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Isolates of *Encephalitozoon cuniculi* from farmed blue foxes (*Alopex lagopus*) from Norway differ from isolates from Swiss domestic rabbits (*Oryctolagus cuniculus*)

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Abstract *Encephalitozoon cuniculi* has a wide host range among mammals, but whether it represents a homogeneous species is a subject of controversy. We have isolated, cultivated (in human MRC-5 cells) and, for the first time, characterized by immunological and molecular biological methods four isolates of *E. cuniculi* from Norwegian blue foxes with a history of encephalitozoonosis. The isolates were compared with nine isolates from domestic rabbits from Switzerland. Two *E. cuniculi* subtypes were identified according to their host species. A 5′-GTTT-3′ tetranucleotide repeat was present twice in the rDNA intergenic spacer in all isolates from foxes as opposed to three times in all isolates from rabbits. Furthermore, random amplified polymorphic DNA analysis showed one polymorphic band among the subtypes, and Western-blot analysis using serum from an infected fox discriminated between the two subtypes on the basis of their banding patterns in the ranges of 31–33 and 38–40 kDa. The 5′-GTTT-3′ tetranucleotide repeat is a valuable genetic marker for these two subtypes of *E. cuniculi* and will be of use in continued studies on the molecular epidemiology of this parasite.

Introduction

The microsporidian *Encephalitozoon cuniculi* is parasitic in different mammals, including rodents, rabbits, guinea pigs, carnivores, goats, and cats as well as primates (Canning and Lom 1986), and has recently emerged as an opportunistic parasite in patients infected with the human immunodeficiency virus (HIV; De Groote et al. 1995; Deplazes et al. 1996; Franzen et al. 1995; Hollister et al. 1995; Zender et al. 1989). Infections with *E. cuniculi* in animals are usually asymptomatic, but severe neurological signs can occur in rabbits and dogs due to granulomatous encephalitis. In Norway, serious outbreaks of encephalitozoonosis have been seen in farmed blue foxes, with great losses of lesions congenitally infected pups. The major are encephalitis, nephritis, and generalized polyartheritis nodosa, leading to reduced growth, neurological disorders, and, often, death (Nordstoga et al. 1974; Mohn 1983). Infections may also cause cataract in minks (Bjerkås 1990).

Recently, two new *Encephalitozoon* species, *E. hellem* (Didier et al. 1991) and *E. intestinalis* (Cali et al. 1993), were discovered in HIV-infected patients. The spores of both species are morphologically indistinguishable from those of *E. cuniculi*. Therefore, earlier reports of *E. cuniculi* diagnosed by microscopy only remain uncertain because species of the genus *Encephalitozoon* need to be identified by immunological and molecular biological techniques.

Furthermore, whether *E. cuniculi* is a homogeneous species is a subject of controversy; no morphological difference among spores from various isolates has been detected, and inoculation experiments have shown that isolates are infective to hosts other than the original host species (see Didier et al. 1995). Moreover, it has been found that several *E. cuniculi* isolates from different hosts do not differ in their ability to develop in various established cell lines (Montrey et al. 1973). On the other hand, several observations indicate that distinct strains of *E. cuniculi* might exist in animals (Canning and Lom 1986); in Europe and the United States, for example, *E. cuniculi* is common in rabbits but is rarely diagnosed in dogs, whereas in South Africa the disease is relatively common in both rabbits and dogs. In Norway, *E. cuniculi* is remarkably pathogenic in the blue fox and the mink, but apparently not in the dog.

By Western-blot analysis and restriction-enzyme analysis of the polymerase chain reaction (PCR)-amplified SSU rRNA gene we have recently isolated and characterized nine *E. cuniculi* isolates from rabbits (from different owners) from Switzerland that show no

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intraspecies variation (Deplazes et al. 1996). Didier et al. (1995) analyzed eight *E. cuniculi* isolates from mice (three isolates), rabbits (three isolates), and dogs (two isolates) and classified them as three subtypes as deduced from slight differences in Western blots as well as from differences in the number of 5′-GTTT-3 repeats present in the sequence of the rDNA intergenic spacer region.

The aim of this study was to isolate and characterize by immunological and molecular biological methods two groups of *E. cuniculi* isolates from different hosts from different geographical regions (Norwegian farmed blue foxes and Swiss domestic rabbits).

Materials and methods

Microsporidian sources

Nine isolates of *Encephalitozoon cuniculi* from rabbits (from different owners) as well as one isolate of *E. hellem* (IPZ:CH-H1) and *E.* (*Septata*) *intestinalis* (IPZ:CH-H7) that had been isolated from HIV-infected patients (Deplazes et al. 1996) were available for comparative purposes.

