

Immunologic and Molecular Characteristics of *Encephalitozoon*-Like Microsporidia Isolated from Humans and Rabbits Indicate That *Encephalitozoon cuniculi* Is a Zoonotic Parasite

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To assess the zoonotic potential of *Encephalitozoon*-like microsporidia, we isolated and cultivated spores from specimens of urine, respiratory secretions, and stool from six patients infected with human immunodeficiency virus and from nine rabbits. Because spores of *Encephalitozoon*-like species are indistinguishable by microscopy, we characterized the isolates by western blot analysis and by restriction enzyme analysis of the small subunit (SSU) rDNA after amplification by the polymerase chain reaction. We identified *Septata intestinalis* in one patient and *Encephalitozoon hellem* in two symptomatic patients. *Encephalitozoon cuniculi* was found in all rabbits and in three patients. One of these patients had clinical manifestations of infection with this parasite (severe interstitial pneumonitis). We observed abatement of symptoms and cessation of parasite excretion when these patients were treated with albendazole. Our findings suggest that *E. cuniculi* may be pathogenic in humans and that it is a zoonotic parasite.

In recent years, microsporidia have emerged as important opportunistic parasites in patients with AIDS. Three *Encephalitozoon*-like microsporidia of animal origin that share most of the morphological features of *Encephalitozoon cuniculi* have been found in humans [1]. *E. cuniculi* is parasitic in birds and mammals including rabbits, rodents, carnivores, and primates [2]. *E. cuniculi* was detected in the CSF and/or urine (in 1959 and 1984) of two children with seizures and, subsequently, in HIV-infected patients with hepatitis, peritonitis, and keratoconjunctivitis [1]. Whether these infections indeed were caused by *E. cuniculi* or by another *Encephalitozoon*-like microsporidian is not known because only results of light microscopical studies and/or electron microscopical studies of the parasites are available.

Results of a western blot analysis performed in 1992 implied that human *Encephalitozoon* isolates belong to a separate species, which was named *E. hellem*. Another *Encephalitozoon*-like microsporidian that caused chronic diarrhea and systemic disease in HIV-infected patients was subsequently described; this organism was named *Septata intestinalis* on the basis of its specific ultrastructural appearance in small intestinal enterocytes (reviewed in [1]). Recently, molecular characterization

of an *Encephalitozoon*-like organism isolated from the urine and respiratory specimens of a single HIV-infected patient has suggested that *E. cuniculi* may also be parasitic in humans [3].

Patients and Methods

Encephalitozoon-like microorganisms were detected in different specimens from six HIV-infected patients (one female and five males) by light microscopical examination of chromotrope-stained specimens [1]. Rabbits with antibodies to spores of *E. cuniculi* detected with use of a routine immunofluorescence assay were selected for the isolation of microsporidia. Urine samples (0.1–2 L) and bronchoalveolar aspirates were centrifuged (1,000g for 20 minutes) and washed twice with distilled water. Unfixed stool specimens were homogenized in water, pressed through sieves (widths of mesh: 200 μm , 50 μm , and 20 μm), and washed five times in distilled water. The sediment was overlaid with a PBS-50% Percoll solution (Pharmacia, Silver Spring, MD) and centrifuged (600g for 30 minutes) at room temperature. Kidney and brain tissue from rabbits were mechanically homogenized, washed, and centrifuged in PBS-30% Percoll (700g for 40 minutes at room temperature).

The following microsporidia reference isolates were available: an *E. cuniculi* isolate of animal origin (courtesy of Professor F. Derouin, Hôpital Saint-Louis, Paris), two *E. hellem* isolates (CDC [Centers for Disease Control and Prevention] 0291:V213 [4] and CH-H1BG [5]), and an *S. intestinalis* isolate (CDC:V297 [6]). In vitro cultivation of spores was done as described [4] with human embryonic lung fibroblast (MRC-5) cells. Antibodies to spores of *E. cuniculi* (isolate CH-R2169 from a rabbit), *E. hellem* (isolate CH-H1BG), and *S. intestinalis* (isolate CDC:V297) were raised in 9-week-old female ICR

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Informed consent was obtained from all patients to perform the examinations described in this paper. Compassionate use of albendazole was reviewed by the ethical committee of the University Hospital of Zürich.

