Two *Encephalitozoon cuniculi* strains of human origin are infectious to rabbits

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SUMMARY

The microsporidian *Encephalitozoon cuniculi* can infect a wide variety of mammals including man. In this study, *E. cuniculi* isolates of animal origin were compared with 6 isolates obtained from HIV-infected patients. Based on results of Western blot analysis, random amplified polymorphic DNA (RAPD) and the sequence of the rDNA internal transcribed spacer (ITS) the isolates were classified into 3 groups with the repeated element 5′GTTT-3′ in the ITS being a reliable genetic marker. Five isolates from Swiss patients were found to be homologous to isolates from Swiss rabbits (strain I). The sixth isolate from a patient from Mexico differed by all methods and could be attributed to *E. cuniculi* strain III that has been described from 2 dogs from the USA. All of these isolates were distinguished from isolates from blue foxes from Norway (strain II). Intraspecific nucleotide divergence of the SSU rRNA gene of *E. cuniculi* belonging to the 3 strains was in the same low range (0·00–0·15 %) as was found for the corresponding sequence of 2 *E. hellem* isolates. Groups of 2 rabbits were infected by oral inoculation of 10^7 *E. cuniculi* spores (2 isolates from strain I of human and rabbit origin, 1 from strain III) as shown by antibody responses and the re-isolation of the parasites from brain material. The results provide further evidence that per oral transmission of the parasite between various hosts is feasible.

Key words: *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, HIV-infected patients, zoonoses, rDNA sequences, experimental infection.

INTRODUCTION

Three species of the microsporidial genus *Encephalitozoon*, *Encephalitozoon cuniculi*, *Encephalitozoon hellem* and *Encephalitozoon* (formerly *Septata*) *intestinalis* have been described as opportunistic parasites in HIV-infected patients (Weber *et al.* 1994). *E. cuniculi* is the only species which has also been found to infect other hosts, particularly a wide variety of mammals (Canning & Lom, 1986). The first report of an *E. cuniculi* infection in a human dates back to 1959 (Matsubayashi *et al.* 1959). However, in this case as well as 3 subsequent cases (2 of them from HIV-infected patients) the diagnoses were based on spore morphology (Bergquist *et al.* 1984; Terada *et al.* 1987; Zender *et al.* 1989) before the discovery of *E. hellem* (Didier *et al.* 1991) and *E. intestinalis* (Cali, Kotler & Orenstein, 1993), which have spores that are not distinguishable morphologically from those of *E. cuniculi* of animal origin. Therefore, in order to identify spores of the *Encephalitozoon*-type, immunological and molecular techniques are required. Recently, we have shown by Western blot analysis of spore antigens, as well as by restriction enzyme analysis of the PCR-amplified small subunit rRNA gene (SSU rRNA), that 3 of 7 human *Encephalitozoon* isolates were indistinguishable from 9 rabbit *E. cuniculi* isolates and from 1 mouse reference isolate (Deplazes *et al.* 1996*a*). These findings as well as 3 recently published case reports of *E. cuniculi* infections in humans (De Groote *et al.* 1995; Franzen *et al.* 1995; Hollister *et al.* 1995) imply a zoonotic nature of *E. cuniculi* infections. A few observations, however, suggest that there might exist different strains of *E. cuniculi* in nature with different host preferences (reviewed by Canning & Lom, 1986). Recently, Didier *et al.* (1995) identified 3 strains of *E. cuniculi* from animals based on subtle differences in SDS–PAGE, immunoblots and on the number of repeats of the tetranucleotide sequence 5′GTTT-3′ in the rDNA internal transcribed spacer (ITS). Thus, strain I of *E. cuniculi*, including mainly isolates from rabbits contained 3 tetranucleotide repeats, isolates from 2 mice (strain II) had 2 repeats, and 2 isolates from dogs (strain III) had 4. Mathis *et al.* (1996) have similarly characterized *E. cuniculi* isolates from Swiss rabbits and farmed blue foxes (*Alopex lagopus*) from Norway and classified them as strain I (all 9 isolates from rabbits) and strain II (all 4 isolates from foxes), respectively. The classification