E. cuniculi was isolated from farmed blue foxes (*Alopex lagopus*) from three different farms in Norway. Isolates N-F120 and N-F220 were isolated from two 10-month-old male foxes from the same litter. Both animals showed lameness and reduced growth and had experienced as pups acute neurological signs suggestive of encephalitozoonosis. Isolate N-F589 was isolated from a 4-month-old male animal with stunted growth and posterior weakness that was suffering from acute encephalitozoonosis. Madin-Darby canine kidney (MDCK) cells infected with isolate N-F82 (isolated as described by Mohn et al. in 1981) had been stored in liquid nitrogen since 1982.

Parasite isolation and cultivation

Kidney and brain tissue from the foxes were cut into small pieces and mechanically homogenized by passage through a sterile metal sieve (mesh width 400 µm) with the help of the plunger of a disposable syringe. These cell suspensions were washed twice with sterile water, and the resuspended sediment (approximately 2 ml) was centrifuged in phosphate-buffered saline (PBS)-30% Percoll (700 *g*, 40 min, room temperature) to remove cell debris.

Before inoculation of the cell cultures the washed pellets were resuspended in 10 ml 5 m*M* HCl, incubated for 10 min at room temperature, and again washed twice with Hanks' balanced salt solution (HBSS). Cultivation was done in 50-ml plastic tissue-culture flasks (Corning) containing a monolayer of human embryonic lung fibroblast cells (MRC-5; Bio-Mérieux, Marcy, France) in minimum essential medium (MEM; Gibco, Basel, Switzerland) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 100 U penicillin/ml, 100 mg streptomycin/ml, and 0.25 mg Fungizon/ml (Gibco). Cultures were grown in a humidified incubator (air supplemented with 5% CO_2 at 37°C). The culture medium was replaced weekly. The cultivation process was visually monitored using an inverted microscope.

Molecular biology

Spores from the culture supernatants were centrifuged (10 min, 1000 *g* at room temperature), the pellets were washed with 3 ml distilled water, and DNA was obtained using the QIAmp tissue kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. DNA concentrations were measured with a fluorometer (Hoefer, San Francisco, Calif.).

PCR was performed with an MJ Research DNA engine. The PCR buffer contained 50 m*M* KCl, 20 m*M* TRIS-HCl (pH 8.4), 2.5 m*M* $MgCl_2$, 0.5% Tween-20, and 200 µ*M* of each deoxyribonucleotide. For amplification of the SSU rRNA gene in 100-µl reactions, 2.5 U Taq polymerase (BRL, Basel, Switzerland) were used along with 1 µ*M* of both primers (5′-CACCAGGTTGATTCTGCCT-3′ and 5′-TTATGATCCTGCTAATGGTTCTC-3′, corresponding to positions 1–19 and 1277–1299, respectively, of the SSU rRNA gene sequence of *E. cuniculi*; Hartskeerl et al. 1993). The "hot start" technique was used (5-min initial denaturation step), and 35 cycles of 94°C for 30 s, 47°C for 30 s, and 72°C for 150 s were performed. For random amplification of polymorphic DNA (RAPD), 10 ng DNA and 1 µ*M* primer (5′-AATCGGGCTG-3′, OPA-4; Operon Technologies, Alameda, Calif.) were subjected to the following cycling regime: 5 cycles of 94°C for 30 s (denaturation), 30°C for 30 s (annealing), and 72°C for 30 s (extension) followed by 35 cycles at an annealing temperature of 36°C for 30 s (50-µl reactions). The ramp rate from 94°C to the annealing temperature was set to 0.5°C/s.

The intergenic spacer region of the rDNA was amplified using the primers described by Katiyar et al. (1995), and its sequence was determined with the cyclist exo Pfu DNA sequencing kit (Stratagene, La Jolla, Calif.) using the internal primer 5′-TCGTAACATGGCTGCTGTTGG-3′ (corresponding to positions 76–96 of the *E. cuniculi* sequence; Katiyar et al. 1995). Restriction-enzyme analysis (REA) of the PCR-amplified SSU rRNA gene was done as previously described by Deplazes et al. (1996).

Western blotting

Antigen analyses of the spores were performed by Western blotting as described by Didier et al. (1991) and Deplazes et al. (1996). In brief, spores from the cultures were washed three times with HBSS, incubated in 10 ml 0.25% sodium dodecyl sulfate (SDS) at 37°C for 20 min, and then washed again three times with HBSS. The pretreated spores were dissolved in electrophoresis sample buffer containing 2% SDS and 5.0% ß-mercaptoethanol. Antigens from 1×10^6 isolated spores per lane were separated by electrophoresis in mini-PROTEAN II ready gels (4%–20% gradient gels; Bio-Rad Laboratories, Glattbrugg, Switzerland). After electrophoretic transfer to nitrocellulose membranes the blots were saturated in PBS supplemented with 0.3% Tween-20 (PBST) and then incubated overnight with a serum sample from an infected blue fox (N-F120; 1:100 in PBST) at 4°C on a rotator platform. After several washes with PBST the blots were incubated with a 1:400 dilution of goat anti-dog IgG / H+L (horseradish peroxidase-labeled; Southern Biotechnology Associates, Birmingham, Ala.) in PBST for 2 h at room temperature. For visualization of the antibody reactions, hydrogen peroxide and diaminobenzidene were used as the substrate and chromogen, respectively.

