

# Serodiagnostic Studies in an Immunocompetent Individual Infected with *Encephalitozoon cuniculi*

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Little is known about the prevalence and clinical significance of infection with *Encephalitozoon* species in immunocompetent individuals. In the present study, by using indirect immunofluorescence technique (IFAT), Western blot, and recombinant antigens of the spore wall (SWP1) and polar tube (PTP1, PTP2, and PTP3), we analyzed the IgG antibody response of a laboratory worker who was infected with *Encephalitozoon cuniculi*. Serum samples were analyzed 1, 20, 32, and 38 months after infection. After 1 month, by use of IFAT, only spore-wall antigens were recognized, an antibody reaction that changed toward both the spore wall and polar tube in the following months. By use of Western blot analysis, a characteristic pattern that recognized multiple bands was noticed. Reaction against SWP1 was present in all 4 serum samples. The IgG response against PTP1, PTP2, and PTP3 was not detectable 1 month after infection, but became evident in the follow-up serum samples. Serum samples showed cross-reactivity with the spore wall of *Encephalitozoon hellem* and *Encephalitozoon intestinalis*, but only little cross-reactivity with the polar tube of these parasites. This is the first study to our knowledge that provides full details about the antibody response against a specified *Encephalitozoon* species in an immunocompetent person. The results strongly encourage the development and use of reliable serodiagnostic methods, which will provide information about the prevalence and clinical significance of *Encephalitozoon* species infection in humans.

Infection with microsporidia of *Encephalitozoon* species, *E. intestinalis*, *E. hellem*, and *E. cuniculi*, is frequently reported in patients with AIDS. Clinically, these infections are associated with diarrhea, rhinosinusitis, keratoconjunctivitis, hepatitis, nephritis, and encephalitis [1, 2]. Compared with patients with AIDS, little is known about the prevalence and clinical significance of infection with *Encephalitozoon* species in immunocompetent individuals. Intestinal infection with *Encephalitozoon* species was reported for inhabitants living in rural areas in Mexico and travelers returning from the tropical regions [3–5]. Systemic infection caused by *Encephalitozoon* species was reported in 2 children with

severe neurological symptomatology, including convulsive seizures [6, 7].

Appropriate diagnostic tests could improve our knowledge of infection with *Encephalitozoon* species in immunocompetent individuals. Detection of spores, which is most effective for diagnosis of microsporidiosis in patients with AIDS, is less useful in immunocompetent cases, because shedding of spores occurs only during a short duration. In these patients, indirect diagnosis with serological methods could be useful.

In earlier serological studies, antibodies to *Encephalitozoon* species were observed in humans residing or returning from the tropics and in 1 of 2 children with systemic *Encephalitozoon* species infection [7–11]. However, proper interpretation of results of these studies always has been hampered by possible cross-reactivity of antibodies to other microsporidia and, especially, by the absence of detailed knowledge of the specific antibody response in an immunocompetent human host infected with a specific *Encephalitozoon* species.

In the present study, we describe for the first time in detail the serological response of an HIV-uninfected

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immunocompetent patient infected with *E. cuniculi*, for whom both the moment of infection and the infecting species were known and long-term follow-up was available.

## PATIENTS, MATERIALS, AND METHODS

**Patients and collection of serum samples.** An HIV-uninfected laboratory worker, previously in good health, was accidentally infected with *E. cuniculi* (type 1 strain), when several drops of culture supernatant containing several millions of spores, were spilled in both eyes. Despite direct rinsing with water, severe conjunctivitis and keratitis developed 3 weeks after the accident. No systemic symptomatology was observed. Treatment with albendazole (400 mg/day) was started and continued for 5 months. In addition, treatment with fumagillin (10 mg/mL) was started 6 weeks after the accident and continued for 4 months. Clinical improvement was observed 8 weeks after the accident. After 1 year, signs of conjunctivitis and keratitis in the left eye disappeared completely. However, the cornea of the right eye remained clouded. Swabs of the right and left conjunctivae were negative for microsporidia when tested with polymerase chain reaction (PCR).

Serum samples were collected 1, 20, 32, and 38 months after infection. Serum samples before the accident and urine or stool samples after the accident were not available for examination. A serum sample from an HIV-infected patient with proven *E. intestinalis* infection was used as a positive control for Western blot with *E. intestinalis*. Spores were detected in maxillary sinus aspirate and in urine samples stained with Uvitex 2B [12]. Confirmation of the species was performed by use of electron microscopy and molecular techniques [13].