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Table 1. Sequence polymorphisms in the SSU rRNA gene of *Encephalitozoon cuniculi* isolates from rabbits, a farmed blue fox and humans

E. cuniculi isolate (host/strain†)	Positions of nucleotide sequence divergence*		
	572	717	798
GenBank L17072* (rabbit/strain N.D.)	T	A	A
IPZ:CH-R2169 (rabbit/strain I)	T	A	G
IPZ:CH-H4 (human/strain I)	C	A	A
IPZ:N-F120 (blue fox/strain II)	T	T	A
IPZ:MX-H5 (human/strain III)	T	T	A

* Pieniazek *et al.*, GenBank accession no. L17072.
† As determined by the number of tetranucleotide 5′-GTTT-3′ repeats in the sequence of the internal transcribed spacer region (Didier *et al.* 1995).
N.D., Not determined.

was confirmed by consistent differences among the isolates of these 2 groups from rabbits and blue foxes by Western blot as well as by RAPD (random amplification of polymorphic DNA) analysis.

The objectives of this study were to compare *E. cuniculi* isolates of animal origin with 6 isolates obtained from HIV-infected patients with local and disseminated infections. Furthermore, we evaluated the infectivity in rabbits of 2 human-derived *E. cuniculi* isolates belonging to different strains.

MATERIALS AND METHODS

Parasite isolates

Since our first report on the identification of 3 *E. cuniculi* isolates (IPZ:CH-H4, IPZ:MX:H5, IPZ:CH-H6) from HIV-infected patients (Deplazes *et al.* 1996*a*), *Encephalitozoon*-like spores have been detected by light microscopical examination of chromotrope-stained specimens (Weber *et al.* 1992) from another 3 patients. These patients were identified in an ongoing study in which urine specimens obtained from HIV-infected patients with and without symptoms were screened for the presence of microsporidial spores. Isolation of spores from urine (in 1 case also from bronchoalveolar fluid) as well as *in vitro* cultivation in human embryonic lung fibroblast cells (MRC-5) was done as described earlier (Deplazes *et al.* 1996*a*). These 3 isolates were identified as *E. cuniculi* by restriction enzyme analysis of the PCR-amplified SSU rRNA gene (isolates IPZ:CH-H9, IPZ:CH-H10, and IPZ:CH-H12). Furthermore, 1 representative *E. cuniculi* isolate from a rabbit from Switzerland (IPZ:CH-K2169, Deplazes *et al.* 1996*a*) and from a farmed blue fox (*A. lagopus*) from Norway (IPZ:N-F120, Mathis *et al.* 1996) were available, as well as 2 isolates of *E. hellem* (IPZ:CH-H1 and IPZ:CH-H2, Deplazes *et al.* 1996*a*).

Western blot analysis (WBA)

WBA was performed basically as described previously (Deplazes *et al.* 1996*a*). The blots were saturated in PBS with 0·3 % Tween 20 (PBST) and incubated overnight at 4 °C on a rotator platform with a serum sample (1:250 in PBST) from a rabbit experimentally infected with the *E. cuniculi* isolate IPZ:CH-H4 (see below). After several washes with PBST the blots were incubated with a 1:400 dilution of peroxidase conjugated to goat anti-rabbit IgG (whole molecule) (Sigma, Buchs, Switzerland) in PBST for 2 h at room temperature. For visualization of the antibody reactions, hydrogen peroxide and diaminobenzidene were used as substrate and chromogen, respectively.

Molecular biology

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

DNA amplifications by polymerase chain reaction (PCR) were performed with a PTC 200 DNA engine (MJ Research, Watertown, MS). The PCR buffer contained 50 mM KCl, 20 mM Tris–HCl (pH 8·4), 2·5 mM $MgCl_2$, 0·5 % Tween 20, and 200 μM of each deoxyribonucleotide. For amplification of rDNA sequences, about 100 ng of genomic DNA was cycled in 100 μl reactions containing 2·5 U *Taq* polymerase (BRL, Basle, Switzerland).

