FULL PAPER *Parasitology*

High Seroprevalence of *Encephalitozoon cuniculi* in Pet Rabbits in Japan

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ABSTRACT. Infection with *Encephalitozoon cuniculi* in rabbits frequently exists as a chronic, latent infection, and only a percentage of infected animals develop clinical disease. This study presents a seroepidemiological study of *E. cunicucli* infection in 337 pet rabbits collected from 20 prefectures in Japan in 2006 and 2007, using enzyme-linked immunosorbent assay (ELISA) capable of measuring IgG and IgM antibodies. These rabbits were divided into the following four groups: healthy and isolated rabbits (n=74, group I), healthy and companioned rabbits (n=121, group II), neurologically diseased rabbits (n=105, group III), and other diseased rabbits (n=37, group IV). Using ELISA for IgG antibodies, the highest detection rate, 81%, was seen in group III, the second highest, 75.2%, in group II, and the lowest, 29.7%, in group I, which was significantly different to the other groups except for group IV (43.2%). On the other hand, when ELISA was used for IgM antibody detection, 14–40% of rabbits in the four groups were also observed to have anti-*E. cuniculi* IgM. This study demonstrated high seroprevalence of *E. cuniculi* in not only neurologically diseased rabbits but also healthy and other diseased rabbits.

KEY WORDS: *Encephalitozoon cuniculi*, IgG, IgM, rabbit, seroprevalence.

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Encephalitozoon cuniculi is an obligate intracellular parasite that belongs to the Microsporidia [1]. The parasite principally affects rabbits but can also be found in other animals such as rodents, dogs, other canids, etc., and its distribution is worldwide [3]. *E. cuniculi* infection in rabbits leads to a disease called encephalitozoonosis, showing clinical signs in the central nervous system, kidney and/or eye. The most commonly observed neurological sign is head tilt [12].

E. cuniculi infections in rabbits in Japan have been reported as sporadic cases [8] and also as intracolonial outbreaks [4, 7, 13], which were confirmed clinically and histopathologically. However, thus far, a systematic survey has not been performed investigating rabbits from a variety of regions of Japan. This study reports on the screening of sera from 337 pet rabbits seroepidemiologically analyzed by ELISA of IgG and IgM antibodies against microsporidial soluble antigens extracted from purified *E. cuniculi* spores, which were collected in 2006 and 2007 from 20 prefectures of Japan.

MATERIALS AND METHODS

Serum samples: Serum samples were taken from 337 pet rabbits at 34 veterinary facilities in different regions of Japan in 2006 and 2007, as shown in Fig. 1. For the seroepidemiological study, these rabbits were divided into four groups: healthy rabbits which had been kept singly (n=74, group I), healthy rabbits which had been reared together with one or more companions (n=121, group II), rabbits with neurological signs (n=105, group III), and rabbits with other clinical symptoms (n=37, group IV). Neurological signs included head tilt, seizures, ataxia, paralysis, behavioral changes, nystagmus, muscular weakness and swaying at rest. Other clinical symptoms embraced tumors and disorders of the digestive tract, respiratory system, kidney, eye, dens, and skin.

ELISA: *E. cuniculi* spores were propagated *in vitro* using RK-13 cell (ATCC CCL-37) cultures [11]. Spores were isolated from culture supernatants and were purified by density gradient centrifugation with Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using a standard procedure [5]. Microsporidial soluble antigen was extracted by treating the purified spores with Laemmli sample buffer (Bio-Rad laboratories, Hercules, CA, U.S.A.) with 5% 2-mercaptoethanol at 95°C. This was desalted through a NAP-5 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) [5]. Micro titer plates were prepared: 96 flat-bottom wells were coated with the extracted soluble antigen at a concentration of 2 μg/m*l* [15]. Subsequently, plates were washed three times with phosphate buffered saline, pH 7.2 (PBS), were treated with blocking buffer (SuperBlock; Pierce, Rockford, IL, U.S.A.) for non-specific sites, and were kept at 4°C until use.

