Encephalitozoon cuniculi in rabbits: serological screening and histopathological findings

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Abstract

Serological prevalence of *E. cuniculi* infection in live and deceased rabbits and histopathological lesions in seropositive deceased rabbits, were evaluated. Blood samples from 183 adult rabbits, including 118 industrial rabbits (IR), 10 family farm rabbits (FR), 16 zoo animals (ZR), 30 laboratory animals (LR) and 9 pet rabbits (PR) were collected, centrifuged, and tested by an enzyme-linked immunosorbent assay. Data were statistically analysed. Tissue samples from brain and kidney of 10 deceased rabbits were fixed in 4% neutrally buffered formaldehyde solution, and subsequently processed for histopathological and immunohistochemical analysis.

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

At histological examination, all serological positive (9) deceased animals resulted positive also to histology with different degrees of pathological lesions. In three deceased rabbits with a history of neurological signs, the severity of the lesions was interpreted as the cause of the death.

Keywords: *Encephalitozoon cuniculi*, rabbit, prevalence, lesions, Italy.

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, 2 aggiungere lavoro di Joakim]. *E. cuniculi* isolates from animal and human hosts are divided into 4 genotypes, named I, II, III and IV, on the basis of the differences in the nucleotide sequences of the internal transcribed spacer (ITS) region of ribosomal RNA (rRNA) gene. Genotypes I, II and III are mainly detected in isolates from rabbits, mice and dogs, respectively, while the human genotype IV has so far been found in humans, cats and dogs (lavoro di Joakim + Talabaniet al., 2010.). Humans have been found to be infected with all known genotypes; for this reason it has been assumed that infections with *E. cuniculi* are predominantly zoonotic [lavoro di Joakim]. In humans, *E. cuniculi* is a possible cause of fever and multi-organ involvement in severely immunocompromised patients [3, 4] and one of the major microsporidial agents causing latent infections in immunocompetent individuals [5]. 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 [6]. In farm rabbits, especially in industrial animals, the infection can cause considerable financial loss, due to mortality, reduced carcass weight and increased number of reformed animals [7]. 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 [8-10]. Phacoclastic uveitis is the consequence of intrauterine infection, when the spores are reported also to reach the anterior lens capsule of the eye [8+ Kunzel et al., 2008]; this feature is characterized by the infiltration of the eye lens by various inflammatory cells (granulocytes, macrophages, giant cells) leading to a rupture of the lens capsule. 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 + Giordano et al., 2005].

Some papers assess the seroprevalence of *E. cuniculi* infection in rabbits in Italy. They are referred to industrially reared animals [11, 12] and pet rabbits [13, 14]. This study was aimed to determine the seroprevalence of *E. cuniculi* infection in rabbits, considering industrial and family farm animals, pets, 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 were examined to assess *E. cuniculi* infection and lesions. More precisely, 118 industrial rabbits (commercial hybrids), 10 family farm rabbits (commercial hybrids), 16 zoo animals (15 coloured dwarf rabbits and 1 giant grey rabbit), 30 laboratory animals (New Zealand), and 9 pet rabbits (4 angora, 3 coloured dwarf and 2 English lop rabbits) 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 showed clinical signs suggestive of encephalitozoonosis, mostly represented by torticollis. Among deceased rabbits, 4 pets and 2 laboratory animals had a clinical history of neurological signs, while the remaining 4 rabbits (laboratory animals) were asymptomatic.

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 and centrifuged at 1,500 rpm for 15 minutes 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 [15] to find significant differences among the groups such as animal breeding (industrially or family farm reared, zoo animals, laboratory rabbits 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), Acid-fast trichrome (AFT), and Gram methods.

Selected sections were chosen for immunohistochemistry (IHC) and put onto polylisinated glass slides (Superfrost®). 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).

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 animals (2) 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 typology 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 AFT 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 (Fig. 1), 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 (Fig. 2). Granulomatous lesions were scattered and mainly located in the inner and outer cerebrocortical layers of all cerebral lobes and particularly in the pyriform lobe. Positive immunolabelling was frequently detected in the brain, in which cyst-like aggregates of *E. cuniculi* were observed within parasitophorous vacuoles close to granulomata and within the cytoplasm of perivascular macrophages. Histological examination confirmed the negativity found at serology of one deceased rabbit.