The full length of the SSU rRNA gene was amplified as described (Deplazes *et al.* 1996*a*) and the sequences from 4 isolates of *E. cuniculi* and 2 isolates of *E. hellem* (Tables 1 and 2) were determined initially with the Sequenase II kit (United States Biochemicals, Cleveland, OH) after cloning the PCR products into the plasmid pCR-script (Stratagene). Later, the PCR products were directly sequenced using Stratagene's Cyclist Kit. From all isolates, the sequence of both strands was determined from the fragments of 2 independent amplification reactions and aligned to the sequences published by Pieniazek *et al.* (GenBank accession number L17072 for an *E. cuniculi* isolate from a rabbit or GenBank accession number L19070 for an *E. hellem* isolate from a HIV-infected patient).

The rDNA internal transcribed spacer region was

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Table 2. Sequence polymorphisms in the SSU rRNA gene of 3 *Encephalitozoon hellem* isolates from HIV-infected patients

	Positions of nucleotide sequence divergence*								
E. Hellem isolate	119	160	171	505	517	538	539	952	1013
GenBank L19070*	A	G	G	A	G	T	A	A	G
IPZ:CH-H1	A	T	T	T	A	G	G	A	A
IPZ:CH-H2	T	T	T	T	A	G	G	G	A

* Pieniazek *et al.* GenBank accession no. L19070.

amplified using the primers described by Katiyar, Visvesvara & Edlind (1995) and its sequence determined with Stratagene's (Heidelberg, Germany) Cyclist Exo Pfu DNA Sequencing Kit (using the internal primer 5′-TCGTAACATGGCTGCTGT-TGG-3′, corresponding to positions 76–96 of the *E. cuniculi* sequence, Katiyar *et al.* 1995).

For random amplification of polymorphic DNA (RAPD), 10 ng of DNA with 1 μM primer (5′-AATCGGGCTG-3′, OPA-4, Operon Technologies, Alameda, CA) and 1 U *Taq* polymerase were subjected to the following cycling regimes: 5 cycles of 94 °C 30 sec (denaturation) 30 °C for 30 sec (annealing), 72 °C 30 sec (extension) followed by 35 cycles with the annealing temperature raised to 36 °C (50 μl reactions). The ramp rate from 94 °C to the annealing temperature was set to 0·5°/sec.

Cross-contamination problems were avoided by following good laboratory practice, including the use of aerosol-guarded pipette tips. The areas of DNA extraction, amplification and amplicon analysis were strictly separated.

Experimental infections of rabbits

The SPF New Zealand white rabbits were obtained from Charles River Savo (Kisslegg/Allgäu, Germany) and were kept singly in steel cages. All animals were serologically tested for *E. cuniculi* twice (with a 3-week interval) and were negative. Groups of 2 rabbits were inoculated with 3 *E. cuniculi* isolates by administering *per os* 10^7 spores (in 2 ml of PBS) obtained freshly from *in vitro* cultures. The rabbits inoculated with 2 human *E. cuniculi* isolates (IPZ:CH-H4, IPZ:MX-H5) were kept together with 2 control animals in a room separate from the one housing the 2 animals inoculated with the isolate from rabbit (IPZ:CH-K2169) and another 2 control animals.

Blood was taken once a week until the end of the experiments at day 168 p.i., when 1 animal of each group was killed. Serum was tested by IFAT using spores from *in vitro* cultures as antigen for the presence of specific IgG antibodies. Isolation of parasites from brain material and subsequent *in vitro* cultivation were done as previously described (Deplazes *et al.* 1996*a*).