To measure anti-*E. cuniculi* IgG antibodies in serum samples using the IgG-ELISA test, a 100 μl volume of rabbit sera diluted at 1:400 in PBS containing 0.05% Tween 20 (PBS-T) was added to each well and incubated at room temperature for 1 hr. After incubation, plates were washed five times with PBS-T. Bound antibodies were probed by incubating at room temperature for 1 hr with a 100 μl volume of horseradish peroxidase-conjugated protein A (Kirkegaad &

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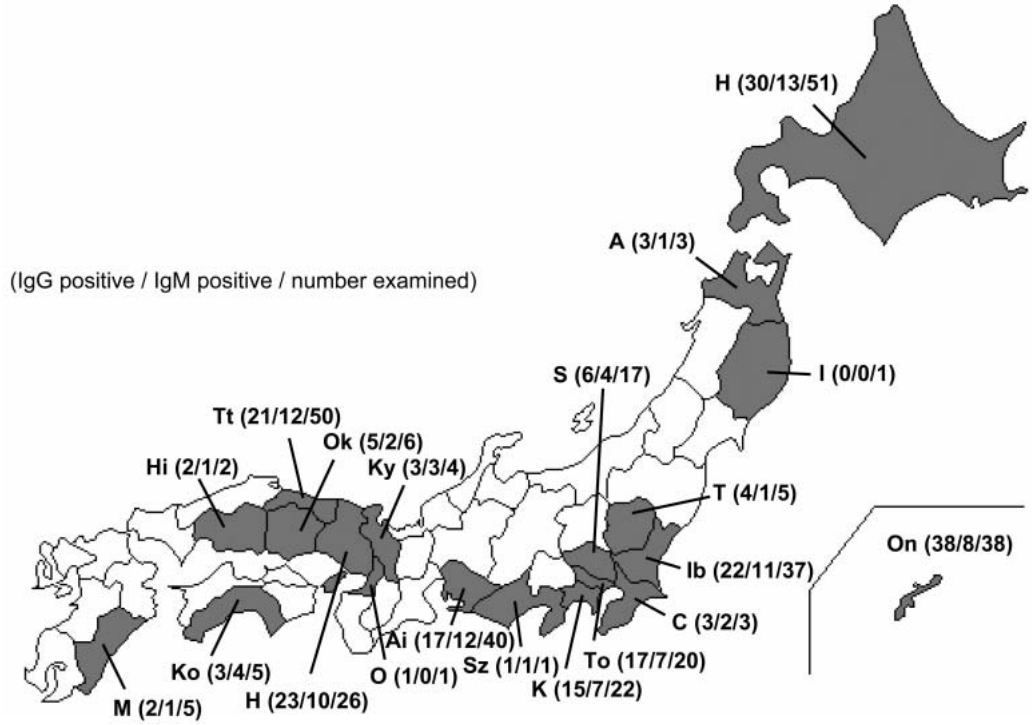

Fig. 1. Geographic distribution of *Encephalitozoon*-seropositive pet rabbits. Shaded regions indicate prefectures where serum samples were collected. Seropositive rabbits were found in Hokkaido (H), Aomori (A), Ibaraki (Ib), Tochigi (T), Chiba (C), Saitama (S), Tokyo (To), Kanagawa (K), Shizuoka (Sz), Aichi (Ai), Kyoto (Ky), Osaka (O), Hyogo (H), Tottori (Tt), Hiroshima (Hi), Okayama (Ok), Kochi (Ko), Miyazaki (M) and Okinawa (On). In Iwate (I), one rabbit was examined, but it was seronegative.

Perry Laboratories, Gaithersburg, MD, U.S.A.) diluted at 1:4,000 in PBS-T. Finally, plates were washed five times with PBS-T, and the enzyme activity of bound peroxidase was revealed by adding 100 μl of the ABTS substrate (Zymed Laboratories Inc., San Diego, CA, U.S.A.) to each well. Optical density (OD) at 415 nm in each well was measured using a TP-500 microplate reader (Corona Electrical, Japan).

In this study, in either the IgG-ELISA test or the IgM-ELISA test described below, ELISA results were regarded as positive when absorbance was 1 or more. OD values below 1 were regarded as negative or quasi-positive, according to the extent of absorbance. As far as could be checked, it was found that serum samples showing OD values over 1.0 generated distinct multiple bands between 6 and 96 kDa in immunoblot analysis, whichever class it detected, i.e., IgG or IgM. On the other hand, in the cases of serum samples showing OD values below 1.0, some showed no bands stainable by immunoblotting, and others revealed a few bands or several but weak bands (unpublished data).