4. Discussion

Serology was chosen considered that in rabbits with chronic *E. cuniculi* infections excretion of the spores into urine and feces is short and intermittent [10, 16], and direct methods could lead to underestimate the real prevalence. Could the authors provide any data about sensitivity and specificity of the commercial ELISA kit used? No but for ELISA test a sensitivity of 94-98% and a specificity of 97-99% has been reported (Boot R., Hansen C., Nozari N., Thuis H. (2000) “ Comparison of assays for antibodies to *Encephalitozoon cuniculi* in rabbits.” Lab. Anim. 34(3): 281-289.)

The examined animals showed an overall seroprevalence of 70.5%, confirming the high rate of infection of the pathogen in these lagomorphs. Rabbits reared in industrial farms appeared

significantly more frequently infected. These results agree with the work of Neumayerova et al. [17] that reported 85.9% of seroprevalence in commercial farms versus 56.3% in house farms, supporting the hypothesis that the direct transmission of *E. cuniculi* by spores excreted in infected animal's urine is a factor that facilitates the spreading of the infection in rabbit industrial flocks, due to the high density of animals. These data are also supported by Lonardi et al. [12], who found infected 75.4% of industrially reared rabbits from Italy. Fifty-seven percent of laboratory animals resulted infected, suggesting a large spread of this infection in such animals. Data about infection with *Nosema cuniculi* in laboratory rabbits date back to the middle of last century. Encephalitozoonosis is reported to be a frequent problem, affecting the health status of laboratory animals and interfering with experiments [18-21], but to the best of our knowledge there is a lack of recent epidemiological studies in this typology of animals. Considered that *E. cuniculi* targets organs of interest in toxicological studies and impacts on the immune system, infected laboratory animals are of questionable utility. Moreover, lesions associated with this microsporidium observed in apparently healthy subjects also in this study, can confound histologic evaluation in such animals [22, 23] and a thorough check of laboratory rabbits to exclude this infection is mandatory. Although all zoo rabbits showed seropositivity, data concerning these animals do not allow us to draw conclusions because a single zoo and a small number of rabbits were examined. Furthermore, 44% of pet rabbits resulted seropositive. This prevalence is lower than that (67.2%) reported by Dipineto et al. [13] and that (59.5%) reported by Lavazza et al. [14] but higher than 22.6% referred by Shin et al. [24], indicating a strong variability probably due to the different size of the samples, but confirming a substantial occurrence of this zoonotic agent in rabbit living in close contact with humans in Italy.

Lymphoplasmacytic meningoencephalitis associated with granulomatous lesions and chronic interstitial nephritis and fibrosis are considered typical pathological findings of cerebral and renal encephalitozoonosis, respectively [8-10, 25]. Results from this study confirm these data, but

188 differently from what previously reported [2, 8], in one case only cerebral lesions without renal
189 involvement were observed. As evidenced in a previous study [9], both acute and chronic lesions
190 were observed in the kidneys of 6 deceased positive rabbits. In 3 out of the 6 deceased symptomatic
191 ones, the severity of the lesions, comparable to the most severe grades (II and III) according to
192 Rodríguez-Tovar et al. [10], was interpreted as the cause of the death of the infected rabbits. ZN
193 method was confirmed as one of the histochemical gold standard for detection of intracellular *E.*
194 *cuniculi* antigen [8, 25].

