

Surveillance for an Outbreak of *Encephalitozoon cuniculi* Infection in Rabbits Housed at a Zoo and Biosecurity Countermeasures

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ABSTRACT. An outbreak of encephalitozoonosis occurred in a rabbit colony at a zoo in Japan. Throughout the two years after the onset, all 42 rabbits were investigated clinically, pathologically and serologically for prevention and control of the disease. Eleven rabbits (11/42, 26.2%) showed clinical symptoms. Of 38 rabbits examined to detect specific antibodies against *Encephalitozoon cuniculi*, 71.1% (n=27) were found seropositive; 20 out of 30 clinically healthy rabbits (except for 8 clinical cases) were seropositive. The infection rate was 76.2% (32/42), including 5 pathologically diagnosed cases. The results of serological survey revealed that asymptomatic infection was widespread, even among clinically healthy rabbits. However, encephalitozoonosis was not found by pathological examination in any other species of animals kept in the same area within the zoo. Isolation and elimination of the rabbits with suspected infection based on the results of serological examination were carried out immediately; however, encephalitozoonosis continued to occur sporadically. Therefore, all the remaining rabbits were finally slaughtered. Then, the facility was closed, and all the equipment was disinfected. After a two-month interval, founder rabbits were introduced from encephalitozoonosis-free rabbitries for new colony formation. Since then, encephalitozoonosis has not been seen in any animals at the zoo. In this study, biosecurity countermeasures including staff education, epidemiological surveillance and application of an "all-out and all-in" system for rabbit colony establishment based on serological examination were successfully accomplished with regard to animal hygiene and public health for the eradication of *E. cuniculi*.

KEY WORDS: asymptomatic infection, biosecurity, *Encephalitozoon cuniculi*, rabbit, serological examination.

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Encephalitozoon cuniculi is an obligate intracellular microsporidian parasite [6, 24, 36]. Spontaneous *E. cuniculi* infections have been documented in various host species including laboratory and domestic rabbits (*Oryctolagus cuniculus*), guinea pigs (*Cavia porcellus*), goats, sheep, horses, carnivores and primates [4, 6, 24, 36]. Guinea pigs housed with rabbits are reported to have a particularly high risk of infection with *E. cuniculi* [4]. In recent years, severe diseases have become recognized more frequently in pet rabbits [18, 22, 33].

Furthermore, *E. cuniculi* has emerged as a significant opportunistic pathogen of immunocompromised humans, particularly acquired immunodeficiency syndrome (AIDS) patients. It has been indicated as a zoonotic pathogen [8, 9, 24, 29], and the number of records of patients with *E. cuniculi* infection has increased worldwide with the increase in human immunodeficiency virus (HIV)-infected patients.

E. cuniculi is listed in a 1996 WHO report as an emerging infectious agent [38]. There was also a report documenting that an HIV-uninfected immunocompetent laboratory worker was accidentally infected with *E. cuniculi* when drops containing spores were spilled in his eyes [34]. Moreover, *E. cuniculi* spores were detected in the urine of one seropositive animal caretaker who was immunocompetent and worked with infected rabbits [27]. In addition, in Japan, specific immunoglobulin G (IgG) and IgM antibodies against the polar tube of *E. cuniculi* were detected in HIV-infected individuals and even in healthy people [26]. Nevertheless, only one case of natural *E. cuniculi* infection in a child has been reported in Japan [25]. However, this case was considered not to have been unambiguously attributed to *E. cuniculi*, as species differentiation was not possible at that time [24]. Thus, the prevalence of *E. cuniculi* in humans is still unclear in Japan.

In Japan, rabbits have become a popular companion animal, and a variety of improved breeds, especially dwarf-breeds, have been imported from the United States and Europe, where high seroprevalence of *E. cuniculi* in rabbits has been shown by serological surveys [15, 16, 18, 20, 22, 33]. Furthermore, in Japan, high seroprevalence of *E. cuniculi* in pet rabbits was reported [17]. *E. cuniculi* infection has also been reported recently in squirrel monkeys (*Saimiri*

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sciureus) [1] and domestic dogs [30] living around humans in Japan. This background is associated with the need for an increased focus on public health issues and has prompted us to perform systemic studies on *E. cuniculi* [12]. Pathological studies and case reports of encephalitozoonosis in laboratory and pet rabbits have also been increasing in number in Japan [10, 12, 17, 28, 32]. However, there is still little information based on comprehensive evaluation of the disease by systemic epidemiological surveys in conjunction with clinical, histopathological and serological studies in Japan.