The procedure for measurement of IgM antibodies to *E. cuniculi* in the IgM-ELISA test was primarily the same as that for IgG-ELISA described above, except for the concentration of the tested sera and the secondary antibody employed. Serum samples were tested at a 1:50 dilution in PBS-T and horseradish peroxidase-conjugated anti-rabbit IgM (mu-chain specific; Southern Biotechnology Associate, Birmingham, AL, U.S.A.) was used at a 1:4,000 dilution.

Statistical analysis: A Chi-squared test was used to investigate significant differences among groups of rabbits. P values of <0.01 were considered statistically significant.

RESULTS

Table 1 summarizes the results of serological tests using ELISA. Sera from a total of 337 rabbits were examined for IgG and IgM antibodies raised by natural infection with *E. cuniculi*; 63.5% (214/337) and 28.5% (96/337) of examined sera showed positive OD values of 1 or more by IgG-ELISA and IgM-ELISA, respectively. Seropositive rabbits were distributed in most prefectures (from Hokkaido to Okinawa) from which pet rabbits were sampled for this study (Fig. 1).

These rabbits were divided into four groups: group I, II, III, and IV (Materials and Methods). IgG ELISA-positive results were found in 22 (29.7%) of the 74 rabbits in group I and in 91 (75.2%) of the 121 rabbits in group II. Eleven (14.9%) of the 74, group I rabbits and 31 (25.6%) of the 121, group II rabbits were positive for the IgM ELISA. On the other hand, when the 105 rabbits in group III and 37 rabbits in group IV were examined using IgG-ELISA, 85 (81%) and 16 (43.2%) were positive, respectively. In IgM-ELISA tests, 40% (42/105) of group III rabbits and 32.4% (12/37)

Table 1. Detection rates of IgG and IgM antibodies in sera from pet rabbits by ELISA with *E. cuniculi*-soluble antigens

Group	Number of rabbits examined	Number of rabbits testing positive for:		Number of rabbits with IgG Abs (OD)[a)]			Number of rabbits with IgM Abs (OD)*[a)]		
		IgG Abs (%)	IgM Abs (%)	<1	1–2	2<	<1	1–2	2<
I*[b)]	74	22 (29.7)	11 (14.9)	52	10	12	63	10	1
II*[c)]	121	91 (75.2)*[f)]	31 (25.6)	30	38	53	90	29	2
III*[d)]	105	85 (81.0 *[f)]	42 (40.0)*[f)]	20	26	59	63	34	8
IV*[e)]	37	16 (43.2)	12 (32.4)	21	10	6	25	11	1
total	337	214 (63.5)	96 (28.5)	123	84	130	241	84	12

a) OD values of 1 or more were regarded as positive in this study.
b) Healthy rabbits which had been kept singly.
c) Healthy rabbits which had been reared together with one or more companions.
d) Rabbits with neurological signs.
e) Rabbits with other clinical signs.
f) $P<0.01$.

of the group IV rabbits were positive. In all groups, most IgM ELISA-positive rabbits were also IgG ELISA-positive. However, the rate of IgG ELISA-positive rabbits (63.5%) was 2.2 times higher than that of IgM ELISA-positive rabbits (28.5%). Statistical analyses of detection rates of anti-*E. cuniculi* IgG antibodies revealed that the rate for group I, 29.7%, was significantly lower than that for group II, 75.2%, or for group III, 81%. On the other hand, significant differences were also evident between group I (14.9%) and group III (40%) rabbits, when detection rates using the IgM-ELISA test were compared.

Two grades of positivity were prepared for this study as indicated in Table 1–60.7% (130/214) of IgG antibody-positive rabbits and 87.5% (84/96) of IgM antibody-positive rabbits were distributed among OD values over 2 and OD values of 1–2, respectively. It should be emphasized that the number of animals with IgG activity over 2 in group-III was 2.3 times higher than that of animals with IgG activity of 1–2 in the same group.