195 **5. Conclusion**

196 The present study reports data about the high seroprevalence of *E. cuniculi* in different rabbit
197 breeding typologies in central Italy, suggesting possible impacts on the health of other animals and
198 the risk of a possible interference with research. Also possible effects on human health cannot be
199 ruled out. Our findings seem to confirm that many animals are seropositive without showing
200 clinical signs [26, 27]. Moreover, seropositive animals may demonstrate typical histopathological
201 changes [8] without showing clinical signs, even if symptomatic rabbits are more likely to be *E.*
202 *cuniculi* antibody-positive than healthy ones [14, 28] and frequently show the most severe brain
203 lesions [10].
204 Serological check of rabbits is recommended, considered that *E. cuniculi* infection suppresses both
205 humoral and cell-mediated immunity [29], making seropositive animals more likely to become
206 unwell from any supervening pathological cause. Furthermore, such routine screening tests would
207 reduce the diffusion of this potential zoonotic microorganism allow the establishment of *E.*
208 *cuniculi*-free colonies [30].
209

210 **Conflict of interest**

211 None of the authors has any financial or personal relationships that could inappropriately influence
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Authorship

Stefania Perrucci concepted and designed the study. Stefania Perrucci, Giovanni Maestrini, Emanuele Ricci, Carlo Cantile, Francesca Mancianti, Gisella Paci and Carlo D'Ascenzi contributed to the acquisition of data. Riccardo Mannella performed the statistical analysis. Stefania Perrucci, Giovanni Maestrini, Emanuele Ricci, Carlo Cantile, Francesca Mancianti and Riccardo Mannella analysed and interpretated the results. Stefania Perrucci, Giovanni Maestrini, Emanuele Ricci, Carlo Cantile and Francesca Mancianti drafted the article. All authors revised it critically and approved the submitted final version of the manuscript.

References

1. P. Deplazes, A. Mathis, R. Baumgartner, I. Tanner, R. Weber, Immunologic and molecular characteristics of *Encephalitozoon*-like microsporidia isolated from humans and rabbits indicate that *Encephalitozoon cuniculi* is a zoonotic parasite, Clin. Inf. Dis. 22 (1996) 557‑559.

2. F. Künzel, A. Joachim, Encephalitozoonosis in rabbits, Parasitol. Res. 106 (2010) 299-309. doi: 10.1007/s00436-009-1679-3.

3. B. Hinney, B. Sak, A. Joachim, M. Kvàc, More than a rabbit's tale e Encephalitozoon spp. in wild mammals and birds, International Journal for Parasitology: Parasites and Wildlife 5 (2016) 76 – 87.

4. H. Talabani, C. Sarfati, E. Pillebout, T. van Gool, F. Derouin, J. Menotti, Disseminated infection with a new genovar of Encephalitozoon cuniculi in a renal transplant recipient. J. Clin. Microbiol. 48 (2010) 2651e2653.

5. F, Künzel F, A. Gruber, A. Tichy, R. Edelhofer, B. Nell, J. Hassan, M. Leschnik, J.G. Thalhammer, A. Joachim, Clinical symptoms and diagnosis of encephalitozoonosis in pet rabbits, et Parasitol. 151(2008):115-24. doi: 10.1016/j.vetpar.2007.11.005.

3. A. Mathis, R. Weber, and P. Deplazes, Zoonotic potential of the microsporidia, Clin. Microbiol. Rev. 18 (2005) 423–445.

4. S. Fournier, O. Liguory, C. Sarfati, F. David-Ouaknine, F. Derouin, J. M. Decazes, and J. M. Molina, Disseminated infection due to *Encephalitozoon cunic*uli in a patient with AIDS: case report and review, HIV Med. 1 (2000) 155–161.

5. B. Sak, M. Kváč, Z. Kučerová, D. Květoňová, K. Saková, Latent microsporidial infection in immunocompetent individuals - a longitudinal study, PLoS Negl. Trop. Dis. 5 (2011), e1162. doi: 10.1371/journal.pntd.0001162.

250 6. M. Igarashi, E. Oohashi, G. Dautu, A. Ueno, T. Karya, K. Furuya, High seroprevalance of
251 *Encephalitozoon cuniculi* in pet rabbits in Japan, J. Vet. Med. Sci. 70 (2008) 1301-1304.

252 7. M. Saviotti, Tamba M., Gallazzi D., Lavazza A. Further data on the diffusion of *Encephalitozoon*
253 *cuniculi* in italian rabbitries, J. World Rabbit Sci Assoc. 8 (2000) 355-362.

