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Encephalitozoon cuniculi in rabbits: Serological screening and histopathological findings

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ABSTRACT

Serological prevalence of *E. cuniculi* infection was assessed in 183 rabbits from central Italy. In seropositive deceased rabbits, histopathological lesions were also evaluated. Sera from 118 rabbits from 6 intensive farms, 10 rabbits from 6 family farms, 16 rabbits from a zoo, 30 rabbits from 5 research laboratories and 9 pet rabbits from 9 different owners, were tested by an enzyme-linked immunosorbent assay. Data were statistically analysed. Tissue samples from brain and kidney of 10 deceased rabbits were formalin-fixed and subsequently analysed by histopathology and immunohistochemistry.

Anti-*E. cuniculi* antibodies were found in 129/183 (70.5%) analysed sera. At statistical analysis, *E. cuniculi* seropositivity was significantly higher ($p < 0.05$) in industrial and zoo rabbits.

At histology, different degrees of pathological lesions were found in serological positive (9) deceased animals. In three rabbits deceased after showing neurological signs, the severity of the lesions was interpreted as a likely cause for their death.

1. Introduction

Encephalitozoon cuniculi is a worldwide mammalian microsporidian pathogen that can affect a number of different species of animals as well as humans [1–3]. On the basis of the differences in the nucleotide sequences of the internal transcribed spacer (ITS) region of ribosomal RNA (rRNA) gene [3,4], *E. cuniculi* isolates from animal and human hosts are divided into 4 genotypes, named I, II, III and IV. Although host preference in each strain is not strict [4], genotypes I is mainly detected in isolates from rabbits, genotype II in rodents and genotype III in dogs. The human genotype IV has been found in humans and in cats and dogs [4,5]. However, humans have been found to be infected with all known genotypes and for this reason it has been assumed that infections with *E. cuniculi* are predominantly zoonotic [3]. In humans, *E. cuniculi* is a possible cause of fever and multi-organ involvement in severely immunocompromised patients [6,7] and one of the major microsporidial agents causing latent infections in immunocompetent individuals [8]. In rabbits, infections usually have a chronic and latent course and only a low percentage of infected animals develop clinical disease, characterized by neurological and ocular signs and symptoms linked with renal failure [9]. In farm rabbits, especially in industrial animals, the infection can cause considerable financial loss, due to mortality, reduced carcass weight and increased number of discarded animals at the slaughterhouse [10]. In laboratory rabbits, encephalitozoonosis is a frequent problem affecting the health status of the animals and interfering with experiments [2].

Granulomatous meningoencephalitis and chronic interstitial nephritis and fibrosis are the typical lesions observed in infected deceased rabbits [11–13]. Phacoclastic uveitis, characterized by the infiltration of the eye lens by various inflammatory cells (granulocytes, macrophages, giant cells) leading to a rupture of the lens capsule [11,14], is the consequence of intrauterine infection, when the spores are reported also to reach the anterior lens capsule of the eye. First tissue changes are known to be present in the kidneys, liver and lung while the brain is affected after about 3 months post-infection [2,15].

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Some papers assessed the seroprevalence of *E. cuniculi* infection in rabbits in Italy. They are referred to industrial animals [16,17] and pet rabbits [18,19]. This study was aimed to determine the seroprevalence of *E. cuniculi* infection in rabbits, considering industrial and family farm, pet, zoo and laboratory rabbits. Cerebral and renal histopathological lesions in necropsied serologically positive deceased rabbits, were also evaluated.

2. Materials and methods

2.1. Animals

One hundred and seventy-three alive and 10 deceased adult rabbits from central and northern Italy were examined to assess *E. cuniculi* infection and lesions. More precisely (Table 1), 118 industrial rabbits (commercial hybrids) from 6 intensive farms with a number of animals ranging from 300 to about 700, 10 rabbits (commercial hybrids) from 6 family farms of about 8–10 animals each, 30 rabbits (New Zealand) from 5 research laboratories of about 10 rabbits each, 16 zoo animals (15 coloured dwarf rabbits and 1 giant grey rabbit) from a zoo, and 9 pet rabbits (4 angora, 3 coloured dwarf and 2 English lop rabbits) from 9 different private owners, were included in the study. The clinical status of the 173 live subjects was assessed by physical and neurological examination. Twelve live rabbits (4 pet, 5 laboratory and 3 zoo rabbits) out of 173 (12/173, 6.9%) showed clinical signs suggestive of encephalitozoonosis, mostly represented by torticollis. Among the 10 deceased rabbits, 4 pets and 2 laboratory animals had a clinical history of neurological signs, while the remaining 4 rabbits (laboratory animals) were asymptomatic (Table 1).

Blood samples were collected by the permission of the owners. The study was carried out in accordance with the guidelines given by the European law on the use of animals in research and was approved by the animal ethics and welfare committee of Pisa University (n. 2A-13374).