**Growth of parasites.** *E. cuniculi* (type I and III strains), *E. intestinalis*, *E. hellem*, and *Vittaforma corneae* were grown in human lung mucoepidermoid cells (NCI-H292 and ATCC-CCL 34), in Dulbecco's modified Eagle medium (Gibco BRL; no. 041-01095) supplemented with 10% heated-inactivated FCS and erythromycin (2.4 mg/L) in 5% CO<sub>2</sub> at 37°C [14].

**Immunological studies.** The indirect immunofluorescence technique (IFAT) method used has been described elsewhere [14]. In brief, cells containing *E. cuniculi* were grown on an 18-well glass slide. Serum samples were tested in a 2-fold dilution from 1:20 to 1:320 with FITC-labeled anti-human IgG (Fc and Fab) as conjugate. Slides were examined at a magnification of ×630 under a Leitz fluorescence microscope. Antibody reaction against the spore wall and polar tube were expressed as dilution fractions. For cross-reactivity studies, IFAT slides with *E. cuniculi* type III strain, *E. intestinalis*, *E. hellem*, and *V. corneae* were processed and studied in a similar procedure. For SDS-PAGE and Western blot culture, supernatant was harvested and centrifuged at 100 g for 10 min to remove cell debris. Spores in the resulting supernatant were pelleted

and were washed 3 times with 140 mmol/L PBS (pH 7.2) by centrifuging at 2600 g for 20 min at 4°C. Spores were counted and adjusted to a concentration of  $1 \times 10^9$ /mL and were stored at -20°C. For antigen preparation, spores were concentrated by centrifugation, and the pellet was resuspended in Laemmli sample buffer and boiled for 5 min, vortexed, and boiled for an additional 5 min. Insoluble material was removed by centrifugation, and the cleared supernatant was used as antigen in Western blot experiments. Denatured nonreduced proteins were electrophoretically separated by a 12.5% polyacrylamide gel (Mini-Protean Electrophoresis Cell; Biorad Laboratories). Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). The membrane was incubated for 30 min at room temperature with a blocking solution (50 mmol/L Tris-HCl [pH 10], 150 mmol/L NaCl, and 0.05% Tween 20 [TNT] containing 2% nonfat dry milk) and was incubated for 60 min at room temperature with the patient's serum sample (1:250) in TNT containing 0.9% nonfat dry milk (TNTW). The membrane was washed 3 times with TNTW, and bound antibodies were detected by incubation for 60 min at room temperature with a peroxidase-conjugated rabbit anti-human IgG (1:1000; Dako) in TNTW, followed by washing 3 times with TNTW. Antibody-binding bands were visualized by use of peroxidase activity using diaminobenzidine (DAB) substrate kit (ICN Biomedicals).

**Recombinant protein expression in *E. coli*.** DNA was released by boiling purified *E. cuniculi* spores at 100°C for 10 min in sterile water. Primers for PCR amplification were as follows: SWP1D (5'-CGGGATCCAACAGAAGGGGGAATTGC-3'), PTP1D (5'-CGGGATCCGCAACCGCACTGTGCA-GC-3'), PTP2D (5'-CGGGATCCACGATGGTCCATCCTTC-AG-3'), and PTP3D (5'-GGGGGATCCATTCCCGGA-3'), with a *Bam*HI restriction site in 5'; and SWP1R (5'-CGGAATTCT-TGAAGAGCATTAGTGATG-3'), PTP1R (5'-CGGAATTCCA-GTGATCCGAGGTTTGG-3'), PTP2R (5'-CGGAATTCCTCT-AGACCCTCGCCGTCC-3'), and PTP3R (5'-CGGAATTCCTTGGCC-3') with an *Eco*RI restriction site in 5'. These primers were designed to amplify DNA fragments corresponding to the amino acid regions 21–295 aa of SWP1, 23–330 aa of PTP1, 14–277 aa of PTP2 and 141–574 aa of PTP3. PCR amplifications were performed by use of a Perkin-Elmer DNA thermal cycler 2400 apparatus, according to standard conditions (Eurobio). After denaturing the DNA at 94°C for 3 min, 35 cycles were run with 20 s of denaturation at 94°C, 30 s of annealing at 54°C, and 90 s of extension at 72°C. PCR products were digested with the restriction endonucleases *Eco*RI and *Bam*HI and were cloned in frame with glutathione-S-transferase (GST) at the N-terminus and an 8-His tag at the C-terminus into the modified prokaryotic expression vector pGEX-4T1-His (Pharmacia). The resulting recombinant plasmids were introduced in the *E. coli* BL21<sup>+</sup> strain. After induction

