# THE USE OF HETEROGENOUS (MURINE) HYPERIMMUNE ANTISERA IN B-SA IMMUNOHISTOCHEMICAL EVIDENCE OF *ENCEPHALITOZOON CUNICULI* ANTIGENS IN RABBITS

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**Abstract**

Štefkovič M., J. Rosocha, M. Halánová, M. Horváth: *The Use of Heterogenous (Murine) Hyperimmune Antisera in B-SA Immunohistochemical Evidence of Encephalitozoon cuniculi Antigens in Rabbits.* Acta vet. Brno 1997, 66: 95–99.

Immunocompetent mice of the $C_{57}BL_6$ colony were experimentally infected with *Encephalitozoon cuniculi* to produce hyperimmune antisera. The mice were six times immunized intraperitoneally with doses of $6 \times 10^6$ agent spores in a single 0.5 ml volume of PBS at three-day intervals. An indirect immunofluorescence of antibodies was performed for the detection of immunological responses to the infection. The IFAT IgG titres obtained were from 1: 1,024 up to 1: 4,096 on the 19th day after the first inoculation. Clinical signs and pathological changes in the mice were observed.

Histological sections of the liver, kidneys, spleen, brain, and lymph nodes from the rabbits infected naturally and experimentally with *E. cuniculi* were examined immunohistochemically. The spores were demonstrated by means of the Biotin-Streptavidin Amplified peroxidase detection system using heterogenous murine hyperimmune antisera. Numerous microsporidia were found in the inflammatory granulomas of the rabbits examined. Spores were mostly localized inside the macrophages within parasitophorus vacuoles.

B-SA immunohistochemistry using biotinylated heterogenous murine anti-*Encephalitozoon cuniculi* IgG, described in this work, appears to be a suitable method of confirmation intravital diagnosis of the infection by detection of the parasite antigens in tissues.

*Microsporidial infections, Encephalitozoon cuniculi, hyperimmunization, immunohistochemistry, mice, rabbits*

Microsporidia are strict, intracellular, spore-forming protozoa that appear to be ubiquitous in nature, infecting a variety of cell types in a broad range of both invertebrate and vertebrate hosts (Bryan 1995). *Encephalitozoon cuniculi,* Levaditi, Nicolau and Schoen, 1923 (syn. *Nosema cuniculi*) is a relatively frequent mammalian parasite occurring in colonies of laboratory animals (rabbits, mice, rats, guinea pigs, hamsters), and in farmed rabbits. Moreover, encephalitozoonosis has also been observed in several species of carnivores - in dogs (Plowright 1952), farmed blue foxes (Nordstoga et al. 1972) and in the mink (Bjerkås 1990). Furthemore, microsporidia are increasingly recognized as causing opportunistic infections in human patients with AIDS (Shadduck 1989; Orenstein 1991).

Immunocompetent hosts infected naturally or experimentally by the microsporidia usually show only a few clinical signs of the disease, yet the microsporidia persist to cause chronic infections that last for the lifetime of the host (Shadduck and Pakes 1971). In contrast, the inoculation of immunodeficient animals, such as athymic mice, resulted in lethal systemic disease after infections (Gannon 1980; Schmidt and Shadduck 1983).

Brain and kidneys are considered as target organs with the typical pathological lesions caused by *E. cuniculi.* In these organs the microsporidia usually caused the inflammatory

microgranulomas (Pakes and Gerrity 1994) that can be observed in histological sections. These are commonly being stained in the standard ways of processing, predominantly with Haematoxylin-Eosin (HE), Giemsa stain, and Luxol Blue.

The aim of the study reported here was an immunohistochemical demonstration of the *E. cuniculi* spores in the histological sections from organs of infected rabbits using the specific heterogenous hyperimmune antisera of mouse origin.

### Materials and Methods

In the experiment, eight four-month-old males of an inbred conventional mice colony ($C_{57}BL_6$) were used for producing hyperimmune antisera against *E. cuniculi*. Three groups of 4 mice per standard plastic cage were housed in pathogen-free environment. Mice were offerred mouse diet and water *ad libitum*. Animals were originally obtained from the laboratory colony (Ing. Tomašovičová, Parasitological Institute of the Slovak Academy of Sciences, Košice, Slovak Republic), that proved negative in IFAT test.

*Encephalitozoon cuniculi* organisms isolated from mice were grown in „E6" cells of Vero Green Monkey Kidney Culture. The cells were cultivated in modified RPMI 1640 medium with an addition of 5 % foetal calf serum. The spores freshly collected from the culture supernatants according to Koudela et al. (1993) were used for both animal inoculation and IFAT serological examinations as an antigen.