Furthermore, efforts to eradicate *E. cuniculi* from a rabbit colony were reported to be successful by using a serological survey in facilities overseas in which outbreaks occurred [3, 5, 7]. However, there have been no reports on hygiene measures to assist in the prevention and control of disease after an outbreak of encephalitozoonosis in Japan.

This paper describes the results of clinical, pathological and serological surveys of an encephalitozoonosis outbreak that occurred in a rabbit colony at a zoo in Japan, where encephalitozoonosis had never been seen for the 40 years since the opening of the zoo. In addition, biosecurity countermeasures that successfully prevented the spread of and eradicated this disease within the zoo are also reported.

MATERIALS AND METHODS

Animals: In an educational facility at a zoo in Japan, 42 rabbits were housed and exhibited for animal contact experiences. The zoo kept approximately 150 species of animal (750 individuals), including 50 mammals, 90 birds and 10 reptiles.

Initially, two juvenile rabbits showed symptoms of central nervous system dysfunction and were presented to the animal hospital of the zoo on the 5th and the 8th of May, 1999. They died on the 9th and the 20th of May, respectively. They were pathologically diagnosed with encephalitozoonosis. From the onset until the 15th of March, 2001, all rabbits were examined for an epidemiological surveillance study of *E. cuniculi* infection.

The rabbit breeds were as follows: Nineteen Lop-eared rabbits (Lops), seven Angora lops, five Lion lops, five Lions, three Netherland dwarfs, a Flemish giant, a Lion-Angora mixed breed and a mixed breed. Thirty-eight of these rabbits were subjected to serological examination. Twenty-one rabbits, including four that died and 17 cases euthanized due to suspected encephalitozoonosis on the basis of clinical or serological diagnosis, were examined pathologically. All examined animals were handled according to the guidelines for laboratory animal handling published by the Fund for the Replacement of Animals in Medical Experiments (FRAME) [2]. Euthanasia was performed humanely by intravenous administration of saturated potassium chloride solution after anesthesia by injection of an excess of ketamine hydrochloride.

Clinical and pathological examination: The histories of each rabbit were taken from zookeepers, and general physical conditions such as behavior, appetite and body-weight changes were checked. A complete physical examination

including inspection, auscultation and palpation was performed. In addition, further diagnostic procedures, such as blood test, urine analysis, radiography of tympanic bullae and abdominal ultrasound examination, were performed to rule out other differential diagnoses, if necessary. Complete necropsy and histopathological examination were performed on all of the 21 individuals mentioned above. Organs including brain, kidneys, heart, lungs, liver, spleen, duodenum, ileum and colon were collected and fixed in 20% neutral-buffered formalin. Then, the tissues were embedded in paraffin, sectioned (5 μ m) and stained with hematoxylin and eosin. The brain sections were also stained with gram or Giemsa solution, and examined under light microscopy.

Serological examination: Specific antibodies against *E. cuniculi* were detected using two methods, enzyme-linked immunosorbent assay (ELISA) capable of measuring IgM and IgG antibodies in 15 cases and western blot assay (WB) in all of the 38 rabbits, using purified spores as an antigen [13]. The ELISA procedures were performed as described previously [17]. In either the IgG-ELISA test or the IgM-ELISA test, optical density (OD) at 415 nm in each well of microtiter plates was measured using a microplate reader, and the results were regarded as positive when OD values were 1.0 or more according to the extent of absorbance. In addition, WB procedures were performed as described previously [13, 37]. A positive judgment was evaluated according to the presence of band formation, and 1+, 2+ and 3+ scores were defined depending on the number of bands. To rule out toxoplasmosis, serum assays for anti-*Toxoplasma gondii* antibodies were carried out on seven individuals using the latex agglutination method (Toxocheck-MT "Eiken", Tanabe, Osaka, Japan).

