FULL PAPER *Parasitology*

Detection and Genotype of *Encephalitozoon cuniculi* DNA from Urine and Feces of Pet Rabbits in Japan

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ABSTRACT. A newly developed nested polymerase chain reaction (PCR) assay targeting the internal transcribed spacer (ITS) region of the ribosomal DNA was applied to detect and characterize *Encephalitozoon cuniculi* DNA from pet rabbits in Japan. The analysis was carried out using 257 urinary samples and 314 fecal samples collected from 307 pet rabbits in the age group of 1 month to 12 years from 30 different prefectures of Japan and 107 fecal samples and 3 urinary samples collected from 1-month-old rabbits from 3 breeding facilities in Japan. We detected 840-bp amplicons in 20 urinary samples (7.78%) from the pet rabbits of the 13 prefectures and in 1 urinary (33.3%) and 6 fecal (5.6%) samples from the rabbits of the 2 breeding facilities. The sequences (803 bp) of the 27 amplicons had no variations and completely coincided with the sequence of *E. cuniculi* genotype I. To the best of our knowledge, this is the first report on the detection and genotype characterization of *E. cuniculi* DNA from pet rabbits in Japan.
KEY WORDS: *Encephalitozoon cuniculi*, genotype, Japan, nested-PCR, pet rabbits.

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Encephalitozoon cuniculi is an obligate intracellular parasite that is classified as a microsporidium, although this group is currently considered to comprise highly derived fungi descended from a zygomycete ancestor [15]. The organism mainly parasitizes rabbits and causes encephalitozoonosis with clinical signs of the disorders in the central nervous system, kidney and eye, which sometimes causes lethal damage to the animals. Furthermore, the organism has also been detected in other mammalian hosts, including rodents, horses, carnivores, non-human primates and humans [2, 16]. *Encephalitozoon cuniculi* infections in rabbits show worldwide prevalence, and the seroprevalence has been reported to be 7.0% in breeding colonies in the U.S.A. [9], 59.2% in pet rabbits in England [10], 67.2% in pet rabbits in Italy [5] and 15% in farmed rabbits in Egypt [1]. In Japan, occasional cases of the infections have been reported since the 1990s [6, 12, 13, 17], and a high seroprevalence of *E. cuniculi* has been recently reported in pet rabbits [11].

Encephalitozoon cuniculi isolates from animal and human hosts are divided into 3 genotypes I, II and III on the basis of the differences in the nucleotide sequences of the internal transcribed spacer (ITS) region of ribosomal RNA (rRNA) gene [4]. Genotypes I, II and III are mainly detected in isolates from rabbits, mice and dogs, respectively. Genotypes I and III appear to have zoonotic potential, because these genotypes have been detected from humans as well as animals [16]. In Japan, although *E. cuniculi* genotype I has been isolated from a rabbit reared in a zoo and rabbits reared at a laboratory in an institute [7, 8], there is no report on genotype of *E. cuniculi* DNA in pet rabbits. This study was designed to detect *E. cuniculi* DNA and to characterize the genotype using urinary and fecal samples obtained from pet rabbits in Japan.

MATERIALS AND METHODS

Urinary and fecal samples of rabbits: We collected 257 urinary samples and 314 fecal samples from 307 healthy pet rabbits (age, 1 month to 12 years) in 30 different prefectures of Japan. In addition, 107 fecal samples and 3 urinary samples were collected from 1-month-old rabbits at 3 breeding facilities located in 3 prefectures. These samples were obtained between 2009 and 2010.

Total DNA preparation: Fecal samples from the pet rabbits were primarily treated by performing the sucrose floatation technique. Briefly, 1 g of the samples was dissolved in 0.05% Tween 80 solution and centrifuged at 2,000 × *g* for 10 min. Sucrose solution (specific gravity, 1.21) was added to the sediments, and the mixture was left undisturbed for 15 min. The surface layer possibly containing the *E. cuniculi* spores was collected using a looped needle and subjected to total DNA extraction by using the polymerase chain reaction (PCR) Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). One hundred microliters of total DNA was obtained from each sample. Fecal samples from young rabbits in the breeding facilities were used for total DNA extraction with no treatment of the sucrose floatation technique. The urinary samples (0.5–5 m*l*) were centrifuged at 3,000 × *g* for 20 min, and the pellets were used for the

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total DNA.