254 8. J. Csokai, A. Gruber, F. Kunzel, A. Tichy, A. Joachim, Encephalitozoonosis in pet rabbits
255 (*Oryctolagus cuniculus*): pathohistological findings in animals with latent infection versus clinical
256 manifestation, Parasitol. Res. 104 (2009) 629-635.

257 9. M. Leipig, K. Matiasek, H. Rinder, D. Janik, D. Emrich, K. Baiker, W. Hermanns, Value of
258 histopathology, immunohistochemistry, and real-time polymerase chain reaction in the
259 confirmatory diagnosis of *Encephalitozoon cuniculi* infection in rabbits, J. Vet. Diagn. Invest. 25
260 (2013) 16-26.

261 10. L. E. Rodríguez-Tovar, A. M. Nevárez-Garza, A. Trejo-Chávez, C. A. Hernández-Martínez, G.
262 Hernández-Vidal, J. J. Zarate-Ramos, U. Castillo-Velázquez, *Encephalitozoon cuniculi*: grading the
263 histological lesions in brain, kidney, and liver during primo infection outbreak in rabbits, J. Pat.
264 (2016) article ID 5768428, 9 pages, http://dx.doi.org/10.1155/2016/5768428

265 11. C. Giordano, A. Weigt, A. Vercelli, M. Rondena, G. Grilli, C. Giudice, Immunohistochemical
266 identification of *Encephalitozoon cuniculi* in phacoclastic uveitis in four rabbits, Vet Ophthalmol. 8
267 (2005) 271-275.

268 11. A. Santaniello, L. Dipineto, L. Rinaldi, and L. F. Menna, Serological survey of *Encephalitozoon*
269 *cuniculi* in farm rabbits in Italy, Res. Vet.. Sci. 87 (2009) 67-69.

270 12. C. Lonardi, G. Grilli, V. Ferrazzi, M. Dal Cin, D. Rigolin, A. Piccirillo, Serological survey of
271 *Encephalitozoon cuniculi* infection in commercially reared rabbit does in Northern Italy, Res. Vet.
272 Sci. 94 (2013) 295-298. doi: 10.1016/j.rvsc.2012.09.020.

13. L. Dipineto, L. Rinaldi, A. Santaniello, M. Sensale, A. Cuomo, M. Calabria, L. F. Menna, A. Fioretti, Serological survey for antibodies to *Encephalitozoon cuniculi* in pet rabbits in Italy, Zoonoses Public Health 55 (2008) 173-175.

14. A. Lavazza, M. Chiari, C. Nassuato, D. Giardiello, C. Tittarelli, G. Grilli, Serological investigation on *Encephalitozoon cuniculi* in pet rabbits in North-Central Italy, J. Ex. Pet Med. 25 (2016) 52–59.

15. R Development Core Team, R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, 2011, http://www.R-project.org/

16. M. Kimura, M. Aoki, M. Ichikawa-Seki, K. Matsuo, K. Yagita, T. Itagaki, Detection and genotype of *Encephalitozoon cuniculi* DNA from urine and feces of pet rabbits in Japan, J. Vet. Med. Sci. 75 (2013) 1017-1020.

17. H. Neumayerová, J. Juránková, E. Jeklová, H. Kudláčková, M. Faldyna, K. Kovarčík, E. Jánová, B. Koudela, Seroprevalence of *Toxoplasma gondii* and *Encephalitozoon cuniculi* in rabbits from different farming systems, Vet. Parasitol. 204 (2014) 184-90. doi: 10.1016/j.vetpar.2014.04.020.

18. M. Petri, Studies on *Nosema cuniculi* found in transplantable ascites tumours with a survey of microsporidiosis in mammals, Acta Pathol. Microbiol. Scand. Suppl. 204 (1969) 1-91.

19. J. A. Shadduck, S. P. Pakes, Encephalitozoonosis (nosematosis) and toxoplasmosis, Am. J. Pathol. 64 (1971) 657-72.

20. J. Chalupský, J. Vávra, P. Bedrník, Encephalitozoonosis in laboratory animals-a serological survey, Folia Parasitol. (Praha) 26 (1979) 1-8.

