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Prevalence of anti-microsporidial antibodies in randomly examined dogs in Eastern Slovakia

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Summary

Sixty-eight dogs were examined for the presence of Encephalitozoon spp. antibodies. Twenty-one dogs (30.9%) were healthy without any clinical signs of diseases. Forty-seven animals (69.1%) developed clinical symptoms of diseases such as chronic otitis externa, conjunctivitis, upper respiratory tract inflammation, status epilepticus, pyodermatitis, skin hypersensitivity, demodicosis, flea allergy. Different detection methods of encephalitozoonosis including IFAT (Indirect Immunofluorescence Antibody Assay), in vitro cultivation, SDS PAGE electrophoresis, Western blot and PCR were applied. There were 33 (48.5%) positive reacting sera to E. cuniculi II (mouse type) antigen using IFAT, including 9 positive samples obtained from clinically healthy dogs. Sixteen samples with the antibody titer equal to 1 : 256 were then tested by Western Blot. Most of the samples reacted with E. cuniculi II and III antigens. The presence of E. intestinalis antibodies was lower and just a few samples reacted with E. hellem antigen. The electrophoretic analysis of the encephalitozoon strains used as antigens confirmed that they differ primarily in the molecular size. The strain of type II (mouse) expressed a double strip at 54 and 58 kDa level. The strain of type III (dog) expressed a wide strip at 59 kDa. E. cuniculi types II and III are more related in protein structure in comparison to the other analyzed strains. When PMP1/PMP2 primers were used in PCR, the size of the amplified product was 268 bp for E. cuniculi and 270 bp for E. intestinalis. A species-specific primer pair for E. cuniculi ECUNF/ECUNR gave a 549 bp fragment and V1/SI-500 primers specific for E. intestinalis gave a 370 bp fragment.

Keywords: microsporidia, encephalitozoonosis, IFAT, antibody, dog

Encephalitozoonosis is an opportunistic microsporidial infection and its clinical manifestation depends on external factors and on immunocompetence of the susceptible organism. The latent form of encephalitozoonosis is preserved until the reproduction of the parasite and immune response of the host are in balance. Interest about the diseases induced by microsporidia is growing, mainly because these infections are considered as zoonosis. Application of genetic analyses showed the zoonotic potential of microsporidia, which has been clearly documented for several *Encephalitozoon* species including *E. cuniculi* (15).

Microsporidia are obligatory intracellular parasites characterized as typical eucarionts. However, they also have some features of prokaryotic organisms, e.g. small rRNA, lack of mitochondria, peroxysomes and Golgi's complex (23). They can survive outside of the host cell only in the form of infectious spores, which are preserved by cellular wall composed of proteins and chitin.

The most common microsporidial pathogens for mammals are: *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, *Nosema corneum*, *Encephalitozoon intestinalis* and *Enterocytozoon bieneusi* (21). Encephalitozoonosis in domestic dogs, which is caused by the strain III of *E. cuniculi*, has been described in Tanzania, South Africa, and USA (18).

Spores are the most typical stages according to which the microsporidial pathogens are identified and differentiated from other microorganisms (2). However, the diagnostic techniques used for identification of encephalitozoonosis have some specific features, especially because these pathogens are not clearly morphologically distinguishable in non-sporulated form. The immunoflourescence staining techniques and immunological methods play an important role in screening and early diagnosis of microsporidial infections. The successful application of the molecular biology methods in diagnostic procedures is conditioned by revealing the microsporidial genome (8).

The goal of the study was to perform random screening of antibodies against *Encephalitozoon* spp. and the application of other diagnostic methods for the characterization of particular microsporidial strains in dogs.

Material and methods

Animals. Sixty-eight dogs of different age, sex and race, without any clinical signs of encephalitozoonosis were included in the study. Dogs were divided into three groups: group A – 12 clinically healthy dogs, group B – 47 dogs with different diseases: chronic otitis externa, conjunctivitis, inflammation of upper respiratory tract, status epilepticus, pyodermatitis, skin hypersensitivity, demodicosis, flea allergy, group C – 9 animals from a dog shelter without any clinical signs of disease.