RESULTS

The *E. cuniculi* isolates from HIV-infected patients were characterized after *in vitro* mass cultivation of spores by immunological (Western blot) and molecular biological (riboprinting) methods and compared with isolates from rabbits and farmed blue foxes. No differences were seen in the riboprinting patterns among all isolates. Subtle differences were observed in Western blot analysis using serum of a rabbit experimentally (*per os*) infected with *E. cuniculi* spores (Fig. 1). The isolate IPZ:CH-K2169 (from a rabbit) was indistinguishable from 4 isolates from HIV-infected patients (Fig. 1, lanes 1–5) with a band at about 33 kDa varying considerably in intensity between these isolates. The antigen profile of the isolate IPZ:CH-H12 (Fig. 1, lane 6) resembled these isolates except for the double band in the 50–55 kDa range which is slightly more widely separated (confirmed in a repeated WB, not shown). The isolate IPZ:MX-H5 (from a human; Fig. 1, lane 7) differed from the former isolates by the band characteristics in the ranges 50–55 kDa (double bands more widely separated), 40 kDa (slightly broader double bands) and 29 kDa (lower band). The isolate IPZ:N-F120 (from blue fox) resembled this latter isolate except for the 50–55 kDa range where narrower double bands were observed (Fig. 1, lane 8). The *E. hellem* isolate IPZ:CH-H1 could clearly be differentiated from all *E. cuniculi* isolates (Fig. 1, lane 9).

Random amplification of polymorphic DNA with primer OPA-4 reproducibly gave a very homogenous pattern among all *E. cuniculi* isolates (Fig. 2). However, subtle differences were observed between a group including the rabbit and 5 isolates from humans and the isolates IPZ:MX-H5 (from human) and the 1 from blue fox: in the rabbit-derived isolate as well as in the 5 isolates from humans (Fig. 2, lanes 1–6), a band at about 1350 bp (Fig. 2, arrow) is seen which is absent in the isolate from the blue fox (Fig. 2, lane 8). In the latter, on the other hand, slightly above the bright band at 1200 bp, there is a faint band detectable at about 1250 bp which is absent in the former 6 isolates. The banding pattern of the sixth isolate from human (Fig. 2, lane 7) contains both of these bands as well as an additional unique

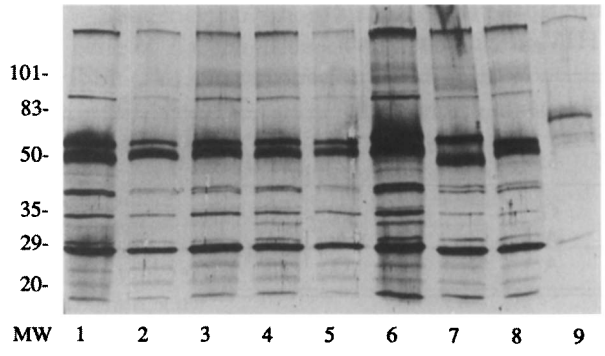

Fig. 1. Western blot analysis of purified spores from *in vitro* cultures: lane (1) *Encephalitozoon cuniculi* IPZ:CH-K2169 (isolated from a rabbit), (2) IPZ:CH-H4, (3) IPZ:CH-H6, (4) IPZ:CH-H9, (5) IPZ:CH-H10, (6) IPZ:CH-H12 (7) IPZ:MX-H5 (2–7 isolated from humans), (8) IPZ:N-F120 (isolated from a blue fox), (9) *E. hellem* reference isolate (IPZ:CH-H1). A serum sample of a rabbit experimentally infected with isolate IPZ:CH-H4 was used. (MW) Molecular weights (in thousands).