295 21. J. C. Cox, D. Pye, Serodiagnosis of nosematosis by immunofluorescence using cell-culture-
296 grown organisms, Lab. Anim. 9 (1975) 297-304.

297 22. D. G. Baker, Natural pathogens of laboratory animals: their effects on research, ASM Press,
298 Washington DC, USA, 2003.

299 23. D. H. Percy, S. W. Barthold, Rabbit, in: Percy, D. H., and S. W. Barthold (eds), Pathology of
300 Laboratory Rodents and Rabbits, 3rd edn, 253-307, Blackwell Publishing Professional, Ames,
301 Iowa, USA, 2007.

302 24. J. C. Shin, D. G. Kim, S. H. Kim, S. Kim, K. H. Song, Seroprevalence of *Encephalitozoon*
303 *cuniculi* in pet rabbits in Korea, Korean J. Parasitol. 52 (2014) 321-3. doi:
304 10.3347/kjp.2014.52.3.321.

305 25. J. Csokai, A. Joachim, A. Gruber, A. Tichy, A. Pakozdy, F. Künzel, Diagnostic markers for
306 encephalitozoonosis in pet rabbits, Vet. Parasitol. 163 (2009) 18-26. doi:
307 10.1016/j.vetpar.2009.03.057.

308 26. F.M. Harcourt-Brown, H. K. R. Holloway, *Encephalitozoon cuniculi* in pet rabbits, Vet. Rec.
309 152 (2003) 427-431.

310 27. E. Keeble, D. J. Shaw, Seroprevalence of antibodies to *Encephalitozoon cuniculi* in domestic
311 rabbits in the United Kingdom, Vet. Rec. 158 (2006) 539-544.

312 28. J. Hein, U. Flock, C. Sauter-Louis, K. Hartmann, *Encephalitozoon cuniculi* in rabbits in
313 Germany: prevalence and sensitivity of antibody testing, Vet. Rec. 174 (2014) 350. doi:
314 10.1136/vr.102126.

315 29. A. Valencakova, M. Halanova, Immune response to *Encephalitozoon* infection, Review. Comp.
316 Immunol. Microbiol. Inf. Dis.35 (2012) 1-7.

30. M. Varga, Questions around *Encephalitozoon cuniculi* in rabbits, Vet. Rec. 174 (2014) 347-8. doi: 10.1136/vr.g2494.

Table 1. Breeding typology, breed, areas of provenance and number of rabbits from Italy serologically examined for *Encephalitozoon cuniculi*.

Breeding	Provenance	N. Animals	Breed	N. Farms/ Laboratory/ Private owners	N. Symptomatic
Industrial Farm	Pisa (Central Italy), Lucca (Central Italy), **Forlì (Northern Italy)**	118	Commercial ibrids	**18**	0
Family Farm	Pisa (Central Italy), Carrara (Central Italy)	10	Commercial ibrids	6	0
Pet*	Pisa (Central Italy), Rome (Central Italy	9	4 Angora, 3 coloured dwarf 2 English lop	9	8
Laboratory**	Pisa (Central Italy)	25	New Zealand	5	7
Zoo	Pistoia (Central Italy)	16	15 coloured dwarf 1 giant grey	1	3

*: 4/9 deceased animals
**: 6/25 deceased animals

- Materials and Methods: The categories of the examined rabbits are confusing, maybe a Table would be helpful, stating the origin of the animals, including the number of farms/households/zoos they were derived from, their health status (healthy or diseased) and the breeds. This information is a requirement for understanding results, esp. statistics!

Figure Legends

Figure 1. Kidney. Positive immunolabelling of *Encephalitozoon cuniculi* in a parasitophorous vacuole within the tubular epithelium. IHC, x500.

Figure 2. Brain. Granulomatous lesion with presence of Ziehl-Neelsen positive *Encephalitozoon cuniculi* within the cytoplasm of macrophages. ZN, x500.