***Encephalitozoon* spp. antigen.** Spores of *Encephalitozoon cuniculi II* (mouse type) and *Encephalitozoon intestinalis* were cultivated on VERO E6 cell cultures (monkey's kidney cells). *Encephalitozoon cuniculi III* (dog type) and *Encephalitozoon hellem* were cultivated on RK-13 cells (rabbit's kidney fibroblasts). For cultivation of infected cells we used the RPMI 1640 medium (Biocom, Slovakia) with 25 mM of HEPES buffer (N-2-hydroxyetylpiperazine-No--2-etansulphonic acid), 0.3 g/L L-Glutamine, 2.0 g/L sodium bicarbonate enriched with 5% fetal bovine serum (Biocom, Slovakia), antibiotics and antimycotics – Penicilin G 10 000 U/ml, Streptomycin 10 mg/ml and Amphotericin B 25mg/ml (Biotech Bratislava). After cultivation spores were released to the RPMI medium and centrifuged for 30 min at 10 000 r.p.m. Next isolated spores were washed 3 times with PBS, counted in a Bürker chamber and diluted to a desired concentration of $3\text{-}4 \times 10^7.ml^{-1}$.

In-direct immunofluorescence antibody test (IFAT). The indirect immunofluorescence antibody test was used for the analysis of anti-*E. cuniculi* antibodies in sera according to (3). Fresh suspension of *E. cuniculi* III (dog type) obtained from the tissue culture was dropped on a special slide for fluorescence microscopy. The slides were air-dried for 24 hours, fixed in absolute acetone for 15 min, and air--dried again. The samples of sera were diluted on the micro-titration plates in PBS at the ratio 1 : 2 to 1 : 8192. Addition of serum was followed by incubation at 37°C for 30 min. The slides were then washed with water and with PBS for 10 min. Finally, the slides were rinsed with water and air-dried at room temperature. The samples were incubated with rabbit anti-dog IgG conjugated with FITC (Sigma, UK) for 30 min at 37°C. After incubation the slides were washed, dried and stained with Evans blue for 2 min. Stained slides were mounted to buffered glycerin (9 parts of glycerin + 1 part of Kawamuri buffer solution). Reaction was examined by the Olympus (Olympus, Japan) fluorescence microscope at 200 × magnification using 510 nm colored light, excitation filters of 405-409 nm and barrier filter of 550 nm. Positive and negative sera were included in the test as controls. All the animals with antibody titers equal to or higher than 1 : 64 were considered as positive.

Western blot. 1-3 mm wide strips were prepared from the nitrocellulose membrane. The strips were saturated with blocking buffer (5% solution of skim milk in phosphate buffer pH 7.2 with 0.05% Tween 20 – PBS-T) for 1 hour at 37°C. The strips were incubated with examined sera in dilution 1 : 150 in 3% blocking buffer for 1 hour at 37°C and washed for 3-5 min in PBS-T. Anti-dog-IgG conjugate RHP (Sigma, UK) was added on strips at the ratio 1 : 300 in 3% blocking buffer and then the strips were incubated for 1 hour at 37°C. Reaction was visualized by the application of the substrate – 4-chloro-l-naphtol (Fluka, Germany). Visualisation was stopped by washing with water.

SDS-PAGE electrophoresis. SDS-Page electrophoresis was performed according to (13) on the Mini-Protean II system (Biorad, UK). Briefly, the suspension of fresh spores 10^8 in 100 µl of sampling buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) was heated for 3 min at 100°C. For separation of proteins, 12% separating gel and 4% stacking gel were used. The sample volume was 10 µl. The electrophoresis was performed in the vertical apparatus (Biorad, UK) with Tris glycine buffer pH 8.3, by 200 V, for 45 min. Polypeptides were stained with Coomassie blue and transferred on the nitrocellulose membrane using the Mini-Trans blot system (Biorad, UK) by 250 mA for 2 h 30 min.