Results

Encephalitozoon cuniculi was cultivated from brain tissue from three blue foxes. In addition, the parasite was successfully isolated from the kidney of one fox (N-F589) suffering from acute encephalitozoonosis, but not from the two chronically infected animals. During in vitro cultivation of the samples in MRC-5 cells, spores and infected cells were directly detected at 1–3 weeks after inoculation by inverted light microscopy.

A PCR product of the expected size (1300 bp) was obtained from all four isolates from foxes. Double digestion of this fragment with Mbo I/Hpa II, which had been shown to discriminate between the species *E. cuniculi*, *E. hellem*, and *E.* (*Septata*) *intestinalis* (Deplazes et al.

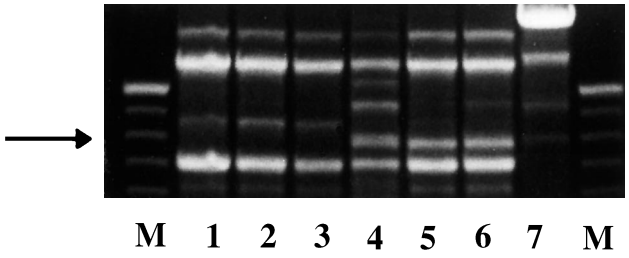

Fig. 1 RAPD banding patterns obtained with primer OPA-4 (*M* DNA size marker – 100-bp ladder in which the brightest band corresponds to 1500 bp, *1 Encephalitozoon cuniculi* CH-K1762, *2* CH-K1881, *3* CH-K2373 – *1–3* isolated from rabbits, *4* N-F82, *5* N-F120, *6* N-F589 – *4–6* isolated from blue foxes, *7 E. hellem* reference isolate IPZ:CH-H1, *arrow* position of the polymorphic amplified DNA band)

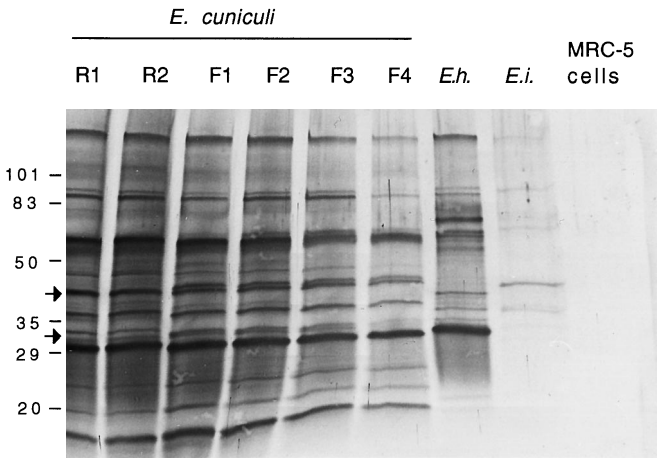

Fig. 2 Western-blot analysis of purified spores (*R1 E. cuniculi* CH-K1762, *R2* CH-K1881 – *R1* and *R2* isolated from rabbits, *F1* N-F82, *F2* N-F120, *F3* N-F220, *F4* N-F589 – *F1-4* isolated from blue foxes, *E.h. E. hellem* reference isolate IPZ:CH-H1, *E.i. E.* (*Septata*) *intestinalis* reference isolate IPZ:CH-H7). A serum sample from an infected fox (N-F120) was used. Molecular weights are given in kDa *on the left*

1996), identified all four isolates from foxes as *E. cuniculi* (data not shown).

The sequence of the intergenic spacer region of the rDNA was identical to the one described by Didier et al. (1995) in all nine *E. cuniculi* isolates from rabbits and in four isolates from blue foxes except for the number of a 5′-GTTT-3′-repeat, which was present twice in all fox isolates and three times in all rabbit isolates.

RAPD with primer OPA-4 allowed the differentiation of rabbit- and fox-derived *E. cuniculi* isolates by reproducibly obtained differences in the banding pattern in the size range of 1300 bp (Fig. 1, arrow). A band of about 1350 bp that occurred in the rabbit isolates was absent in the fox isolates, all of which showed a unique band at about 1230 bp. In addition, there was some heterogeneity in the banding pattern observed among the fox isolates, with the isolate IPZ:N-F82 (Fig. 1, lane 4) having a unique band at slightly above 1500 bp and a band at about 1400 bp that was much weaker in the other fox isolates.