Biosecurity countermeasures: Biosecurity countermeasures were carried out for animal hygiene and public health after the first two cases of encephalitozoonosis from 1999 until 2001. The procedures included monitoring, surveillance, isolation, transport limitation, elimination, eradication and prevention. Encephalitozoonosis was monitored by clinical examination, serological examination and post-mortem by histopathology in the rabbit colony. Rabbits with suspected *E. cuniculi* infection were immediately isolated and humanely euthanized for epizootic prevention. Epidemiological surveillance, including analysis of the history of rabbit introduction, was carried out to estimate the source of the infection. All clinical cases of rabbits in this colony presented to the animal hospital were evaluated retrospectively over two years.

Besides rabbits, three guinea pigs with suspected encephalitozoonosis were pathologically examined during this period; two cases showed fatal weakness, and one euthanized case showed stunted growth and neurological signs like convulsion. A Hokkaido mountain hare (*Lepus timidus aimu*), which was housed separately from the rabbit colony, showed neurological symptoms such as ataxia and hind-limb asthenia (partial paralysis), and was pathologically investigated after its death. One goat, one pig, three ferrets and three hedgehogs that were housed in the same area as the rabbit colony died after various stages of progression and

were also pathologically examined.

The animal care staff were informed that *E. cuniculi* is a potential zoonotic risk and were told to avoid direct contact with the urine of even healthy animals to prevent spreading the infection. Hygiene measures such as hand-washing and disinfection were thoroughly performed.

RESULTS

Clinical findings: Eleven out of 42 rabbits (26.2%) showed various clinical symptoms attributed to encephalitozoonosis (Table 1). They were aged from 40 days to four months (juveniles; n=5) and over four months (adults; n=6). The most affected breed with clinical symptoms was Angora lops (n=5/7). The affected rabbits showed depression (n=9), ataxia (n=7), torticollis (n=5), stunted growth (n=5), death (n=4) and hind-limb asthenia (n=3). There were two major groups that presented characteristic clinical signs: (a) The first two juvenile cases showed stunted growth, depression, ataxia and hind-limb asthenia, followed by eventual death (Fig. 1); (b) five cases showed sudden torticollis and ataxia.

Serodiagnosis: Of 15 rabbits examined by ELISA, 93.3% (14/15) and 60.0% (9/15) were specifically IgG- and IgM-positive, respectively. Twenty-seven out of 38 rabbits (71.1%) were seropositive by WB: three were 1+, five were 2+ and 19 were 3+. It was found that all the serum samples showing OD values over 1.0 in the IgG-ELISA test generated distinct multiple bands in WB, whereas one sample showing OD values below 1.0 revealed no bands stainable by WB. All seven rabbits (Cases 3–9) tested for anti-*Toxoplasma gondii* antibodies were negative.

Pathological findings: As shown in Table 1, all 21 individuals revealed infection with *E. cuniculi*; pseudocysts were detected scattered throughout the brain in 16 cases, in which many gram-positive organism bodies were observed. At necropsy, all cases showed small white spots or focal depressions of several millimeters in diameter on the kidney



Fig. 1. Rabbits infected with *E. cuniculi* (Case 3, left; Case 5, right). The rabbit on the left was found to have stunted growth compared with the rabbit of the same age on the right.

cortex. Histological examination showed that 20 cases had mild to moderate chronic interstitial nephritis and nonpurulent encephalitis with glial nodules or granulomas. The remaining one case had an anemic infarct in the kidneys, encephalomalacia and moderate perivascular mononuclear cell cuffs in the brain. The heart (61.9%), lungs (85.7%), liver (81.0%) and spleen (71.4%) were also shown to be involved in proliferating inflammatory changes with mononuclear cell infiltration. In addition, severe coccidia infection was found in the intestines of one case.

Comprehensive evaluation: Of 38 rabbits serologically examined, 71.1% (n=27) had anti-*E. cuniculi* antibodies, and 21.2% (n=8/38) showed clinical symptoms; 20 out of 30 clinically healthy rabbits were seropositive in this colony. In addition, five cases revealed infection with *E. cuniculi* pathologically, although one symptomatic case was seronegative (Case 3) and four cases were not tested serologically (Cases 1, 2, 10 and 13). In total, 32 out of 42 (76.2%) rabbits in the colony were found to be pathologically and/or serologically infected with *E. cuniculi*.