PCR primers. The sequences of the pan-microsporidial primer pair used for DNA amplification of SSU rRNA gene region of *E. bieneusi*, *E. cuniculi*, *E. intestinalis* and *E. hellem* (6) were as follows: PMP1 5'-CACCAGGTTGATTCTGCCTGAC-3', PMP2 5'-CCTCTCCGGAACCAAACCTG-3' (Invitrogen, USA).

The sequences of the primer pair used for DNA amplification of SSU rRNA gene region of *E. cuniculi* (22) were as follows: ECUN-F 5'-ATGAGAAGTGATGTGTGTGTGCG-3' and ECUN-R 5'-TGCCATGCACTCACAGGCATC-3' (Invitrogen, USA).

In case of *E. intestinalis*, amplification of the SSU rRNA gene was performed with: V1 5'-CACCAGGTTGATTCTGCCTGC-3', and SI500 5'-CTCGCTCCTTTACACTCGA-3' primer pair (4). The Gen Bank program BLAST was used to ensure that the proposed primers were complementary with the target species sequence.

Sample preparation and PCR amplification procedure. DNA isolation was performed with QIAamp DNA Stool Mini Kit (Qiagen, UK) according to the manufacturer instructions. Briefly, spores (approx. 6×10^6) from the cell culture were mixed up and lysed with 1.4 ml of ASL buffer. The possible PCR inhibitors were adsorbed using InhibitEX tablets. Residual proteins were digested under denaturing conditions during 95°C incubation with Proteinase K. DNA was bound to the QIAmp silica-gel membrane and washed in two centrifugation steps. Purified, concentrated DNA was eluted from the QIAamp spin column with water.

E. coli DNA isolated by the same kit was used as the negative control template DNA. DNA isolated from the known pure microsporidial cell cultures served as the positive control. 5 µl of purified chromosomal DNA was added to the subsequent PCR reaction.

PCR mixture contained 0.5 µM each primer, 0.2 mM each deoxynucleoside (dATP, dTTP, dCTP, dGTP) (Promega, USA), 2.5 mM $MgCl_2$ (Promega, USA), 1 × PCR

buffer (Promega, USA), 1.25 U Taq polymerase (Promega, USA) and H_2O to the total volume of 50 µl. Amplification conditions were as follows: initial denaturation at 95°C for 10 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, final elongation at 72°C for 10 min. All amplifications were performed on Techne PTC termocykler (Techne, UK). PCR products (10 µl of each) were separated by electrophoresis in 1% or 1.5% agarose gels buffered with 1X TAE (Merck, Germany) containing 1 µg/ml ethidium bromide (Promega, USA). The gels were photographed and analyzed using the Kodak EDAS 120 system (Kodak, USA). The molecular mass standard (Promega, USA) was used according to the manufacturer's instructions and showed one additional band at 1500 bp and 10 bands at 1000 to 100 bp.

Results and discussion

A total of 33 (48.5%) positive reacting sera to *E. cuniculi* II (mouse type) antigen were found from 68 samples examined by IFAT. 9 samples from clinically healthy dogs were also positive. This represents 42.8% of the dogs without any clinical signs of diseases. In group A, 6 positive samples were found with titres from 1 : 64 to 1 : 128. In group B, 24 positive sera were found with antibody titres from 1 : 64 to 1 : 256. In group C, 3 sera reacted as positive with titres from 1 : 64 to 1 : 128 (tab. 1).

