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STUDY ON ANTIBODY PREVALENCE TO MICROSPORIDIAN *ENCEPHALITOZOON CUNICULI* IN DOGS (*CANIS FAMILIARIS*) USING INDIRECT IMMUNOFLUORESCENCE

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Several species of mammals including the dog seem to be potential sources of encephalitozoonosis for animal as well as human hosts. The presence of specific serum antibodies to Encephalitozoon cuniculi was studied in a group of 178 dogs from Eastern Slovakia. The indirect fluorescence of antibodies was chosen as a diagnostic method. Entire cell corpuscular antigen of the in vitro grown microsporidia and swine anti-dog FITC-conjugated immunoglobulin were used in the IFA test.

The animals whose sera reacted by immunofluorescence in a titre of at least 1: 20 were considered positive. Specific anti-E. cuniculi antibodies were found in 53 out of the 178 dogs investigated (29.8 %). Two thirds of the serum positive samples (37 out of 53) were in the lower titre range of 1: 20 to 1: 40. Sixteen dogs were seropositive at a dilution from 1: 80 to 1: 320. The other 125 dogs (70.2 %) showed seronegative reactions.

Our screening results indicated that the examination of canine sera for the presence of anti-E. cuniculi antigens is of great importance especially in dogs with health problems. The IFA test is a very sensitive method for detection of early microsporidian infection in dogs and for indication of very small amounts of specific post-infectious antibodies.

Key words: microsporidia, Encephalitozoon cuniculi, dogs, serological survey, indirect immunofluorescence

INTRODUCTION

Encephalitozoon cuniculi (phylum *Microspora*) is an obligate intracellular protozoan parasite invading a variety of cell types in a broad range of mammalian hosts including man. The parasite is well known as an agent causing encephalitozoonosis, a chronic disease with mostly latent progression. From the latent course the disease may progress into an active form of the infection that is usually influenced by suppression of the host immune system (Levkut *et al.*, 1997).

Kantorowicz and Lewy (1923) in Germany firstly reported microsporidiosis in the dog (*Canis familiaris*). Since that time, canine encephalitozoonosis has been

diagnosed in numerous countries including France, England, Tanzania, the Republic of South Africa, Czechoslovakia, Zimbabwe, as well as in the USA (Van Dellen *et al.*, 1989).

In the susceptible individual *E. cuniculi* prefers to invade central and peripheral nervous and renal tissues. The infection in dogs is occasionally connected with clinical signs of nonsuppurative encephalitis combined with weight loss in puppies (Plowright, 1952) and recurrent nephropathies in young and geriatric individuals (Stewart *et al.*, 1986), well known as an *encephalitis-nephritis* syndrome. Subsequent uraemia and death may also occur. Moreover, various ophthalmic lesions such as cataract, retinitis, and superficial or deep keratitis have been reported in blue foxes - *Alopex lagopus* (Arnesen and Nordstoga, 1977) and domestic cats - *Felis catus* (Buyukmihci *et al.*, 1977).

Natural modes of transmission of encephalitozoonosis are uncertain. Generally, it is believed that the disease is spread horizontally through infectious excrement by the oro-faecal, but above all the oro-urinal routes (Cox *et al.*, 1979). Vertical - transplacental transmission of the infection may also play a role in the epizootology and pathogenesis of encephalitozoonosis especially in rodents (Hunt *et al.*; 1972) and carnivores (Mohn *et al.*, 1982).

Recently, encephalitozoonosis has been confirmed as a zoonosis (Eckert, 1989; Deplazes *et al.*, 1996). Some species of mammals including the dog seem to be eligible as potential animal sources of the microsporidial infection. Therefore, we decided to determine the actual serological status in a group of our usual patients using indirect immunofluorescence of antibodies as the diagnostic method.

MATERIAL AND METHODS

Serum samples. The presence of specific antibodies to *E. cuniculi* was investigated in a group of 178 dogs that came from the East Slovakia region. Blood samples were taken from dogs with various symptomatologies and diagnoses at the 1st Internal Disease Clinic of the UVM in Košice. Blood was withdrawn from the antebrachial vein (*vena antebrachii*) in the standard way. After allowing the clot to form, the blood was spun in a centrifuge at 600 g for 15 minutes. Decanted sera were stored frozen at -20 °C until used in the serological assay.