DISCUSSION

In this study, we found that *E. cuniculi* was serologically prevalent among many pet rabbits in Japan. Of interest was the number of healthy rabbits which had anti-*E. cuniculi* IgG antibodies. When rabbits in groups I (n=74) and II (n=121) were combined and regarded as samples of healthy rabbits, the detection rate of anti-*E. cuniculi* by IgG-ELISA was estimated to be 57.9% (Table 1). This rate is higher than the detection rates of anti-*E. cuniculi* IgG antibodies in asymptomatic rabbits in other countries. For example, in the United Kingdom, Harcourt-Brown and Holloway [6] reported a rate of detection of anti-*E. cuniculi* IgG antibodies among 38 asymptomatic rabbits of 36.8%. In Switzerland, Mathis *et al.* [10] reported that anti-*E. cuniculi* IgG antibodies were detectable in 7.5% of 292 asymptomatic rabbits. Nevertheless, in this study of the 74 rabbits in group I, regarded as examples of healthy and isolated rabbits, a detection rate of anti-*E. cuniculi* IgG antibodies of 29.7% was found. This was clearly lower than the detection rates of the other groups and indicates that the situation in Japan is comparable to that for rabbits in the United Kingdom, as above. Thus, detection rates of anti-*E. cuniculi* IgG antibodies in healthy rabbits differed between those that had been kept singly (group I rabbits) and those reared with one or more companions (group II rabbits). Regarding the result that anti-*E. cuniculi* IgG antibodies were detected at 75.2% in group II, a finding similar to this was also found in a survey of 100 New Zealand White rabbits maintained in a research colony, in which 72% of the animals showed renal lesions and in which parasites were identified in 9% [14], supporting our decision for having grouped our 337 healthy rabbits into two groups, i.e., groups I and II. The suggestion that *E. cuniculi* can be transmitted by ingestion of spores excreted in urine [10] seemed plausible in positive rabbits in group II.

We next compared the detection rate for anti-*E. cuniculi* IgG antibodies in group III with that for rabbits in group IV. In this case, however, we did not divide each group into two subgroups based on rearing (e.g., subgroups for isolated rabbits and subgroups for companioned rabbits), because of a shortage of companioned rabbits with other clinical signs. The detection rate (81%) for group III (85/105) was found to be 1.88 times higher than that (43.2%) for group IV (16/37). More than half the positive animals in group III revealed OD values over 2. Such a high rate of detection of anti-*E. cuniculi* IgG in neurologically diseased rabbits seemed to be comparable to the rate in other countries. In the United Kingdom, 74.1% of 58 rabbits with neurological signs possessed IgG antibodies against *E. cuniculi* [6]. In Switzerland, anti-*E. cuniculi* IgG antibodies were found in 85% of 72 rabbits with neurological signs [10]. Thus, it appears that anti-*E. cuniculi* antibodies are highly detectable in neurologically diseased rabbits, suggesting that serological testing will be helpful in diagnosing rabbit encephalitozoonosis. However, it should be borne in mind that the present ELISA cannot provide a definitive diagnosis of the disease in Japan, since many other diseased rabbits (e.g., group IV rabbits) as well as healthy rabbits have been found to have anti-*E. cuniculi* IgG, as noted above. It is most unlikely that the severity of the clinical signs is related to how high antibody levels are in the blood [9].

Didier and Bessinger [2] reported that immunologically competent hosts experimentally infected with microsporidia generally expressed an early serum IgM response followed by expression of serum IgG and IgA, and also noted that the IgG response persisted indefinitely in most of these, usually for the entire life of the host, because the inducing immunogens (i.e., microsporidia) persisted. Thus, IgM antibodies may be regarded as early in primary responses. In this study, however, it was found that 40% of group III rabbits, which had neurological signs, were IgM antibody-positive (Table 1) and that most of these were clearly IgG antibody-positive. This rate was significantly higher than that for healthy rabbits (14.9%) in group I. Considering that *E. cuniculi* is a spore-forming intracellular parasite and can cause a disseminated infection [2, 12], the spread of pathogenic organisms in infected hosts may also persistently induce antibody responses of the IgM class. Further research of IgM-ELISA results needs to be done.

There is little existing information on natural *E. cuniculi* infection in rabbits in Japan. Two isolated colonial outbreaks have occurred in Hokkaido [4, 13], which is located at the northern—most part of Japan, and two sporadic cases have appeared in Kyushu [7], which is located at the southern part, of which one was an imported rabbit. This study has demonstrated seropositivity for *E. cuniculi* in many pet rabbits from 20 prefectures, as shown in Fig. 1, suggesting that *E. cuniculi* infections are now widespread in commercial and pet rabbitries. Further studies are necessary to substantiate these findings in conjunction with a microbiological, histopathological, immunological and other studies.

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