was replaced weekly, and the cultivation process was visually monitored using an inverted microscope.

**Light microscopy**

A kidney smear was prepared for Gram staining. In addition, after the culture was maintained for about 1 mo through several passages, a 6-well plate was inoculated with the culture medium containing free spores, incubated for 4 d, and stained with a Masson’s trichrome staining kit (Right Tech; Changchun Thermo Right, Changchun, China) according to the manufacturer’s instructions. The stained slides and plates were observed under a light microscope.

**Confocal microscopy**

Freshly harvested *E. cuniculi* was added to glass slides coated with 1% poly-o-lysine and used for IFAT. The slides were incubated at 55 °C for 40 min and briefly rinsed in PBS, fixed with 4% PBS-buffered paraformaldehyde for 15 min, and then rinsed in PBS again. After blocking in 5% BSA in PBS (blocking solution) overnight at 4 °C, the slides were incubated with positive serum (collected from the fox used for the parasite isolation) diluted in the blocking solution at 37 °C for 1 h. The slides were then washed three times with PBS and incubated with a rabbit anti-fox polyclonal antiserum (prepared in our lab) for 1 h at 37 °C. After washing three times with PBS, a goat anti-rabbit antibody conjugated to Alexa 488 (Molecular Probes, Inc., Eugene, OR) was applied to the slides for 1 h at 37 °C. After washing three times with PBS, the slides were mounted with a mounting medium (Vector Laboratories, Inc., Burlingame, CA).

**PCR assay**

The DNA was extracted from the kidneys and the spores from cultured cells using a TIAN amp Genomic DNA Kit (TIANGEN, Beijing, China) according to the manufacturer’s instructions. The extracted DNA was stored at −30 °C until use. The SSU rRNA gene sequence and the ITS region of the rDNA of *E. cuniculi* were amplified using the primers described by Mathis et al. (1996). The full length sequence of the polar tube protein gene 1 and 2 (PTP1and PTP2) without signal peptides, the partial sequence of polar tube protein gene 3 (PTP3) and the partial sequence of spore wall protein 1 (SWP1) were also cloned with the specific primers shown in Table 1. PCR was performed in a 50 µl-reaction mixture, containing 2 µl of the extracted DNA, 25 pmol of each primer, 200 µM of each deoxyribonucleoside triphosphate, and 1 U of KOD–Plus DNA polymerase (Toyobo Co., Osaka, Japan). The PCR reactions included: an initial 5 min at 95 °C to activate the KOD DNA polymerase; 30 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and extension at 68 °C for 1 min; and a final 7-min extension at 68 °C.

**DNA sequencing**

The PCR products were purified using a QIA quick Gel Extraction Kit (QIAGEN, Hilden, Germany) and ligated into
PMD 18T vector (TaKaRa Biotechnology (Dalian) Co., Dalian, China) for subsequent transformation into *Escherichia coli* DH5α competent cells. Three plasmid vectors with the insert were purified from different clones using the QIA prep Spin Miniprep Kit (QIAGEN) and then sequenced. GENETYX version 7.0 software (Software Development, Tokyo, Japan) was used for preliminary sequence alignment and analysis.

The sequences used for comparison by ITS analysis include two reference sequences from *E. cuniculi* strain II (accession number: GU213880 and JF792398); three sequences from genotype I (accession number: GU198947, AL391737, and GU198946); two sequences from genotype III (accession number: EU001242 and JQ340012); and two sequences of unknown genotype (accession number: GU735480 and JQ340013).

A phylogenetic tree of PTP1 was constructed using the neighbor joining method with bootstrap confidence using MEGA version 5.0 (Tempe, AZ). The sequences used for the PTP1 phylogram include three sequences from genotype I (accession number: AF310677, NM001041403, and AJ005666), three sequences from genotype II (accession number: EU282236, EU282237, and AF310678), and two sequences from genotype III (accession number: AB182700 and AF310679).