All mice were immunized intraperitoneally (i.p.) with six inoculations of $6 \times 10^6$ *E. cuniculi* spores in a single 0.5 ml volume of phosphate-buffered saline (PBS) at three-day intervals. The whole dose given to each mouse during hyperimmunization was $3.6 \times 10^7$ of the spores. Hyperimmune antisera were obtained from blood collected by decapitation on the 3rd day after the last injection. Sera were stored frozen at –20 °C until used.

The indirect immunofluorescent antibody test in the mice was performed according to Chalupský et al. (1973). The animals whose sera reacted at a dilution of 1: 64 or higher, were considered to be positive (Levkut et al. 1996).

The commercial swine-anti-mouse conjugates (SwAM/FITC) used in the test were obtained from the SEVAC a.s., Prague, Czech Republic.

Sixty three samples of internal organs (liver 10, kidneys 27, spleen 10, brain 8, and lymph nodes 8) of the 17 rabbits infected naturally (Levkut et al. 1997) and 10 rabbits infected experimentally (Horváth et al. 1996) were examined for demonstration of *E. cuniculi*. The samples were fixed in 10 % neutral formalin and processed by common paraffin technique. The sections of thickness 5-6 µm obtained were stained by Giemsa stain, PAS, HE and Luxol Blue.

Immunohistochemical procedure was performed on undigested paraffin histological sections using our murine hyperimmune antiserum to *E. cuniculi* diluted in ratio 1: 1,000 in PBS. The slides were incubated at 4 °C for 18 hours. Afterwards, the sections were incubated with the biotinylated anti-mouse immunoglobulins and finally with peroxidase-labelled streptavidin in PBS (Biogenex, San Ramon, CA, USA). Visualization of the immunological reactions was performed by means of diaminobenzidine (DAB). The sections were finally counter-stained by Mayer's haematoxylin.

## Results

The infected mice developed the first clinical signs after the third inoculation at eight to nine days. First, there were lethargy, dermatitis characterized by hyperkeratosis and crusts followed by hypotrichosis observed in most of the mice. Signs of ascites and facial oedema of different degree appeared five to six days later in some of them (Tab. 1). None of the animals infected died until the end of hyperimmunization on the 19th day.

The examination of mice serum samples collected by decapitation just prior to euthanasia showed high positivity to *E. cuniculi*. The levels of specific antibodies corresponded to IgG titres of 1: 1,024 up to 1: 4,096 (Tab. 1). Four control animals were serologically negative.

At the mice necropsy, the most important pathological changes were observed in abdominal cavity, peritoneum, and liver. Accumulation of the straw-coloured ascites fluid in the abdominal cavity, peritonitis of different degree (serosa, granulomatosa), and oedematous infiltration of the facial subcutaneous tissue were found in most of the mice (Tab. 1). The presence of granulomatous peritonitis with several greyish foci scattered was detected in mice No. 5 and 7, respectively. Moreover, mouse No. 5 displayed splenomegaly

Table 1
**Results of serological examination, clinical signs and pathological changes in the mice hyperimmunized with *E. cuniculi***

| Mouse | IFAT titer | Clinical signs | | | | Pathological findings | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| M 1. | 1 : 1.024 | L | D | – | Fo | – | – | O | | |
| M 2. | 1 : 1.024 | L | D | A | Fo | Af | P | O | | |
| M 3. | 1 : 4.096 | L | D | A | – | Af | P | O | | |
| M 4. | 1 : 1.024 | L | D | – | – | – | | – | | |
| M 5. | 1 : 2.048 | L | D | A | Fo | Af | P* | O | Sm | Hm* |
| M 6. | 1 : 2.048 | L | – | A | Fo | Af | P | – | | |
| M 7. | 1 : 2.048 | L | D | A | Fo | Af | P* | O | | |
| M 8. | 1 : 1.024 | – | D | – | Fo | – | – | O | | |
| M 9. C | – | | | – | | | | | | |
| M 10. C | 1 : 16 | | | – | | | | | | |
| M 11. C | – | | | – | | | | | | Hm |
| M 12. C | 1 : 8 | | | – | | | | | | |

Notes:
C – Control animal
L – Lethargy
D – Dermatitis
A – Ascites
Fo – Facial oedema
Af – Ascites fluid
P – Peritonitis
O – Oedema of facial hypodermic tissue
Sm – Splenomegaly
Hm – Hepatomegaly
* – granuloma lesions

and mild hepatomegaly with several yellowish nodular lesions. It is worth mentioning that none of the mice kidneys was macroscopically changed.

Histologically, peritoneal foci were constituted by inflammatory oedema and cell infiltration with predominance of macrophages and lymphocytes. Facial subcutaneous oedema was filled with inflammatory cell infiltration of similar structure. Microlesions in the liver and some kidneys predominantly showed fibrotization and focal granulomatous inflammation without central necrotization. The granulomas consisted of numerous macrophages, several plasma cells, lymphocytes, and a few eosinophils.