Biosecurity countermeasures: Although isolation and elimination of the suspected rabbits based on the results of serological examination were carried out immediately, encephalitozoonosis continued to occur sporadically until the 15th of March, 2001. During the surveillance period, 25 rabbits with any disorders were presented at the animal hospital, and 44.0% (11/25) were diagnosed with encephalitozoonosis. On the other hand, there were no findings regarded as reflecting encephalitozoonosis in any other species of animals examined pathologically.

Since *E. cuniculi* can also infect other animal species including humans, we finally slaughtered all the remaining rabbits in the colony, followed by sterilization of all the equipment, such as floors, cages and feeding devices with a burner, 70% ethanol or boiled water. After a two-month interval, new founder rabbits, five Lop-eared, three Rexes and five mixed breed, which are considered to be relatively resistant to this disease, were introduced from an encephalitozoonosis-free rabbitry. These founder rabbits were quarantined for a week and subjected to inspections. As a result of long-term epidemiological monitoring, ten years after the outbreak, no repeat of the encephalitozoonosis outbreak has been seen at the zoo.

Epidemiologically, new rabbit introductions before the first occurrence of infection were surveyed retrospectively. Encephalitozoonosis had never been identified over the 40 years since the opening of the zoo. Four rabbits each were purchased from a pet shop and newly introduced to the colony without quarantine at the zoo on April 1998 and 1999. Three in 1998 (Cases 11, 18 and 19; one was not examined) and two in 1999 (Cases 6 and 7; the other two were negative) were found to be infected with *E. cuniculi* pathologically. They were suspected as being the source of the infection.

DISCUSSION

This is the first report of an outbreak of encephalitozoonosis occurring in a rabbit colony at a zoo where a variety of

Table 1. Results of the clinical, serological and pathological examinations in 21 rabbits diagnosed as having *Encephalitozoon cuniculi* infection

Case	Breed	Age	Clinical findings	Serological diagnosis ^{a)}			Pathological findings ^{b)}		
				ELISA (IgG)	ELISA (IgM)	WB	Organism detection	Main lesion	Involved organs
1	Angora lop*	40 days	Depression, stunted growth, ataxia, death	NT	NT	NT	3+	Glial nodule in brain 2+	Kidneys, CNS**
2	Angora lop	40 days	Depression, stunted growth, ataxia, death	NT	NT	NT	3+	Glial nodule in brain 3+	Kidneys, CNS, heart, lungs, liver
3	Angora lop	2.5 months	Depression, stunted growth, ataxia	-	-	-	3+	Glial nodule in brain 3+	Kidneys, CNS, lungs
4	Angora lop	2.5 months	None	+	+	3+	2+	Glial nodule in brain 2+	Kidneys, CNS, heart, lungs, liver, spleen
5	Angora lop	2.5 months	None	+	+	3+	2+	Glial nodule in brain 2+	Kidneys, CNS, lungs, liver, spleen
6	Angora lop	>1 year	Depression	+	+	3+	1+	Granulomatous encephalitis 2+	Kidneys, CNS, lungs, liver, spleen
7	Lop	>1 year	None	+	-	3+	-	Granulomatous encephalitis 2+	Kidneys, CNS, lungs, liver, spleen
8	Lion lop	3 months	Depression, severe torticollis, ataxia, hind-limb asthenia	NT	NT	3+	1+	Granulomatous encephalitis 2+	Kidneys, CNS, heart, lungs, liver, spleen
9	Netherland dwarf	50 days	Depression, light torticollis, stunted growth, ataxia	+	+	1+	2+	Granulomatous encephalitis 3+	Kidneys, CNS, lungs, liver, spleen
10	Lion lop	1 year/1 year and 10 months	Light torticollis, ataxia, hind-limb asthenia / death	NT	NT	NT	1+	Granulomatous encephalitis 2+	Kidneys, CNS, heart, lungs, liver, spleen
11	Angora lop	>1.5 years	Depression, stunted growth	+	+	3+	1+	Granulomatous encephalitis 1+	Kidneys, CNS, heart, lungs, liver, spleen
12	Lion	>3.5 years	Depression, head shaking	+	+	3+	1+	Granulomatous encephalitis 2+	Kidneys, CNS, heart, lungs, liver, spleen
13	Lion lop	4 months	None	NT	NT	NT	1+	Granulomatous encephalitis 2+	Kidneys, CNS, lungs, spleen
14	Lion - Angora mixed	3.5 years	Depression, severe torticollis	+	-	3+	1+	Granulomatous encephalitis 2+	Kidneys, CNS, heart, lungs, liver
15	Lion	2 years	Severe torticollis, ataxia, hind limb-asthenia, death	+	-	3+	-	Kidneys infarctus 2+	Kidneys, CNS
16	Lop	>3.5 years	None	+	+	2+	1+	Glial nodule in brain 1+	Kidneys, CNS, heart, lungs, liver
17	Netherland dwarf	>4 years	None	+	-	3+	-	Granulomatous encephalitis 1+	Kidneys, CNS, heart, liver, spleen
18	Lion lop	>3 years	None	+	+	3+	-	Glial nodule in brain 1+	Kidneys, CNS, heart, lungs, liver, spleen
19	Lion lop	>3 years	None	NT	NT	3+	1+	Granulomatous encephalitis 2+	Kidneys, CNS, heart, lungs, liver, spleen
20	Lion	>3 years	None	+	-	3+	-	Granulomatous encephalitis 2+	Kidneys, CNS, heart, lungs, liver, spleen
21	Lion	10 months	None	+	+	3+	1+	Granulomatous encephalitis 2+	Kidneys, CNS, heart, lungs, liver, spleen