Sixteen sera from group B with anti-*E. cuniculi* II (mouse type) antibodies titre equal to 1 : 256 were examined using the Western blot. All the examined sera reacted with 55-60 kDa proteins of *E. cuniculi II* (mouse type), 4 samples reacted with 45 kDa strip, 2 with 43 kDa strip and one with 35 kDa (fig. 1). 3 samples reacted with *E. intestinalis* antigen with 50-63 kDa strip, 10 sera with 45 kDa strip, 2 with 100 kDa, 2 with 35 kDa, one sample with 25 kDa and 2 with 20 kDa. In case of *E. hellem* antigen, 3 sera reacted with 45 kDa strip, 2 sera with 66 kDa, one serum with 96 kDa and 3 with 100 kDa. All sera reacted with *E. cuniculi III* (dog type) proteins at the molecular mass 55-60 kDa and 100 kDa, respectively. 15 samples were positive in the reaction with 43 and 30 kDa strips. Two sera reacted with 25 and 20 kDa strips (fig. 2).

The protein composition of *E. cuniculi II* (mouse type), *E. intestinalis*, *E. hellem* and *E. cuniculi III* (dog type) was analyzed by PAGE (fig. 3). The similarities in the protein profiles of *E. cuniculi* strains were recorded.

A primer pair PMP1/PMP2 by amplification of DNA that was isolated from the cell cultures of *E. cuniculi* gave a 268 bp fragment and in case of *E. intestinalis* a 270 bp fragment. Species-specific primers (ECUNF/ECUNR) gave a 549 bp fragment when *E. cuniculi* DNA was used. A 370 bp fragment was obtained when DNA isolated from the culture of *E. intestinalis* was amplified with V1/SI-500 primers.

Diagnosis of microsporidial infection in animals can be performed by different methods. A variety of serological tests (carbon immunoassay, indirect immunofluorescence test, enzyme-linked immunosorbent assay and Western blotting) have been developed to detect IgG and IgM antibodies to microsporidia, espe-

Tab. 1. Presence of anti-*E. cuniculi* antibodies in the sera from 68 dogs examined by IFAT

	Antibody titer					
Group	Sum	1 : 64	1 : 128	1 : 256	Positive	Negative
A	12	4	2	0	6	6
B	47	4	4	16	24	23
C	9	2	1	–	3	6
Total	68	10	7	16	33	35
Percentage	100.0	29.4	20.6	23.5	48.5	51.5

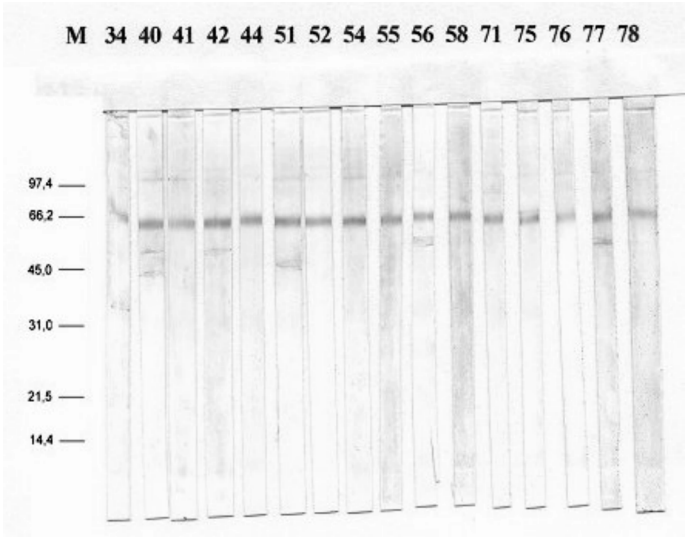

Fig. 1. Western blot of selected sera samples in reaction with *E. cuniculi* II (mouse type) antigen. All samples reacted with the 55-60 kDa strip, 4 samples with 45 kDa, 2 with 43 kDa and one with 35 kDa (M – molecular mass standard)