with 2 mmol/L isopropyl b-D-thiogalactopyranoside for 4 h, bacterial proteins were solubilized in 6 mol/L guanidine hydrochloride, and recombinant proteins were purified on Ni-NTA columns by use of affinity chromatography, according to the manufacturer's protocol (Qiagen). Recombinant proteins then were analyzed by SDS-PAGE and were transferred to PVDF membranes for immunoblot analysis.

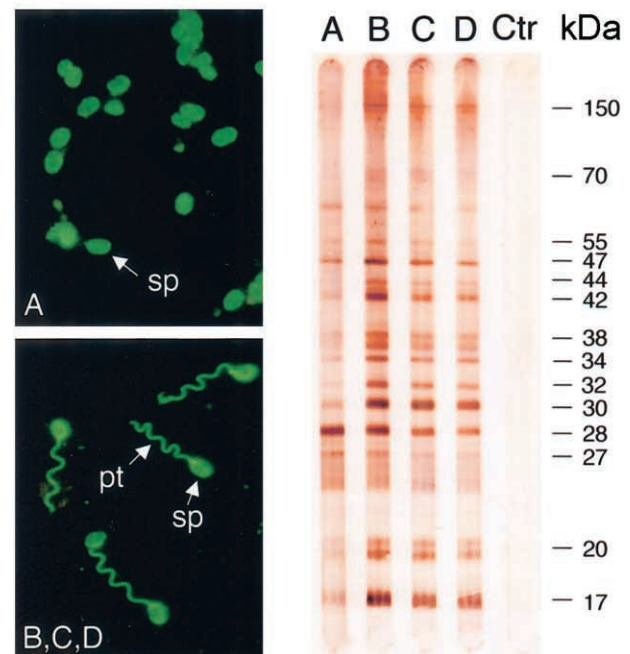
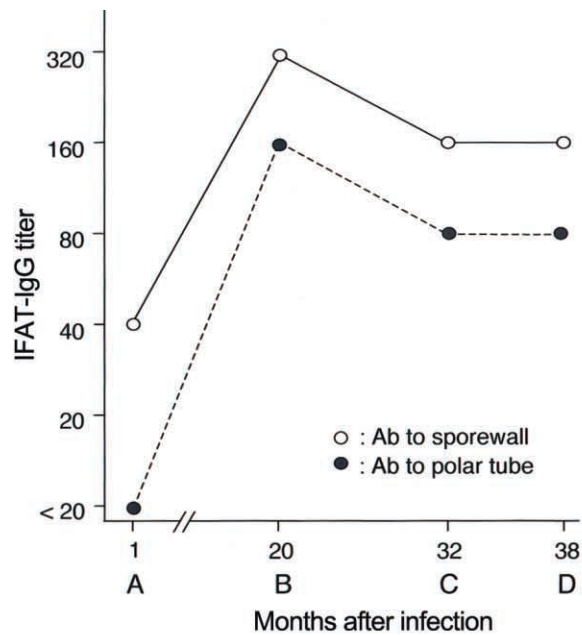
## RESULTS

**IFAT.** By use of IFAT, a serum sample taken 1 month after infection showed a moderately strong IgG antibody response against the spore wall of *E. cuniculi* (figure 1A; table 1), but no reaction was observed against the polar tube. After 20 months, the IgG titer against the spore wall had increased 3-fold with a strong IgG antibody response against the polar tube of the parasite (figure 1B; table 1). Antibody titers against the spore wall and polar tube declined slowly after 20 months (figure 1C and 1D; table 1). Cross-reactivity was observed with the spore wall of *E. intestinalis* and *E. hellem*, but was low with the polar tubes of these species (table 1). No cross-reactivity was observed with the spore wall or polar tube of *V. corneae*. No significant differences were observed with *E. cuniculi* type III strain (data not shown).