*lop: lop-eared rabbit. **CNS: central nervous system. a) Serological diagnosis: NT: not tested, ELISA: enzyme-linked immunosorbent assay, antibody values are expressed as optical density, 1.0 or more was regarded as positive. WB: western blot assay, a positive judgment was evaluated according to the presence of band formation, and 1+, 2+ and 3+ scores were defined depending on the number of bands. b) Detection of *E. cuniculi* organisms and main lesion: 3+: severe; 2+: intermediate; 1+: mild.

animals were kept and the public visited in large numbers, and of the subsequent biosecurity countermeasures. Our surveillance revealed that asymptomatic infection with *E. cuniculi* was widespread among apparently healthy rabbits in this colony. On the other hand, the disease was not found in any other species of animal kept in the same area within the zoo facility. In countries in which outbreaks have been reported, serology-based testing and slaughter have been implemented to eliminate *E. cuniculi* from industrial or laboratory-based rabbit colonies [4, 5, 7]. However, in Japan, such preventative methods have not been applied at sufficient intensity. In the present study, biosecurity countermeasures were successfully accomplished with regard to animal hygiene and public health; in particular, staff education, epidemiological surveillance and “all-out and all-in” colony establishment were key factors for the eradication of *E. cuniculi*.

We attempted isolation of *E. cuniculi* organisms from an infected rabbit showing neurological symptoms (Case 14) using primary tissue culture techniques of the kidneys according to procedures reported previously [13, 14]. *E. cuniculi* spores were successfully isolated from the collected sample, identified as *E. cuniculi* by polymerase chain reaction (PCR) with a species-specific primer set and by direct DNA sequencing of the PCR products, and then coded as 2008FF by searching through the GenBank internet service [13]. The isolate was found to belong to genotype I on the basis of the number of 5'-GTTT-3' repeats in the internal transcribed spacer (ITS) of the ribosomal RNA genes [11].

The dwarf-breed rabbits that were purchased from a pet shop and newly introduced into the colony were considered as carriers of *E. cuniculi*, because the pet shops imported rabbits from the United States and Europe, where high seroprevalence of *E. cuniculi* has been reported [15, 16, 20]. Moreover, the pure-blooded dwarf-breeds tended to be more easily affected by clinical symptoms than the others, suggesting that these breeds are predisposed to encephalitozoonosis, as reported previously [21, 23].