E. cuniculi organisms. The entire cell corpuscular antigen of the microsporidia was used in the IFA test. The agent spores of murine origin (Vávra *et al.*, 1972) were grown within E6 cells of VERO - Green Monkey Kidney Culture. The infected cell culture was cultivated in modified RPMI 1640 media supplemented with 5 % of foetal calf serum and antibiotics (penicillin, streptomycin and amphotericin B).

Spore isolation. After bursting of the infected cells mature spores were released into the media. The organisms were collected from the culture supernatants after centrifugation at 450 g for 20 minutes. The sediment was resuspended in non-buffered Percoll (density 1.30 g/ml, pH 8.8) and following centrifugation at 750 g for 20 minutes spores were separated. Subsequently, the organisms were once washed by centrifugation in sterile phosphate-buffered saline (PBS, pH 7,2). Freshly cleared spores were resuspended in PBS added antibiotics. The spores were counted within Burkers chamber in 10 rectangles, then diluted to a concentration of 8×10^8 spores per ml and stored at 4 °C.

Indirect fluorescent antibody test. The IFAT method was performed to determine specific antibodies to *E. cuniculi*. The test was done as described in detail by Chalupsky *et al.* (1973). Both a positive and a negative serum was included in all tests as controls. An *E. cuniculi* spore suspension in 20 µl PBS was spotted into each well of alcohol-degreased glass slides. After allowing drying the slides were fixed in cold acetone for 20 min and air-dried again. To control antigen preparation quality, some slides were unspecifically stained with optical brightener Calcofluor White M2R (SIGMA) and observed under blue fluorescence conditions (Figure 1).

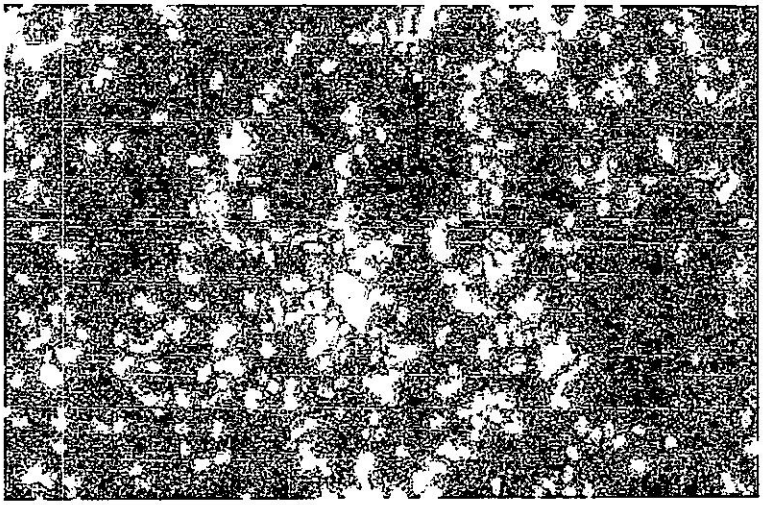

Fig. 1. Control staining of *E. cuniculi* spores without adding of the tested sera using optical brightener Calcofluor White M2R (SIGMA), x 1000. Blue fluorescence, 450 nm colour light, 390-420 nm excited filters and 470 nm barrier filter.

The antigen spots were flooded with 10 µl of sera diluted from 1: 20 to 1: 320 and then incubated for 30 min at 37 °C within a moist chamber. The slides were subsequently washed twice in distilled water and PBS for 10 min each. Following air-drying, the slides were covered with 20 µl of fluorescein isothiocyanate-conjugated swine anti-dog immunoglobulin (SwAD/FITC; SEVAC a.s., Czech Republic) in 1: 160 dilution. After 30 min incubation at 37 °C the slides were washed in PBS and air-dried. Dry slides were counter stained with Evans blue and coverslips mounted with buffered glycerine. The immunofluorescent reaction was evaluated in fluorescent microscope ZEISS JENALUMAR using 510 nm coloured light, excitation filters of 405-409 nm and a barrier filter of 550 nm.