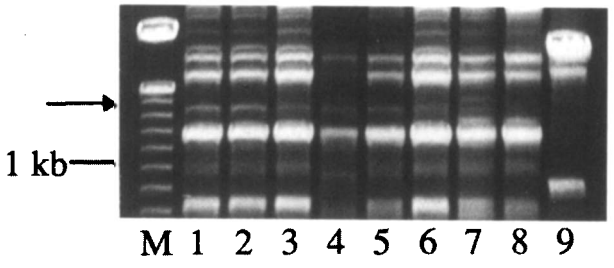

Fig. 2. RAPD banding patterns of *Encephalitozoon cuniculi* isolates obtained with primer OPA-4. Lane M: DNA size marker (100 bp ladder); lane (1) *E. cuniculi* IPZ:CH-K2169 (isolated from a rabbit), (2) IPZ:CH-H4, (3) IPZ:CH-H6, (4) IPZ:CH-H9, (5) IPZ:CH-H10, (6) IPZ:CH-H12 (7) IPZ:MX-H5 (2–7 isolated from humans), (8) IPZ:N-F120 (isolated from a farmed blue fox), (9) *E. hellem* reference isolate (IPZ:CH-H1). The arrow marks the position of the polymorphic amplified DNA band.

band at about 1400 bp. *E. hellem* (isolate IPZ:CH-H1) again could easily be discriminated from *E. cuniculi* (Fig. 2, lane 9).

The sequence of the rDNA internal transcribed spacer was identical to the one described (Didier *et al.* 1995) in all 6 *E. cuniculi* isolates from humans except for the number of the 5′-GTTT-3′ tetranucleotide element which was present 3 times in 5 isolates but 4 times in isolate IPZ:MX-H5.

The SSU rRNA sequences that were determined from 4 isolates of *E. cuniculi* and from 2 isolates of *E. hellem* differed by 11·4% between the two species. The SSU rRNA sequences of the 4 *E. cuniculi* isolates IPZ:CH-H4, IPZ:MX-H5 (from humans), IPZ:CH-K2169 (from a rabbit) and IPZ:N-F120 (from a farmed blue fox from Norway) were aligned with the one published by Pieniazak *et al.* (GenBank accession no. L17072) for an *E. cuniculi* from a rabbit. Three polymorphic sites were identified as shown in Table 1. The sequences of 2 isolates (IPZ:MX-H5 and IPZ:N-F120) were identical; identity among all other sequence pairs was 99·85% (2 differences). The sequences of the 2 *E. hellem* isolates IPZ:CH-H1 and IPZ:CH-H2 (from 2 HIV-infected patients living in Switzerland) differed at 2 positions (99·85% identity, Table 2). As compared to a published sequence of *E. hellem* (Pieniazek *et al.*, GenBank accession no. L19070), sequence divergence was 0·55% (7 different nucleotides) and 0·7% (9 differences), respectively.

To test the infectivity of *E. cuniculi* of human origin, 2 rabbits each were inoculated with spores of the *E. cuniculi* isolates IPZ:CH-H4 (from a human, indistinguishable from the rabbit-derived isolate), IPZ:MX-H5 (from a human, differing from the former isolate as shown above) and IPZ:CH-K2169 (originating from a rabbit, indistinguishable from the first isolate). All rabbits showed a comparable immune response to the infection as assessed by IFAT. Specific antibody reactions occurred between weeks 3 and 4 after inoculation. The antibody titres increased until the end of the experiment at day 168 p.i. The control rabbits remained serologically negative during the whole experiment. No clinical symptoms were observed at the end of the experiment. At day 168 p.i., 1 animal of each group was killed. Most importantly, *E. cuniculi* was successfully cultivated from brain material from all 3 animals. The number of the tetranucleotide repeats of these re-isolated *E. cuniculi* corresponded to the inoculated isolates.

The clinical manifestations associated with *E. cuniculi* in the 6 HIV-infected patients are summarized in Table 3. They range from no detectable symptoms to symptoms detected in different organs due to disseminated infections. All patients were severely immunodeficient with CD4 lymphocyte counts below $0{\cdot}05 \times 10^9$/l. Three patients had clinical manifestations that were associated with microsporidial infection. Of note, patient H5 from whom the *E. cuniculi* strain III was isolated from urine and broncho-alveolar lavage fluid, presented with severe interstitial pneumonitis. Patient H9 presented with seizures of unknown origin (computed tomographic scan of the brain, EEG, cerebrospinal fluid and neurological examination were normal), but no microsporidial spores were detected in the cerebrospinal fluid. Patient H10 was found to shed microsporidial spores in urine when she was asymptomatic. After a 2-week treatment with albendazole (2×400 mg/day) no spores were detected anymore. Seven months later, however, she started again to excrete microsporidial spores and subsequently developed renal insufficiency despite a new treatment with albendazole. Encephalitozoon spores also were detected in a stool sample of patient H5