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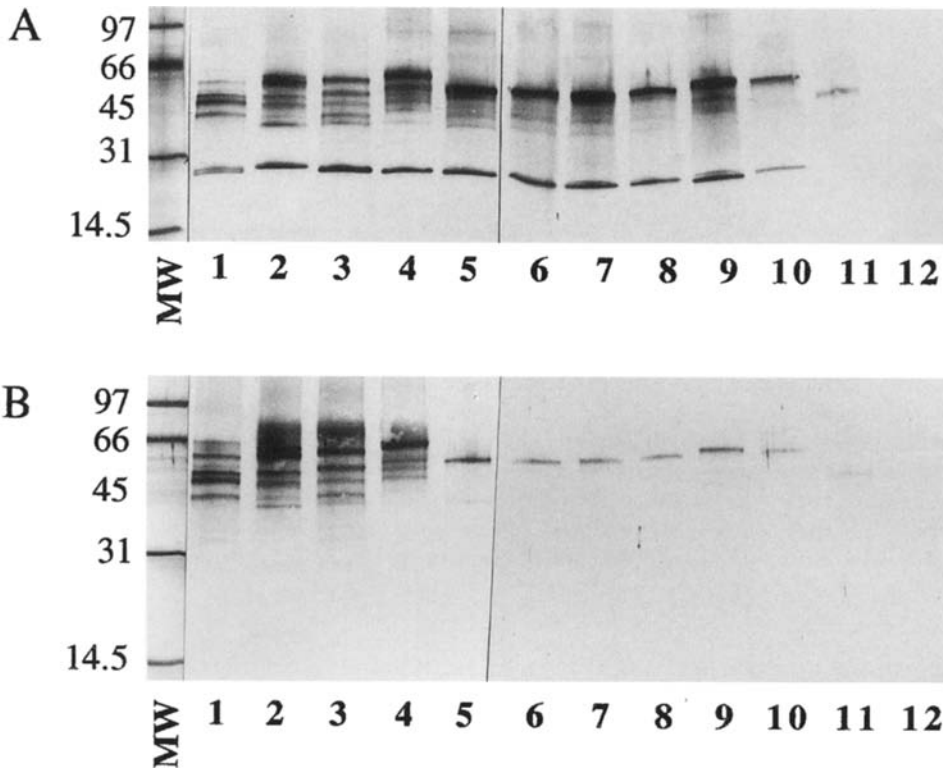


Figure 1. Western blot analysis of purified spores of *Encephalitozoon*-like microsporidia isolated from humans and rabbits. Lanes 1 and 2, *Encephalitozoon hellem* reference strains CDC:0291:V213 and CH-H1BG. Lane 3, strain CH-H2SF, and lane 4, strain CH-H3WR (both isolated from HIV-infected patients). Lanes 5 and 6, *E. cuniculi* strain CH-R2169 from a rabbit. Lane 7, *E. cuniculi* reference isolate. Lane 8, strain CH-H4BJ; lane 9, strain CH-H5RB; and lane 10, strain CH-H6FN (all isolated from HIV-infected patients). Lane 11, *Septata intestinalis* reference strain CDC:V297. Lane 12, strain CH-H7OR isolated from the stool of an HIV-infected patient. Murine antibodies to inactivated and purified spores of *E. cuniculi* (A) or *E. hellem* (B) were used. MW = molecular weights (in thousands).

(non-inbred) mice that were immunized by subcutaneous injection of 2×10^7 spores (inactivated at $+90^\circ\text{C}$ for 5 minutes) in Freund's complete adjuvant. The procedure was repeated 4 weeks later with use of Freund's incomplete adjuvant, and serum samples were collected 2 weeks later.

Western blot analysis was performed as described previously [4]. Antigens from 1×10^6 purified spores per lane were separated by electrophoresis (4%–20% gradient gels). Specific antibody reactions were detected with goat antibody to mouse IgG (H and L chains) (Cappel, Turnhout, Belgium). For riboprinting, DNA was released from the spores through a combination of mechanical disruption (vortexing with glass beads) and proteinase K digestion. The SSU rDNA was amplified under standard PCR conditions with use of primers that corresponded to positions 1–19 and 1277–1299, respectively, of the SSU rRNA gene sequence of *E. cuniculi* [7]. PCR products were cleaved with restriction enzymes and resolved by electrophoresis in 2% agarose gels.

Results

Encephalitozoon-like spores were isolated from five urine sediments and from one bronchoalveolar lavage specimen from five patients and from nine rabbits (three isolates each were from urine, kidney, and brain). All of these isolates were established in culture for further characterization. The human *E. cuniculi* isolate CH-H4BJ was cultured, although strongly

cytopathic BK virus was detected by electron microscopy. Spores directly isolated from stool from a sixth patient were used for further characterization of the *S. intestinalis* isolate CH-H7OR, as the MRC-5 cells were destroyed by adenovirus type 9 that was identified by seroneutralization with polyclonal type-specific antibodies (American Type Culture Collection, Rockville, MD). The isolates were characterized by western blot analysis with use of antibodies to spores of *E. cuniculi* (figure 1A), *E. hellem* (figure 1B), or *S. intestinalis* (data not