RESULTS

The prevalence of specific antibodies to *Encephalitozoon cuniculi* was investigated in a group of 178 dogs using IFAT as the diagnostic method. In the case of a positive immunological reaction, spores were observed as oval fluorescent formations of 1.5-2.5 µm in size. The titre of each serum sample was

defined as the reciprocal value of the highest serum dilution showing strong peripheral fluorescence of 50 % spores (Figure 2).

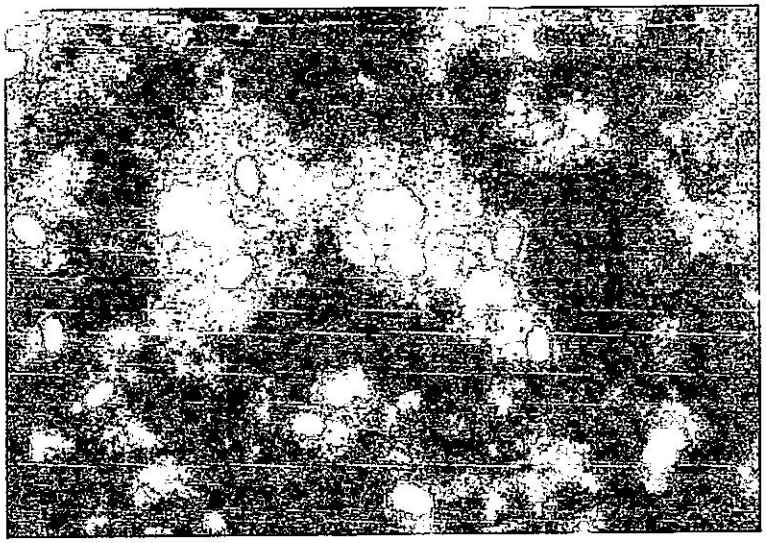

Fig. 2. A positive IIF result fluorescence of the parasite spores after binding of specific antibodies present in the sera of an examined animal, x 1200. Green fluorescence, 510 nm colour light, 405-490 nm excited filters and 550 nm barrier filter.

Both positive and negative control sera as they were detrmined by ELISA (Štefković *et al.*, 1999) were included into the examinations for comparison of the serological reactions.

All canine serum samples were firstly tested at an initial 1: 20 dilution. Sera that positively reacted at this screening titre were subsequently examined also at dilutions from 1:40 to 1:320. The animals whose sera reacted by immunofluorescence in titres at least 1: 20 were considered positive.

Out of 178 dogs investigated, 53 were found to be positive by serological IFAT examination (29,8 %). Blood serum samples of 25 dogs reacted positively at a dilution 1: 20, while an other 28 were positive at a dilution from 1: 40 to 1: 320. The other 125 dogs showed seronegative results (Table 1).

Table 1: Serological positivity to microsporidian *Encephalitozoon cuniculi* in dogs as determined by the IFAT.

Animals	Serological evaluation		Reciprocal IFA titres to *E. cuniculi*				
	negative	positive	1: 20	1: 40	1: 80	1: 160	1: 320
number (*n*)	125	53	25	12	5	8	3
per cent (%)	70.2	29.8	14.1	6.7	2.8	4.5	1.7

DISCUSSION

Encephalitozoonosis is an opportunistic infection of mammals usually with an asymptomatic course in immunocompetent hosts (Koudela *et al.*, 1993). Clinical diagnostics of the disease above all in carnivores, is practically impossible also due to marked similarity of the unique signs to rabies or other diseases affecting the nervous system (Balent *et al.*, 1995). In vivo diagnosis is therefore mostly based upon the results of serological examinations. Several methods have been developed for this purpose, such as the complement fixation test, india immuno-ink reaction, immunoenzymatic assay, the method of indirect microagglutination and the method of radial haemolysis (Balent *et al.*, 1997). However, the most frequently employed method for the evaluation of anti-*Encephalitozoon cuniculi* antibody prevalence is the indirect immunofluorescent test because of its reliability and sensitivity.