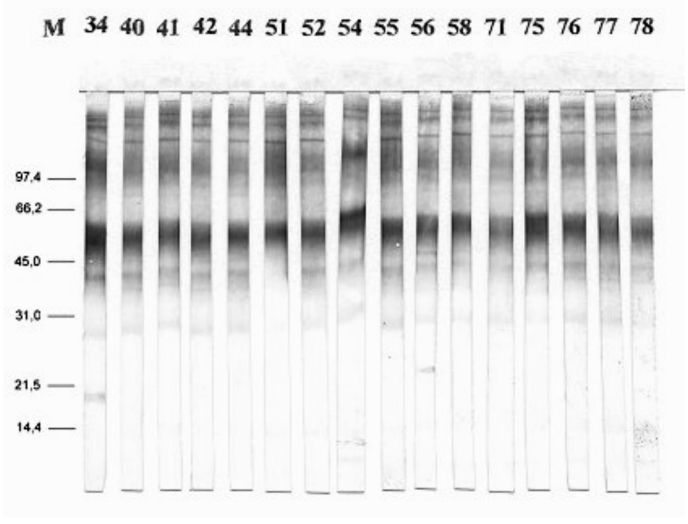

Fig. 2. Western blot of selected sera samples in reaction with *E. cuniculi* III (dog type) antigen. All samples reacted with the 55-60 kDa strip and more than 100 kDa strip respectively, 15 samples with 43 kDa and 30 kDa respectively, one with 25 kDa and one with 20 kDa (M – molecular mass standard)

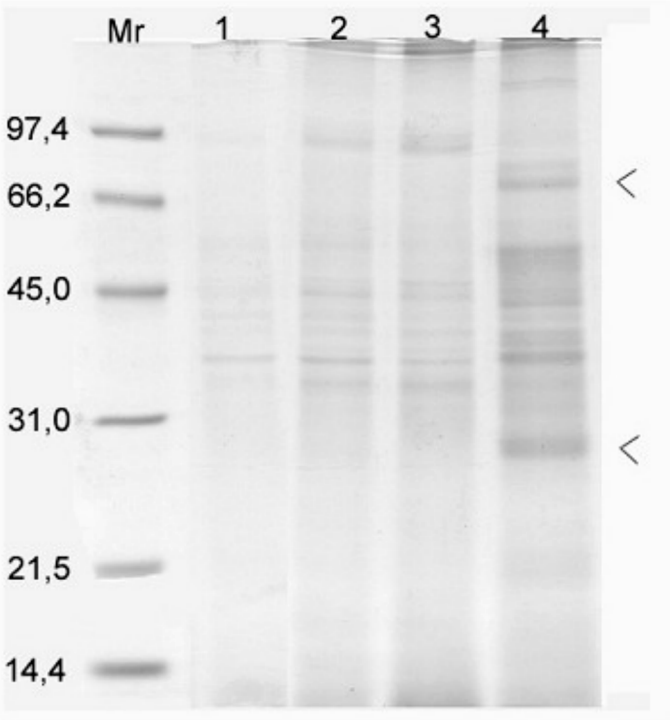

Fig. 3. Protein profile of 1 – *E. cuniculi* II (mouse type), 2 – *E. intestinalis*, 3 – *E. hellem*, 4 – *E. cuniculi* III (dog type) in SDS PAGE. Molecular mass of standard is expressed in kDa. Arrows indicate differences among the strains

cially to *E. cuniculi* (20, 24). Some of these tests are commonly used to detect antibodies in several animal species (11, 16).

Of these assays, the indirect immunofluorescence test and enzyme-linked immunosorbent assay are probably the most useful because they can be easily performed. The specificity and sensitivity of these methods can vary and therefore, they should be used for species differentiation in samples only when the initial diagnosis of microsporidiosis using fluorescent stains with optical brighteners and/or chromotrope-based stains has been performed (6).

Cell culture systems can be used for *in vitro* cultivation of microsporidia and recent success in nucleotide sequencing of various microsporidia genomes has led to the application of molecular biology techniques in the diagnosis of microsporidiosis.