Total DNA extracted from the *E. cuniculi* spores (ATCC 50602), which were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.) and propagated *in vitro* by using Vero cells, were also used to evaluate the sensitivity of a nested PCR assay developed in this study. The spores were serially diluted 10-fold with Milli-Q water to adjust their number from 9×10^4 to 9×10^{-5}, and all the dilutions were separately subjected to DNA extraction using the PCR Template Preparation Kit. The *E. cuniculi* DNA was quantified using the Quant-iT^{TM} dsDNA High Sensitivity (HS) Assay Kit (Invitogen, Tokyo, Japan) and was diluted 10-fold with Milli-Q water to adjust its concentration from 2.2×10^2 to 2.2×10^{-7} *n*g/m*l*. The DNA solutions were then used for a nested PCR assay. Furthermore, *Encephalitozoon intestinalis* DNA (8.45×10^2 *n*g/m*l*) and *Encephalitozoon hellem* DNA (2.2×10^2 *n*g/m*l*) were extracted from the propagated spores of laboratory strains purchased from the American Type Culture Collection, and *Enterocytozoon bieneusi* DNA (2.2×10^2 *n*g/m*l*) was extracted from the spores isolated from feces of an AIDS patient. These DNA samples were then used to evaluate the specificity of a nested PCR assay.

Nested PCR assay and sequencing: PCR primers specific to the ITS region of *E. cuniculi* were designed using GENETYX ver.10 software (Genetics, Tokyo, Japan) on the basis of the nucleotide sequences of ribosomal RNA genes of *E. cuniculi* (accession no. AL391737.2), *E. intestinalis* (CY001952.1), *E. hellem* (AF272836.1) and *Enterocytozoon bieneusi*(AF023245.1). The primer set, used in the first PCR and generated about 1,200-base pair (bp) amplicons, was MK5 (5'-GTTGCGTAAGATGTGAGACCCTT-3') and MK6 (5'-CCTTCTCGCACACCATGCTTAC-3'), and the primers K4 (5'-TAGCGGCTGACGAAGCTGC-3') and K3 (5'-CATGTCATGCCATCGAG-3') were used in the second PCR and generated about 840-bp fragments. The PCR mixture had a total volume of 25 *µl* and contained 2 *µl* of total DNA, 5 *µl* of 5× Go*Taq* buffer (50 mM Tris-HCl, 50 mM NaCl and 5 mM $MgCl_2$), 0.2 *µl* (25 mM) of dNTP, 0.25 *µl* (50 pmol/*µl*) of each primer, 0.125 *µl* (1.25U) of Go*Taq* DNA polymerase (Promega, Madison, WI, U.S.A.) and 17.175 *µl* of Milli-Q water. Conditions for the first PCR were as follows: initial reaction at 96°C for 2 min, followed by 40 cycles at 92°C for 60 sec, at 64°C for 60 sec and at 72°C for 90 sec, and a final extension step at 72°C for 10 min. Conditions of the second PCR were the same as those of the first PCR, except that the annealing was performed at 55°C, and 2 *µl* of the first PCR products were used instead of total DNA. All the amplifications were performed using the GeneAmp PCR Systems 2700 (Applied Biosystems, Tokyo, Japan). PCR products were electrophoresed in 1.2% agarose gels in Tris-acetate-ethylenediaminetetraacetic acid buffer and visualized using ethidium bromide.

PCR amplicons were directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, U.S.A.), K4 and K3 primers, ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) and Sequencing Analysis software ver. 3.7 (Applied Biosystems). The obtained sequences were assembled using the ATGC software ver. 6 (Genetics, Tokyo, Japan) and analyzed using the Basic Local Alignment Search Tool program ver. 2.0 of the National Center for Biotechnology Information for detecting homologous sequences. The determined sequences were aligned together with the reference sequences deposited in GenBank using ClustalX ver. 1.83.

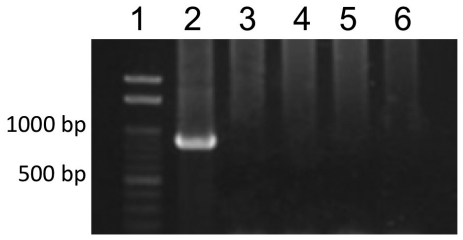

Fig. 1. Specificity of the newly developed nested PCR assay. The nested PCR assay was conducted using the DNA extracted from spores of 4 microsporidia species. Lane 1, 100-bp DNA ladder marker; Lane 2, *Encephalitozoon cuniculi* DNA (2.2×10^2 *n*g/m*l*); Lane 3, *Enterocytozoon bieneusi* DNA (2.2×10^2 *n*g/m*l*); Lane 4, *Encephalitozoon intestinalis* DNA (8.45×10^2 *n*g/m*l*); Lane 5, *Encephalitozoon hellem* DNA (2.2×10^2 *n*g/m*l*); and Lane 6, Milli-Q water (negative control).

RESULTS

Specificity and sensitivity of nested PCR assay: The nested PCR using newly designed primers produced an approximately 840-bp fragment for the *E. cuniculi* DNA and no amplicons for total DNA of *E. intestinalis*, *E. hellem* and *Enterocytozoon bieneusi* (Fig. 1). The 840-bp amplicon was confirmed to have the sequence of *E. cuniculi* DNA by comparison to the reference sequences (AL391737.2, CY001952.1, AF272836.1 and AF023245.1). The 840-bp fragment was also amplified using total DNA extracted from 9×10^1–9×10^4 spores of *E. cuniculi* (Fig. 2) and diluted up to 2.2×10^2–2.2×10^{-1} *n*g/m*l* (not shown).