In Western-blot analysis using serum from an infected blue fox, no difference in banding pattern was observed among the nine rabbit-derived *E. cuniculi* isolates (data not shown). However, slight but consistent differences between *E. cuniculi* isolated from rabbits and the four isolates from foxes (Fig. 2) were obvious. In the 38–40 kDa range a single band occurred in the rabbit isolates that contrasted with the distinct double band found in all fox isolates. Furthermore, at 31–33 kDa the double bands of rabbit isolates were more widely separated than the corresponding bands obtained with the fox isolates.

Discussion

We succeeded in cultivating three *Encephalitozoon cuniculi* isolates from fox brain and kidney tissue in human lung fibroblast (MRC-5) cells. Prior centrifugation with Percoll (30%) allowed the separation of spores from homogenized tissue of fox kidney and brain for further in vitro cultivation. This procedure was necessary, as preliminary experiments had shown that extracts of such tissues had a strong cytopathic effect when added directly to the cultures. MRC-5 cells survive for up to 3 months as a monolayer with low proliferative activity, allowing early microscopic identification of even a single microsporidial focus in the cultures. From two chronically infected foxes, cultivation from brain (but not from kidney) tissue was successful, indicating that this system is very sensitive.

This is the first report of unequivocal identification (by immunological and molecular biological methods) of *E. cuniculi* isolated from farmed blue foxes. This is of importance because the two recently described *Encephalitozoon* species that infect HIV-infected patients (*E. hellem* and *E. intestinalis*) are indistinguishable by their spore morphology from *E. cuniculi* (Weber et al. 1994).

All nine *E. cuniculi* isolates from rabbits (from nine owners) from Switzerland were homologous as assessed by intergenic rDNA sequences, RAPD, and Western blotting but were distinctly different from the four isolates from blue foxes from three Norwegian farms as determined by all three methods. This raises the question as to whether these differences really indicate the existence of different subtypes of *E. cuniculi* with different host-species spectra or whether they merely represent geographically separated isolates.

Didier et al. (1995) and Katiyar et al. (1995) have sequenced the rDNA intergenic spacer region in four *E. cuniculi* isolates from rabbits (one of these being isolated 25 years ago) and have found three repeats in all of them. Thus, all *E. cuniculi* isolates from rabbits characterized thus far (13, including the 9 isolates examined in this study) have contained three repeats of this tetranucleotide. The four *E. cuniculi* isolated from foxes from Norway, however, contained two of these repeats, which corresponds to the findings reported by Didier et al. (1995) for two isolates from mice. Again, the number of repeats seems to be stable over time, as one of our *E. cuniculi* isolates dates back to before 1981 (Mohn et al. 1981).

According to both Western-blot analysis and RAPD analysis, there seems to be subtle heterogeneity among the fox isolates. As determined by the former technique, the weak band below 50 kDa that appears in isolates IPZ:N-82 and IPZ:N-120 (Fig. 2; lanes F1, F2) seems to be absent in isolates IPZ:N-220 and IPZ:N-589 (Fig. 2; lanes F3, F4). This might be a slight but true antigenic polymorphism, but it seems more probable that this polymorphism is due to the apparent loading of less antigen in lanes F3 and F4 (Fig. 2) as compared with lanes F1 and F2. Also, the heterogeneity observed in the RAPD pattern among the fox isolates (particularly between isolate IPZ:N-F82 and the others; Fig. 1, lanes 4–6) might indeed indicate genotypic differences. However, the technique is known to be vulnerable to minor changes in the conditions used, e.g., the amount of DNA employed and the presence of contaminating DNA (from MRC-5 cells) that is also amplified. Nevertheless, considering only the basic profile, the isolates from rabbits could be distinguished from those from foxes. Using another primer for RAPD analysis, Hollister et al. (1995) obtained different banding patterns for their murine and canine *E. cuniculi* isolates. Although the ITS1 sequences of these two isolates were not determined, it is possible that they belong to different subtypes.

Our antigen analysis of the *E. cuniculi* isolates from foxes (with two tetranucleotide repeats) and rabbits (with three repeats) using serum from an infected fox showed the same difference in the range of 38–40 kDa as previously reported by Didier et al. (1995) for isolates of mice (two repeats) and rabbits using murine anti-*E. cuniculi* antibodies. In both investigations the isolates with two tetranucleotide repeats had one band in this range as opposed to the two bands found in the isolates with three tetranucleotide repeats. Hence, the number of the tetranucleotide repeat is a reliable marker for phenotypically distinct subtypes. These data suggest the existence of subtypes of *E. cuniculi*, namely, a mouse-blue fox subtype and a rabbit subtype.

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