In this study, 11 cases showed typical clinical symptoms such as central nervous dysfunction and occasionally fatal prognosis attributed to the encephalitozoonosis [18, 22, 33]. It was interesting that juvenile rabbits showed nonspecific symptoms such as weight loss and stunted growth, finally leading to death. Therefore, encephalitozoonosis should be suspected and differentiated from other diseases in rabbits showing nonspecific and chronic clinical symptoms.

The specific and typical tissue lesions in infected rabbits were the same as those reported previously [21, 23, 31]. The results of serological diagnosis almost correlated with the pathological results. Therefore, the effectiveness of ELISA and WB used in this study for biosecurity countermeasures was confirmed. Antibody detection by serological examination indicates previous exposure to *E. cuniculi*, and it was reported that the presence of IgM antibodies was considered indicative of active infection, even in asymptomatic rabbits [18]. *E. cuniculi* organisms were more likely to be detected in cases that were IgM antibody-positive and pathologically exhibited glial nodules with the focus of necrosis in the brain.

A single seronegative case was found among cases infected with *E. cuniculi* by pathological examination, which might be considered to reflect an early stage of infection, weak humoral immune response or degeneration of antibodies. From these results, we decided that all suspected rabbits in the colony should be eliminated in order to eradicate the *E. cuniculi*, as opposed to individual care.

On the other hand, in rabbits that showed sudden onset of torticollis, the organisms were often found within the brain tissue, which is indicative of active infection. Although severe coccidia infection was also found in one case, it was not observed in the other rabbits. This meant that multiple infections with another pathogen were not always necessary to trigger encephalitozoonosis in this study.

An *E. cuniculi* strain obtained from AIDS patients was classified as type I, which is the same as the rabbit strain detected in the present study, and which is supposed to be an opportunistic zoonotic pathogen [8, 9, 24, 29]. Therefore, eradication of this disease from the rabbit colony was considered necessary as a public health measure, because the zoo staff work with the infected rabbits and also people with weak immunity such as babies or the elderly visit the zoo to enjoy the experience of touching the animals. Firstly, we immediately isolated and eliminated only the rabbits suspected of being infected; however, eradication of *E. cuniculi* was not achieved. Therefore, all the existing rabbits were finally slaughtered. Then, the facility was closed, and all the equipment was disinfected with a burner, 70% ethanol or boiled water, which has been confirmed to be effective against spores of *E. cuniculi* [19, 22, 35]. Fortunately, no staff have shown any symptoms related to *E. cuniculi* infection during the surveillance period, although we have not serologically tested for antibodies against *E. cuniculi* in the animal keepers.

Greenstein *et al.* [15] reported that a significant decline in the incidence of *E. cuniculi* infection in a commercial barrier-maintained rabbit colony was most likely due to a selection process for the breeding program instituted by the supplier, which was based upon the productivity, posture and weight of each animal. In order to establish a healthy rabbit colony, the importance of these selective factors, including breed, was emphasized and the introduction of individuals with suspected infection, such as those presenting with growth abnormality, was avoided. In the present study, after new colony establishment by the introduction of breeds that are considered to be resistant to this disease from encephalitozoonosis-free rabbitries, no repeat of this outbreak has occurred over ten years.

In this study, serological examination has not been applied for the various other species of animal kept at the zoo. The natural infectivity of *E. cuniculi* against other animal species remains unclear. In future study, it might be meaningful to obtain a deeper understanding of *E. cuniculi* ecology to reveal the prevalence of subclinical infection by detection of specific antibodies against *E. cuniculi* in a variety of animals at a zoo facility.

In conclusion, the results of our study show the importance of biosecurity countermeasures for preventing the spread of

E. cuniculi infection, especially considering the potential zoonotic risk. If seropositive rabbits are present, application of an “all-out and all-in” system for rabbit colony establishment based on serological examination with disinfection of the animal-housing facility and an adequate period without rabbit habitation should be successful for eradication of *E. cuniculi*. Rabbits are popular animals, kept not only by individual owners but also in zoos or schools for the purpose of providing children with opportunities to interact with these animals. Therefore, it should be considered necessary to apply biosecurity countermeasures, including serodiagnostic examination, against *E. cuniculi* in order to eradicate it from rabbits owned by zoos, schools or for import quarantine in Japan.

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