Detection and genotypes of E. cuniculi DNA in urinary and fecal samples: The 840-bp amplicons were detected in 20 urinary samples (7.78%) of pet rabbits from 13 prefectures and 1 urinary (33.3%) and 6 fecal (5.6%) samples of rabbits from 2 breeding facilities. No amplicon was detected in the fecal samples from pet rabbits. The sequences (803 bp) of the 27 amplicons had no variations and completely coincided with that of *E. cuniculi* genotype I (AL391737.2), which has 3 repeats of 5'-GTTT-3' in its sequence. The sequence determined in this study was deposited as the accession number, AB713183 to the DNA Data Bank of Japan.

DISCUSSION

To the best of our knowledge, this is the first report on the detection and genotype characterization of *E. cuniculi* DNA from pet rabbits in Japan. The results of this study showed that *E. cuniculi* infections occur widely in pet rabbits in

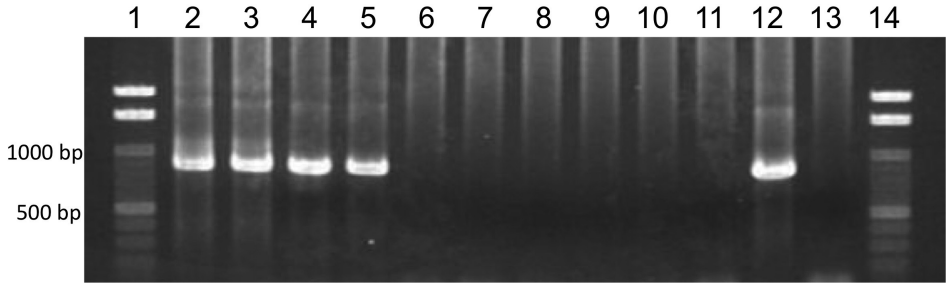

Fig. 2. Sensitivity of the newly developed nested PCR assay. The nested PCR assay was conducted using the DNA extracted from 9×10^{4}–9×10^{-5} spores of *Encephalitozoon cuniculi* (ATCC 50602). Lanes 1 and 14, 100-bp DNA ladder markers; Lanes 2 to 11, 9×10^{4}, 9×10^{3}, 9×10^{2}, 9×10^{1}, 9×10^{0}, 9×10^{-1}, 9×10^{-2}, 9×10^{-3}, 9×10^{-4} and 9×10^{-5} spores; Lane 12, *Encephalitozoon cuniculi* DNA (2.2×10^{2} *n*g/m*l*); Lane 13, Milli-Q water (negative control).

Japan, because *E. cuniculi* DNA was detected in pet rabbits from 13 prefectures of Japan. A high prevalence of this infection in pet rabbits has also been reported in a serological survey targeting anti-*E. cuniculi* IgG and IgM antibodies [11]. However, the detection rate (7.78% in the case of urinary samples) for *E. cuniculi* DNA was significantly lower than that (57.9% in IgG) for the antibodies, which is presumably related to short and intermittent excretion of the spores into urine and feces. Anti- *E. cuniculi* IgG antibody first appears in rabbit sera between days 10 and 17 after infection and is kept at high levels for 400 days [3, 19], whereas *E. cuniculi* spores are excreted in the density of <50 to >500 spores per m*l* into urine between days 38 and 63 after the infection, and thereafter, the spores are intermittently excreted in a very low density [3]. Furthermore, the sensitivity (9×10 spores/sample; total DNA, 2.2×10^{-1} *n*g/m*l*) of the nested PCR assay developed in this study is a possible cause of the low detection rate.

E. cuniculi spores are regularly excreted into urine of infected animals [14], however, *E. cuniculi* DNA was detected in the fecal samples as well as the previous report [18]. Interestingly, the DNA detection from feces was limited in the samples from breeding facilities, which were not treated with the sucrose floatation technique prior to DNA extraction. This may be due to significant reduction of the spores recovered by the floatation technique [20], although contamination of the feces with urinary spores can not be denied absolutely, because of the rabbits reared under crowded situation in breeding facilities.

Genotyping microsporidium isolates is a prerequisite for characterizing their epidemiology [21]. *Encephalitozoon cuniculi* isolates from rabbits have been reviewed to be the genotype I in some European countries, the U.S.A., Australia and Japan [16], however, the number of isolates used for the analysis was very few, except for Switzerland isolates. The results of this study certainly reflect *E. cuniculi* genotype occurring in pet rabbits in Japan. Since genotype I seems to have zoonotic potential, further studies on the spore excretion into urine and feces of pet rabbits will be needed to confirm the possible role of pet rabbits as a source of environmental contamination.

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