The murine anti-*E. cuniculi* IgG isolated from the infected mice reacted immunologically with the microsporidial antigens in histological sections. Consequently, spores were visualized immunohistochemically by means of B-SA peroxidase detection system.

Numerous microsporidial organisms were observed in the liver, kidneys, spleen and brain of the examined rabbits. The spores were localized predominantly inside the macrophages and histiocytes within parasitophorus vacuoles (Fig. 1). Some free spores were observed in inflammatory granulomas and also inside the urine space of the kidney bodies (Fig. 2).

## Discussion

Encephalitozoonosis is a well-known microsporidial infection with chronic, usually latent course in immunologically competent mammals.

The immunosuppressive agents influence the development of clinical and pathomorphological changes in serologically positive individuals (Bálent et al. 1995). An experimental model using *E. cuniculi*-infected BALB/c mice demonstrated that the immune system is responsible for preventing of fatal outcome (Schmidt and Shadduck 1983; 1984).

The development of acute microsporidiosis with clinical signs and histopathological lesions has been reported in SCID mice after infection with $10^7$ *E. cuniculi* spores in a single dose (Heřmánek et al. 1993), while immunocompetent mice (BALB/c) did not show any noticeable signs of the disease. Didier et al. (1994) described that none of the euthymic mice (BALB/c and $C_{57}BL_6$) developed clinical signs of the disease after inoculation with $10^6$ causative spores only. The occurrence of clinical illness in the mice in our experiment was probably caused by the much higher total dose of inoculated microsporidia and also by the way in multiple inoculations.

The intravital diagnosis of the disease consists in serological examinations. Less frequently, methods of direct detection of the microsporidial spores are used, e.g. by means of optical brighteners (Vávra et al. 1993). Occasionally, the causative agents are isolated from urine, kidneys, peritoneal fluid, and macrophages.

*Post mortem* diagnosis particularly consists in demonstration of the pathological lesions caused by microsporidia and in the presence of the agent spores. Although the *E. cuniculi* spores could be stained with Giemsa, Gram's and with silver impregnation methods, there exist some difficulties of their visualization in histological sections (Park et al. 1993).

For detection *E. cuniculi* antigens in tissues, indirect and direct fluorescent as well as immunohistochemical methods have been performed. Park et al. (1993) used homologous rabbit biotinylated anti-*E. cuniculi* IgG prepared from one of the 9 spontaneously infected rabbits in avidin-biotin peroxidase complex (ABC) immunohistochemistry. Using immunohistochemical methods, even one or two spores of *E. cuniculi* present in the affected tissue could be detected.

All our examined rabbits had the IFAT IgG titres of 1.024 to 2.048 (Levkut et al. 1997, Horváth et al. 1996) during their life. To confirm results mentioned above, we examined histological sections taken from the rabbits using indirect (B-SA) immunohistochemistry with heterogenous antisera. The presence of microsporidial spores in the tissues corresponded to the response of rabbit's immune system so that the results of our study have confirmed pathological affecting of the parasite.

## Využitie heterogénneho (myšacieho) hyperimúnneho antiséra v B-SA imunohistochemickom dôkaze antigénov *Encephalitozoon cuniculi* u králikov

Imunokompetentné myši kmeňa $C_{57}BL_6$ boli experimentálne infikované mikrosporídiami druhu *Encephalitozoon cuniculi,* aby vytvorili hyperimúnne antiséra. Myši boli imunizované intraperitoneálne 6-krát v trojdňových intervaloch dávkami $6 \times 10^6$ spór pôvodcu v jednorázovom objeme 0,5 ml PBS. Imunitná odpoveď k infekcii bola testovaná metódou nepriamej imunofluorescencie protilátok. Na 19. deň od prvej inokulácie boli zistené IFAT IgG titre od 1:1 024 do 1:4 096. U myší boli pozorované jednak klinické príznaky, ako aj patologické zmeny.

Imunohistochemicky boli vyšetrené histologické rezy pečene, obličiek, sleziny, mozgu a lymfatických uzlín, získané z králikov prirodzene aj experimentálne infikovaných mikrosporídiami *E. cuniculi*. Spóry pôvodcu boli detekované pomocou B-SA peroxidázového detekčného systému za použitia myšacích hyperimúnnych antisér. U vyšetrovaných králikov bolo v zápalových granulómoch pozorované množstvo mikrosporídií. Spóry boli najčastejšie lokalizované v makrofágoch vo vnútri parazitoforných vakuol.

V tejto práci popísaná B-SA imunohistochemická reakcia, za použitia biotinylovaných heterogénnych myšacích anti-*Encephalitozoon cuniculi* IgG, sa ukázala ako vhodná metóda pre potvrdenie intravitálnej diagnózy infekcie detekciou antigénov parazita v tkanivách.