Some of the above-mentioned methods were used because of their specificity, sensitivity and reliability for detection of encephalitozoonosis in dogs. Positive reactions against *E. cuniculi* III (dog type) antigen were found in almost 50% of the examined sera from different groups of dogs using IFAT. The presence of positive sera in healthy dogs suggests latent infection and possible reservoir of microsporidia in the environment closely related to humans. The dogs infected by ingestion of spores showed moderate clinical symptoms, and the chronically infected animals represented the main source of infections for the offspring.

Western blot is considered to be a rapid and sensitive quantitative method for detection and characterization of particular antigens. Sixteen samples with the highest antibody titer (1 : 256) against *E. cuniculi* II (mouse type) measured by IFAT were tested with different antigens of *Encephalitozoon* spp. by Western blot.

Most of the samples reacted with *E. cuniculi* type II and III antigens. The authors found a lower frequency of *E. intestinalis* presence in the examined samples and just a few samples reacted with *E. hellem* antigen. A strict host specificity of the strains was not demonstrated under the experimental conditions. *E. cuniculi* strains II (mouse strain) and III (dog strain) were also infective to rabbits (14). Non-strict host specificity is also confirmed by the obtained protein profile when a number of similarities were recorded between particular *E. cuniculi* types.

The presence of more bands in the particular sera samples suggests multi-infections with different types of microsporidia. However, the presence of common antigenic structures is also possible in more microsporidial types as found by (17).

The electrophoretic analysis performed by (6) confirmed that strain types differ primarily in molecular size. The strain type I (rabbit) showed a wide strip at 57 kDa, strain type II (mouse) expressed a double strip at 54 and 58 kDa, and strain type III (dog) expressed a wide strip at 59 kDa.

The authors found similarities in the protein profiles of *E. cuniculi* II and *E. cuniculi* III, when compared to those of *E. intestinalis* and *E. hellem*. About 20 representative bands in the range of 14 to 97 kDa for each strain were recorded. (1) recorded more than 50 bands from 14 to 200 kDa of the proteins extracted from the reference and tested strains of *E. hellem*, *E. cuniculi* and *E. intestinalis.*

Phylogenetic classification of microsporidia was originally based on histological and ultrastructural identification (19). Although the diagnosis and identification of microsporidia by light microscopy have been greatly improved during the last few years, species differentiation is usually impossible by this technique. Recently molecular methods have been used for closer characterization of particular species (10, 12). For example, *E. cuniculi* and *E. hellem* are morphologically identical, but molecular biology techniques can identify particular species, their types, and their relationship to clinical signs (5).

Detection of microsporidial species with panmicrosporidial and species-specific primers from the used cell cultures showed their reliability and enough sensitivity for routine screening. For all the primers used the target gene was SSU rRNA. The advantage of panmicrosporidial primers is the possibility of identifying four species with one reaction, including *E. bienusi*, *E. cuniculi*, *E. intestinalis* and *E. hellem*. For specific differentiation of particular strains the application of species-specific primes is more suitable.

Conclusions

As discovered using IFAT, the antibodies against microsporidia can be also present in clinically healthy dogs. This increases a potential risk of human infection, especially for those with a decreased level of immunity. The existence of *E. cuniculi* type III common for dogs and humans was genetically proved. It suggests that the source of human infection can be the dog (9, 15). The non-strict host specificity and close relation between *E. cuniculi* strains can likewise increase a risk for infection of dogs and humans with other types of *E. cuniculi*. Immunocompromised humans were found to be infected with strain I in Europe and with strain III in the Americas (15). As spores of *E. cuniculi* are highly resistant in the environment and can survive several months under humid conditions, direct contact with infected animals or humans is not required. From this point of view it is important to screen the prevalence of anti-*E. cuniculi* antibodies in dogs. PCR could be used as a confirmation tool for the elimination of false positive or false negative results.

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