In Slovakia, animal encephalitozoonosis was first reported in farmed (Hipikova *et al.*, 1995) and laboratory rabbits (Levkut *et al.*, 1996) using IFAT as a diagnostic method. Later the disease was also diagnosed in mice following natural as well as experimental infection (Štefković *et al.*, 1997; El Naas *et al.*, 1998). Seropositivity to *E. cuniculi*, moreover, was also confirmed in an HIV-positive man (Čislakova *et al.*, 1998) and finally the first finding of IFA anti-*E. cuniculi* antibodies in cows was recorded recently in Slovakia (Halánová *et al.*, 1999).

The humoral immune response to the agent causing canine microsporidiosis was first studied by means of the IFA test in South Africa (Stewart *et al.*, 1979). As the minimal antibody titre which would represent positive infection with *E. cuniculi* was not known, they assumed, through experience with experimentally infected dogs, that titres as low as 1: 20 may be significant. Non-specific fluorescence was sometimes detected at a dilution of 1: 10, so that titre was considered negative (Stewart *et al.*, 1979). The serum positivity in our study was evaluated on the basis these previous findings.

Our results showed a 29.8 % prevalence rate of specific antibodies to *E. cuniculi* in the examined dogs. The majority of the serum positive samples (37 out of 53) was in the titre range of 1: 20 to 1: 40. Similarly, Stewart *et al.*, (1979) obtained results that indicated 18 % antibody IFA prevalence in canine serum samples submitted for various clinical pathological examinations in the Pretoria and Durban areas. Most of these positive samples were also in the lower titre range. Moreover, 70 % seropositivity was detected in a group of dogs from previously proven *Encephalitozoon* infected kennels. More than half the positivity was in the titre range of 1:160 to 1:320. Such high prevalence can be expected after outbreaks of encephalitozoonosis-related clinical signs and the high titres obtained would suggest recent infection.

However, it is necessary to note that our study was not a case of invesigating an average sample of the dog population in the area observed. All our dog patients were admitted to the clinic with the aim of solving their health problems. The actual seropositivity in the general canine population in Eastern Slovakia should be much lower.

Another study done in stray dogs indicated 13.3 % prevalence of antibodies to *E. cuniculi* (Hollister *et al.*, 1989). In this survey 33 sera out of the 248 examined reacted positively by ELISA at titres of from 1: 1,000 to 1: 3,200 and were classified as low, moderate or strong positive. Comparison of total IgG and specific IgG showed that specific IgG was greatly increased in the moderately and strongly

positive sera. This finding confirms our previous assumption about the lower general prevalence of anti-*E. cuniculi* antibodies in dogs.

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PROUČAVANJE PREVALENCE ANTITELA NA MIKROSPORIDIJU *Encephalitozoon cuniculi* KOD PASA (*Canis familiaris*) METODOM INDIREKTNE IMUNOFLUORESCENCE

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SADRŽAJ

Nekoliko vrsta životinja, uključujući i pse, predstavljaju potencijalne izvore encefalitozoonoze za ljude i druge životinje. U ovom radu su izneti rezultati ispitivanja prisustva antitela na parazit *Encephalitozoon cuniculi* u serumu 178 pasa iz istočne Slovačke metodom indirektne imunofluorescence. U ispitivanjima su korišćeni kompletni celularni antigeni ove mikrisporidije kultivisani u *in vitro* uslovima i svinjski anti-pseći Ig konjugovani FITC-om. Životinje koje su imale veći titar od 1:20 smatrane su pozitivnim. Specifična antitela protiv ovog paraazita dokazana su kod 53 od 178 ispitivanih pasa (29.8%). Dve trećine pozitivnih uzoraka imalo je vrednosti titra od 1:20 do 1:40. Ukupno 16 pasa je imalo titar od 1:80 do 1:320 dok je 125 pasa pokazalo seronegativnu reakciju (70.2%). Naši rezultati ukazuju da je pregled pasa na prisustvo antitela protiv *E. cuniculi* od velikog značaja za zdravlje ljudi a da je indirektna imunoflorescenca dovoljno osetljiva i pouzdana metoda.