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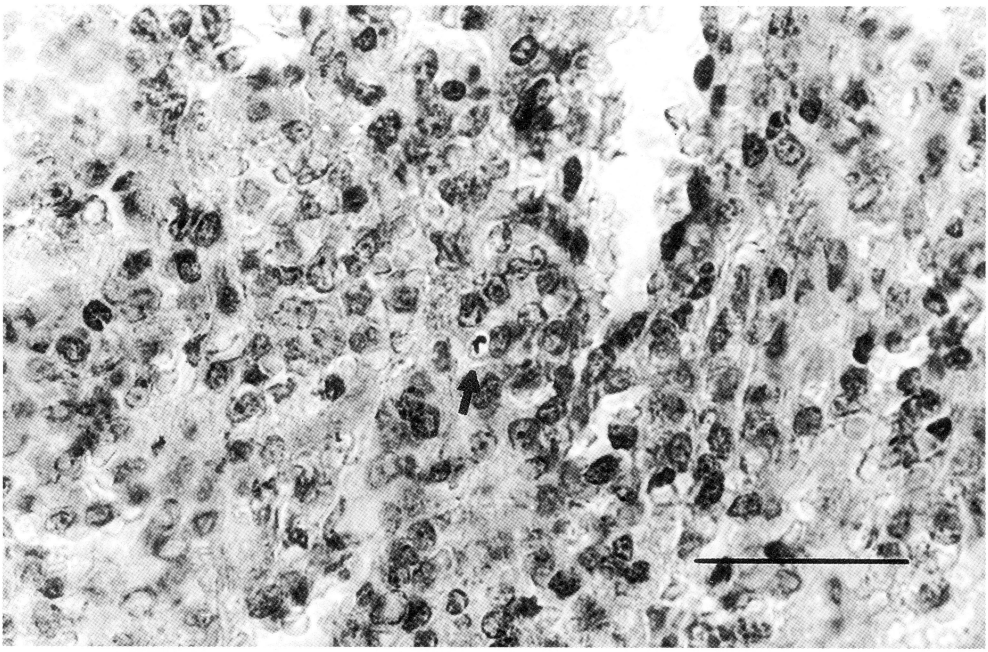

Fig. 1. *E. cuniculi* spores inside the macrophage in red pulp of spleen within parasitophorus vacuole (arrow) are stained positively. Biotin-Streptavidin Amplified peroxidase immunohistochemistry, x 400 (Levkut et al. 1997). Bar = 50 $\mu$m.

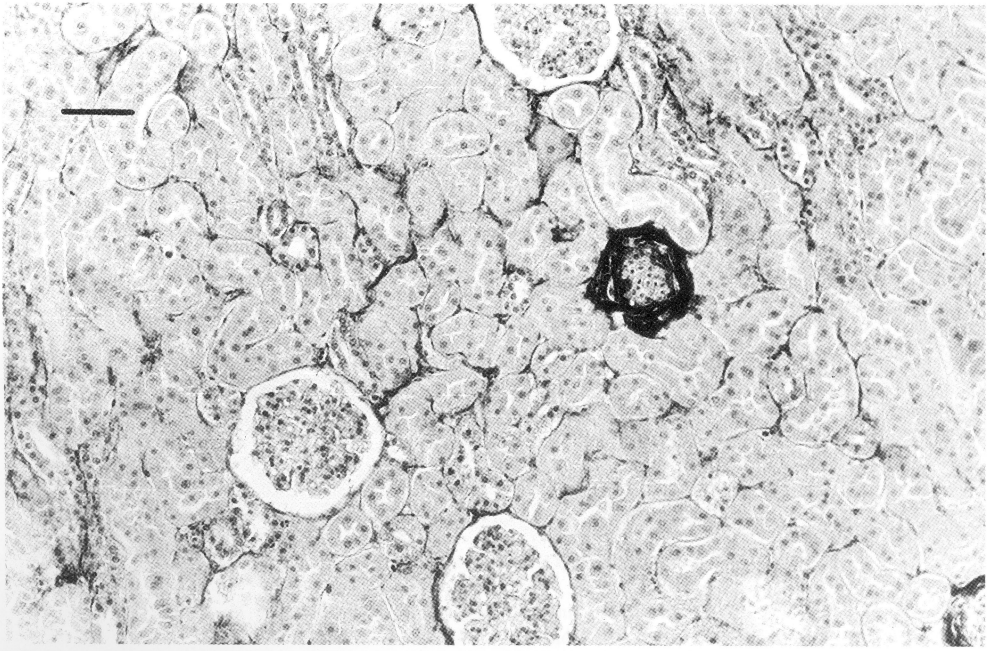

Fig. 2. Numerous microsporidia in the urine space of a renal body of rabbit kidney. B-SA peroxidase immunohistochemistry, x 160 (Horváth et al. 1996). Bar = 50 $\mu$m.