2.2. Serology

From all alive rabbits, 2 ml of blood taken from the marginal ear vein were collected, centrifuged at 1500 rpm for 15 min and tested by a commercial enzyme-linked immunosorbent assay (ELISA, Medicago®, Uppsala, Sweden). From deceased rabbits, serum specimens tested were obtained from intracardiac coagulum.

2.3. Statistical analysis

Data from serology were analysed by a χ^2 test with the Yates correction [20] to find significant differences among the groups such as animal breeding (industrial or family farm, zoo, laboratory or pet rabbits) and clinical status. Significance was set at $P < 0.05$.

2.4. Histopathology and immunohistochemistry

Deceased animals were routinely necropsied. The brain and both kidneys of all animals were fixed in 4% neutrally buffered formaldehyde solution and subsequently embedded in paraffin. Before processing for histology, the brain was sagittally split into two halves and each half was divided into three consecutive parasagittal sections, while only one sagittal section was taken from each kidney. Four μm thick tissue sections were submitted to histochemical staining such as Haematoxylin-Eosin (H&E), Ziehl-Neelsen (ZN), Gomori trichromic (Acid-fast trichrome stain, AFT) and Gram methods.

Selected sections were chosen for immunohistochemistry (IHC) and put onto polylisinated glass slides (Superfrost®). Sections were deparaffinised through xylene and rehydrated with graded concentrations of alcohol (100, 95 and 80%) and finally in water. Endogenous peroxidases were quenched through 30 min incubation in 6% H_2O_2 solution in PBS and subsequently unspecific binding sites were blocked with a 25% solution of normal horse serum at room temperature. A pool of ELISA positive rabbit sera were employed as primary antibody, diluted in PBS (1:100) and incubated overnight at 4 °C. HRP-conjugated Universal ImmPRESS (Vector Labs, Burlingame, UK) was added to sections as secondary antibody for 30 min at room temperature. The reaction was developed with DAB chromogen (ImmPACT DAB, Vector Labs, Burlingame, UK). Sections were counterstained with Haematoxylin, dehydrated through graded concentrations of alcohol (80, 95, and 100%) and mounted.

3. Results

Anti-*E.cuniculi* antibodies were found in 129/183 (70.5%) analyzed sera and 10/12 (83.3%) symptomatic live animals scored positive. In particular, 87 samples out of 118 (73.7%) industrial rabbits, 5 out of 10 (50%) family farm rabbits, all 16 zoo animals (100%), 17 out of 30 (57%) laboratory rabbits and 4 out of 9 (44%) pets resulted positive. Symptomatic seronegative live animals (2/12, 16.7%) presented with clinically evident torticollis resulted affected by otoacariasis. Moreover, 9 out of 10 deceased animals were found seropositive. Among deceased and seropositive rabbits, all animals with a clinical history of neurological signs (6) and all pet rabbits were included.

The results of χ^2 test showed that only the rabbit breeding has a significant effect on *E. cuniculi* seropositivity ($P < 0.05$). In particular, the rate of infection was significantly higher ($P < 0.05$) in the zoo and in industrial rabbits.

Among histochemical staining used for identification of intracellular *E. cuniculi*, ZN showed the higher sensitivity. In particular, H&E allowed only the detection of inflammatory lesions, while compared to Acid-fast trichrome stain (AFT, Gomori trichromic) and Gram staining, ZN allowed to evaluate the histological positivity also in those tissue sections containing very few parasite elements (3–5 spores) and its sensitivity was comparable to that of IHC.

At histological examination of kidney samples, in 8 out of 9 seropositive rabbits lesions were characterised by mesangial proliferative glomerulonephritis, while no histological changes were observed in a seropositive rabbit. In two milder affected cases, there was thickening of the basement membrane of the glomerular capillaries and occasional formation of glomerulo-capsular synechiae. In the six most severely affected cases, marked fibrosis was associated with scar-like tissue causing retraction of the kidney capsule. Histologically, interstitial deposition of collagen was associated with moderate mixed inflammatory infiltration composed of lymphocytes, plasma cells and macrophages. Intraluminal accumulation of proteinaceous material and desquamated cells were observed within the tubules and the tubular epithelium showed both degenerative and regenerative features. In all affected cases, glomerular lesions were characterized by sclerotic atrophy and thickening of the basement membrane. ZN staining revealed the presence of *E. cuniculi* within the cytoplasm of macrophages, mesangial and epithelial cells. In all seropositive rabbits, immunoreactivity to *E. cuniculi* antigen was detected in parasitophorous vacuoles within the tubular epithelium and less frequently free or intracellularly in the sloughed epithelium or within the center of necrotic lesions. In all rabbits, the presence of perivascular cuffs, composed of lymphocytes, plasma cells and rare macrophages, was the main lesion observed in the brain. Granulomata, composed of tightly packed epithelioid macrophages in close proximity of perivascular cuffs, were observed only in the most affected brain areas. Granulo-