### RESULTS

**Clinical, pathological, and morphological observation of a parasite infecting a fox farm in China**

An outbreak of encephalitozoonosis occurred at a fox farm in China whose primary industry was the blue fox (*Alopex lagopus*; also known as the arctic fox). Anepithymia, convulsion, and blindness were typical signs in the affected foxes (Fig. S1). Renal lesions were observed in most foxes that the farmer submitted for inspection (Fig. S2).

To identify the pathogen associated with the outbreak, kidney smears were stained by the Gram staining method. gram-positive microorganisms were observed under a light microscope (Fig. 1A). To further identify the bacterial species, spores were grown in MDCK cells and then were stained with the Masson’s trichrome staining kit. Microsporidia spores displayed a noticeable color reaction (pinkish to red) and exhibited distinguishable shape characteristics under light microscopy (Fig. 1B). These results suggest that the outbreak was caused by protozoa of the phylum Microsporidia.

**Examination of the cultivated parasite by IFAT**

One of the most common pathogenic species of Microsporidia, which causes encephalitozoonosis in foxes and can also be spread to humans, is *E. cuniculi* (Canning and Lorn 1986). To verify that *E. cuniculi* represents the pathogen responsible for the outbreak, IFAT was performed using immobilized purified *E. cuniculi* and the serum from the encephalic animal. Spores with strong green fluorescence and typical shape of *E. cuniculi* were detected under a laser-confocal microscope, further verifying the morphology (Fig. 1C). These results verify the presence of anti-*E. cuniculi* antibodies in the serum of an infected fox.

### Table 1. Primer sequences for the PCR assay

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer sequences</th>
</tr>
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<tbody>
<tr>
<td>SSU rRNA</td>
<td>F (5'-CACCAGTTGATTCTGCTC-3')&lt;sup&gt;1&lt;/sup&gt; R (5'-TTATGATCCTGCTAATTGTTCTC-3')&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ITS1</td>
<td>F (5'-CACCAGTTGATTCTGCTGCTG-3') R (5'-CCTCCCGGAAACCAACCTCG-3')</td>
</tr>
<tr>
<td>SWP1</td>
<td>F (5'-CTGAGACAGGTTGATTCTGCTG-3') R (5'-AGATTTATTAGGAGACTCCACACATT-3')</td>
</tr>
<tr>
<td>PTP1</td>
<td>F (5'-CTGAGACCCTGAGAGCAATTGATGATG-3') R (5'-GAATTCTCTACGAGCTGAGCGATGTT-3')</td>
</tr>
<tr>
<td>PTP2</td>
<td>F (5'-CTGAGACCTGAGCTGAGCTGAGCTGAGCTG-3') R (5'-GAATTCTCTACGAGCTGAGCGATGTT-3')</td>
</tr>
<tr>
<td>PTP3</td>
<td>F (5'-CTGAGATCCGGAGGCAATCCACAG-3') R (5'-GAATTCTCTACGAGGTCCTCGG-3')</td>
</tr>
</tbody>
</table>

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Figure 1 Microscopic observation of *Encephalitozoon cuniculi*. **A.** Gram staining of *E. cuniculi* in a kidney smear. The gram-positive *E. cuniculi* was stain with purple color. **B.** Masson’s trichrome staining of *E. cuniculi* in a cultured MDCK cell monolayer. The pink structures are the microsporidian spores that measured approximately 1–2 μm. **C.** IFAT of *E. cuniculi* under a confocal laser microscope.
animal, which provides further evidence of the role for this pathogen in causing the infections.