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Table 3. *Encephalitozoon cuniculi*-associated clinical manifestations in 6 HIV-infected patients

Isolate/patient identification	Detection of *E. cuniculi* spores*				Clinical manifestations associated with microsporidial infection†
	Urine	Respiratory specimens	Conjunctival swab	Stool	
IPZ:CH-H4	Yes	No	N.D.	No	None
IPZ:MX-H5	Yes	Yes	N.D.	Yes	Pneumonitis, otitis externa (spores detected in swab of external auditory canal
IPZ:CH-H6	Yes	No	N.D.	No	None
IPZ:CH-H9	Yes	No	No	No	Conjunctivitis, sinusitis, seizure disorder of unknown origin (no spores detected in cerebrospinal fluid)
IPZ:CH-H10	Yes	No	No	No	Renal insufficiency, leucocyturia, erthrocyturia
IPZ:CH-H12	Yes	Yes	No	No	None

N.D., Not determined.
* Identified by riboprinting (Deplazes *et al.* 1996*a*) of the cultured isolates obtained from urine and respiratory specimens.
† Other opportunistic infections were excluded by state-of-the-art evaluation at the HIV-outpatient clinic of the University Hospital in Zürich.

who did not have diarrhoea or complaints of abdominal pain. Whether these spores derived from the proven infection of the respiratory tract or from an intestinal infection was not determined. In 3 patients, shedding of microsporidial spores was not associated with any signs or symptoms.

DISCUSSION

This study showed that 4 *E. cuniculi* isolates from HIV-infected patients living in Switzerland were indistinguishable by immunological and molecular methods from the isolates from Swiss rabbits, allowing their classification into *E. cuniculi* strain I (Didier *et al.* 1995), as well as by experimental infections of rabbits. Thus, these *E. cuniculi* isolates can be considered identical and this substantiates the autochthonous zoonotic nature of the parasite. The fifth isolate from a Swiss patient (IPZ:CH-H12) was also classified into strain I (based on ITS sequence and RAPD profile). However, its WB profile differed slightly from those of the other isolates of this strain indicating that even within a strain some antigen variation may occur.

The number of the tetranucleotide repeats in the sequence of the rDNA internal transcribed spacer (ITS) represents an excellent genetic marker for the differentiation of the 3 strains of *E. cuniculi*, reflecting both subtle phenotypic (as shown by WBA) as well as genotypic (RAPD) differences (Didier *et al.* 1995; this study). As microsporidia of vertebrates are asexual (Canning & Lom, 1986), one can expect this genetic marker to be stable over time. Therefore it represents a valuable marker for molecular epidemiological studies.

Hollister *et al.* (1993, 1995) have compared their *E. cuniculi* isolate obtained from the urine of an AIDS patient with 1 isolate each from murine and canine origin. They found differences among all 3 isolates in SDS–PAGE, WBA and RAPD profiles. The 3 strains described by Didier *et al.* (1995) cannot be considered as separate species because the sequences of the SSU rRNA gene among them showed a very high degree of identity (99·85–100%) as we have also found for the sequences from 2 *E. hellem* isolates (99·85%). In contrast, the sequences of the 2 species *E. cuniculi* and *E. hellem* differ by 11·4%. Furthermore, the slight differences in the sequence of the SSU rRNA gene among the different isolates of *E. cuniculi* did not correlate with the strain classification achieved with the other methods (WBA, RAPD, ITS sequence).