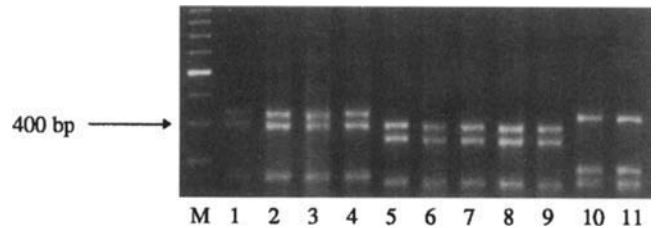


Figure 2. Restriction patterns of the amplified SSU rRNA gene of *Encephalitozoon*-like isolates after cleavage with *Mbo* I/*Hpa* II. Lane M, DNA size marker (100-bp ladder). Lane 1 and 2, *Encephalitozoon hellem* reference strains CDC:0291:V213 and CH-H1BG, respectively. Lane 3, strain CH-H2SF, and lane 4, strain CH-H3WR (both isolated from HIV-infected patients). Lane 5, *E. cuniculi* strain CH-R2169 from a rabbit. Lane 6, *E. cuniculi* reference isolate. Lane 7, strain CH-H4BJ; lane 8, strain CH-H5RB; and lane 9, strain CH-H6FN (all isolated from HIV-infected patients). Lane 10, *Septata intestinalis* reference isolate CDC:V297. Lane 11, strain CH-H7OR.

shown) and by riboprinting (figure 2), with congruent results: all nine isolates from rabbits (not shown) as well as three of six isolates from humans showed banding patterns that were identical to those of the *E. cuniculi* reference isolate (figure 1, lanes 5–10 and figure 2, lanes 5–9). Accordingly, two other isolates were indistinguishable from the two *E. hellem* reference isolates (figures 1 and 2, lanes 1–4), and one was identified as *S. intestinalis* (figure 2, lanes 10 and 11).

Discussion

Diagnosis of human microsporidial infections is presently based on morphological demonstration of the organisms by examination with light microscopy or electron microscopy. These techniques are sufficient to distinguish the species *Enterocytozoon bieneusi* from the *Encephalitozoon*-like microsporidia but are not appropriate for differentiating *Encephalitozoon*-like microsporidia, of which at least three morphologically similar species are parasitic in humans [1]. So far, microsporidial isolates from humans and animals have rarely been compared by means of immunologic and molecular biological techniques. A single HIV-infected patient with confirmed *E. cuniculi* infection and seven patients with systemic *E. hellem* infection have been described [1, 3]. From the epidemiological standpoint, characterization of *Encephalitozoon*-like organisms that are parasitic in animals is of importance, as *E. cuniculi* is known to be prevalent in animals, but animal reservoirs of *E. hellem* and *S. intestinalis* have not been identified so far.

The fact that three of the six *Encephalitozoon*-like isolates from patients and all nine rabbit isolates were characterized as *E. cuniculi* supports earlier suggestions that *E. cuniculi* might be of importance as a zoonotic parasite. In an ongoing epidemiological survey conducted by our laboratory, we found that ~16% of necropsied rabbits proved positive for the organism on serology. Rabbits infected with *E. cuniculi* excrete high numbers of spores in the urine [2]. However, no obvious source of infection was recognized for our six patients, as none owned a pet or reported exposure to animals. As *E. cuniculi* spores are resistant in the environment, infections caused by contaminated water, food, or other sources have to be considered.

All six HIV-infected patients were severely immunodeficient, with CD4 cell counts that ranged from 0 to $0.05 \times 10^9/L$. Two patients with *E. cuniculi* infection had no associated clinical manifestations. The third patient, who had *E. cuniculi* isolated from urine and bronchoalveolar lavage fluid, presented with severe interstitial pneumonitis. Of patients with *E. hellem*

infection, one presented with acute urinary tract infection, and one presented with systemic disease including conjunctivitis, sinusitis, bronchitis, and assumed cystitis. Although organisms were detected in urine specimens of all patients infected with *E. cuniculi* or *E. hellem*, only one patient had significant leukocyturia and erythrocyturia, and none had evidence of renal failure. In contrast to previous reports that described no patients with *E. hellem* and *E. cuniculi* identified in stool specimens, we found that one patient with systemic *E. hellem* infection and one with systemic *E. cuniculi* infection were excreting a few *Encephalitozoon*-like spores in stool. Albendazole (400 mg twice daily for 2–3 weeks) was found to cure infections due to *encephalitozoon* and *S. intestinalis* infections in all six patients. No relapse of *encephalitozoon* infection was diagnosed during the follow-up observation period of 3–14 months, although no secondary prophylaxis with albendazole was given.

Our results strongly suggest that *E. cuniculi* is pathogenic in severely immunodeficient HIV-infected individuals and that the organism is a zoonotic parasite. Modes of transmission have yet to be elucidated.

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