**Genotyping and DNA sequence analysis of cultured *E. cuniculi***

To further characterize the novel strain of *E. cuniculi* infecting the foxes, the sequences of the ITS1 and SSU rRNA were amplified from genomic DNA samples of the new strain (designated LN-1) to produce fragments of 1,300 bp and 1,174 bp respectively. We also amplified the PTP1, PTP2, PTP3, and SWP1 genes to produce 1,038, 786, 1,321, and 1,068 bp fragments, respectively. These sequences were evaluated for homology against previously reported *E. cuniculi* sequences in the GenBank database using a simple BLASTN search. The SSU rRNA sequence of the pathogen shares 99% identity with a sequence (GenBank: L17072) obtained from a rabbit isolate (only one mutation from A to T at 717 bp). The ITS region of this isolate contains four 5'-GTTT-3' repeats, which is characteristic of *E. cuniculi* genotype III strains (Fig. 2A). The isolated PTP1 sequence is most similar to that of CDC. V282, a genotype III strain isolated from a human (Fig. 2B). The PTP2 sequence obtained in this study is identical to that of the GB-M1 strain. The PTP3 sequence contains three synonymous nucleotide substitutions compared to the GB-M1 strain. The amplified SWP1 sequence carries two amino acid differences and is shorter than the reference strain GB-M1 (Fig. S3). The isolated sequences have been submitted to the GenBank database with the accession numbers KF169726 (PTP1), KF169727 (PTP3), KF169728 (SWP1), and KF169729 (SSU rRNA).

**DISCUSSION**

In this study, we isolated a new strain of *E. cuniculi* (LN-1) from the kidney of a blue fox in Northeast China. The parasite cultured in MDCK cells was identified by both Mason's trichrome staining and IFAT. The isolate was confirmed to represent a new strain of *E. cuniculi* by genotyping. The amplified SWP1 sequence harbors more nucleotide substitutions and is shorter than the reference strain. Therefore, the well-conserved PTP2 might be most suitable for use in a serological diagnostic test. Furthermore, the ITS sequence suggests that the pathogen is a genotype III strain. However, the relationship between the isolate and other reported parasites could not be investi-
gated comprehensively because genetic data for *E. cuniculi* have not been well established. Nevertheless, our data might contribute to future analysis of *E. cuniculi* evolution as new strains are revealed.

We isolated the parasite for the kidney of an infected fox, but other possible infection sites were not examined in this study. Little is known about the infective mechanisms of this parasite in the fox, and further examination of the parasite distribution in its host might be helpful to understand the pathogenic mechanism and transmission route.

Spores of *E. cuniculi* are highly resistant in the environment, particularly in water sources, where they can survive for several months to years, and new hosts can become infected without direct contact with infected animals or humans. Therefore, this parasite is very difficult to eliminate from an animal population. For example, efforts to control an outbreak of encephalitozoonosis in a rabbit colony at a zoo in Japan failed. All remaining rabbits were finally slaughtered and the facility was closed (Igarashi et al. 2008). For the current study, the disease may have spread through the fox farm over several years. At first only litters were affected, but more and more pups of new litters became infected. Eventually, at least 50% of pups died, and stunted growth was observed in most of the remaining pups. The farmer finally had to kill all of the female foxes because of a lack of effective control measurements.

Although most of the world’s farmed fur is produced by European farmers, there are still approximately 1.5 million farmed foxes in China. Despite the economic burden of fox disease, little research has examined the disease distribution in the fox. Foxes are highly susceptible to *E. cuniculi* infection (Mohn et al. 1974), but this disease has not been reported over the last few decades in China. An encephalitozoonosis outbreak in the blue fox with a high mortality rate and substantial economic loss was documented in Finland in 2002 (Akerstedt et al. 2002). After the outbreak we reported here, the farmer had to kill all vixens, which give birth to the infected pups. Therefore, the isolation of *E. cuniculi* in a blue fox in China highlights the importance of performing epidemiological surveys of the farmed fox populations.

We have presented the first report of *E. cuniculi* (type III) infecting the blue fox (*A. lagopus*) in China. Genotype III strains have been identified as causing encephalitozoonosis in both dogs and humans, and this pathogen thus has zoonotic potential (Mathis et al. 2005; Snowden et al. 1999). Given the potential detriment to humans and other animals, as well as the potential for economic burden, this information may be useful in understanding the pathogen and preventing its spread.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** A fox with clinical signs of blindness.
**Figure S2.** Renal lesions observed in the kidney of a fox suspected of infection.
**Figure S3.** Alignment of SWP1 amino acid sequences.