The findings that *E. cuniculi* is not a homogenous species raises the questions about the biological and epidemiological relevance of these different strains. There are indeed indications that host specificity of these strains differs under natural conditions. Firstly, all *E. cuniculi* isolates from rabbits characterized so far (9 isolates from Switzerland (Mathis *et al.* 1996), and 4 isolates from the USA (Didier *et al.* 1995; Katiyar *et al.* 1995)) belong to strain I. Secondly, dogs and blue foxes are considered to be the most commonly infected carnivore hosts and, so far, 2 *E. cuniculi* isolates from dogs (from the USA) were characterized as belonging to strain III (Didier *et al.* 1995) and 4 isolates from farmed blue foxes from Norway as belonging to strain II (Mathis *et al.* 1996). *E. cuniculi* is known to have a remarkable pathogenicity for the blue fox and mink in Norway (Canning & Lom, 1986) but encephalitozoonosis of dogs has not been reported from Norway and, in a recent investigation, no *E. cuniculi*-infections were diagnosed by histopathological examinations of dogs with neurological symptoms (J. Åkerstedt, Depart-

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ment of Virology and Serodiagnostics, Central Veterinary Laboratory, Oslo, Norway, personal communication). Thirdly, in Switzerland *E. cuniculi* is a common parasite in rabbits and has been diagnosed by ELISA, or IFAT in 7·5% of 292 healthy rabbits from 126 owners and in 85% of rabbits (total 72 animals from 48 owners) with neurological symptoms (Deplazes *et al.* 1996*b*). The diagnostic value of the tests was confirmed, as parasites could be isolated from brain and/or kidney and cultivated *in vitro* from 18 of 19 serologically positive animals. Nine of these isolates were characterized and all were found to belong to *E. cuniculi* strain I. A sero-epidemiological survey revealed that none from 212 dogs and 82 red foxes was serologically positive (Deplazes *et al.* 1996*b*). Thus, *E. cuniculi* strain I has a high prevalence in rabbits from all over Switzerland and is also infective to HIV-infected patients (5 of 6 of our patients were infected with this strain), but obviously not for carnivores.

On the other hand, a strict host specificity of the *E. cuniculi* strain III was not demonstrated under experimental conditions. The 1 isolate belonging to strain III ('dog strain') that we had obtained from a patient (a Swiss citizen living in Mexico who returned to Switzerland when seeking medical treatment) was also infective to rabbits that were given spores from *in vitro* culture by oral administration. Previous reports have also shown that isolates of *E. cuniculi* from rabbits and dogs are infectious in other hosts: an *E. cuniculi* isolate from a rabbit that was included in the study of Didier *et al.* (1995) and was determined to belong to strain I was previously shown to infect BALB/c mice after intraperitoneal injection of 10^7 spores (Schmidt & Shadduck, 1983). Shadduck *et al.* (1979) infected rabbits, mice, rats and Rhesus monkeys with an *E. cuniculi* isolate from a rabbit after intracerebral inoculation of 10^6 spores. In both reports rather forced inoculation procedures were used, but our experiments showed that infection via the presumed natural oral route also led to brain infections. Using oral inoculation, Van Dellen, Stewart & Botha (1989) infected another primate host, vervet monkeys (*Cercopithecus pygerythrus*), with a canine *E. cuniculi* isolate, as was confirmed by re-isolation of the parasites from kidneys.

The source of human *E. cuniculi* infection and the mode of acquisition and/or transmission remain unclear. None of our patients from Switzerland, all of whom were infected with *E. cuniculi* of the rabbit type, owned a rabbit or recalled some exposure to rabbits during adulthood, but most of the patients had a history of repeated exposure to different mammals. One of the patients grew up on a farm where animals were raised including rabbits. Another 2 owned other pets (cat, mice, dog) of which stool and/or urine samples (but no serum samples) were provided. No microsporidial spores were detected in these specimens by light microscopical examination. Patient-to-patient transmission appears unlikely because our patients neither knew each other nor had obvious personal contacts like hospitalization at the same time. Long-term follow-up observation will have to determine whether the patients without clinical signs associated with *E. cuniculi* infections were asymptomatic carriers or whether microsporidial infections were detected before symptoms had developed.

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