**Western blot.** Western blot analysis with these serum samples, which used an *E. cuniculi* type I strain extract as antigen, showed that, 1 month after infection, 1 strong band was observed at 28 kDa, and other bands were only faintly visible (figure 1A, right panel). After 20 months, a strong reactivity was observed at 17, 20, 28, 30, 32, 34–38, 42, and 47 kDa, with weak bands at 27, 44, 55, 70, and 150 kDa (figure 1B, right panel). Intensity of these bands decreased slightly after 32 months (figure 1C and 1D). By use of *E. cuniculi* type III strain as antigen, the Western blot profile was very similar to the profile of the *E. cuniculi* type I strain, except at 55–65 kDa, at which, by use of the *E. cuniculi* type III strain, 2 double bands were observed (data not shown).

When the serum samples were tested by Western blot with *E. intestinalis* antigen, only a single band at 26 kDa reacted strongly (figure 2A–D). The serum sample of the HIV-infected case patient infected with *E. intestinalis*, which was used as the positive control in the Western blot, did show strong reactivity with several antigens (figure 2). By use of the *E. intestinalis* antigen, IFAT analysis indicated that this sample had a titer of 1:640 to the spore wall and 1:320 against the polar tube. The sample cross-reacted with both the spore wall and the polar tube of *E. hellem*, with titers of 1:640 and 1:160, respectively, but only cross-reacted with the spore wall (1:320) of *E. cuniculi*. No reactivity was observed with *V. corneae* (data not shown).

To study the humoral response against *E. cuniculi* in more detail, the serum samples obtained at the various time intervals



**Figure 1.** IgG antibody (Ab) response against *Encephalitozoon cuniculi* with samples taken at various time points after infection. Indirect immunofluorescence technique (IFAT) results are presented in the top and lower left panels, and Western blot results are indicated in the lower right panel. "A," "B," "C," and "D" correspond to serum samples taken 1, 20, 32, and 38 months, respectively, after infection. "Ctr" indicates the negative serum control. *E. cuniculi* was used as antigen in both IFAT and Western blot analyses.

after infection were tested for reactivity against 4 *E. cuniculi* recombinant antigens expressed in *E. coli*: 1 spore-wall protein (SWP1) and 3 polar-tube proteins (PTP1, PTP2, and PTP3). Reaction against SWP1 was present in all 4 serum samples and

**Table 1. Cross-reactivity studies with different microsporidia species in indirect immunofluorescence technique (IFAT) with the samples of the index case patient infected with *Encephalitozoon cuniculi*.**

Sample time after infection, months	Microsporidian species in IFAT							
	<i>E. cuniculi</i> , type 1 strain		<i>E. intestinalis</i>		<i>E. hellem</i>		<i>V. corneae</i>	
	Spore wall	Polar tube	Spore wall	Polar tube	Spore wall	Polar tube	Spore wall	Polar tube
1	40	<20	40	<20	40	<20	<20	<20
20	320	160	80	40	80	20	<20	<20
32	160	80	40	40	80	20	<20	<20
38	160	80	20	20	20	20	<20	<20

**NOTE.** Data are titers, expressed as reciprocal values.

closely followed the signal and stain against the spore wall in IFAT (figure 3, lane 1). The IgG response against the components of PTP1, PTP2, and PTP3 was not detectable 1 month after infection, but became evident in the follow-up serum samples, of which the stronger reactions were associated with PTP1 and PTP3 (figure 3, lanes 2 and 4). PTP2 was only weakly recognized 20 months after infection (figure 3, lane 3). A specific labeling of a lower migrating band was observed with the different serum samples in lanes 1 and 2. These protein bands that are revealed with the anti-GST antibody also probably correspond to degradation products of the recombinant proteins SWP1 and PTP1.

## DISCUSSION

In the present study, we demonstrated that *E. cuniculi* infection in an HIV-uninfected immunocompetent person produced a strong IgG antibody response against both the spore wall and the polar tube of the parasite. By use of Western blot, a characteristic profile was observed against multiple protein bands with molecular weights ranging from 17 to 150 kDa. By use of recombinant antigens, strong reactivity was observed with SWP1 and PTP1. Specific antibody responses persisted for at least 3 years after infection.

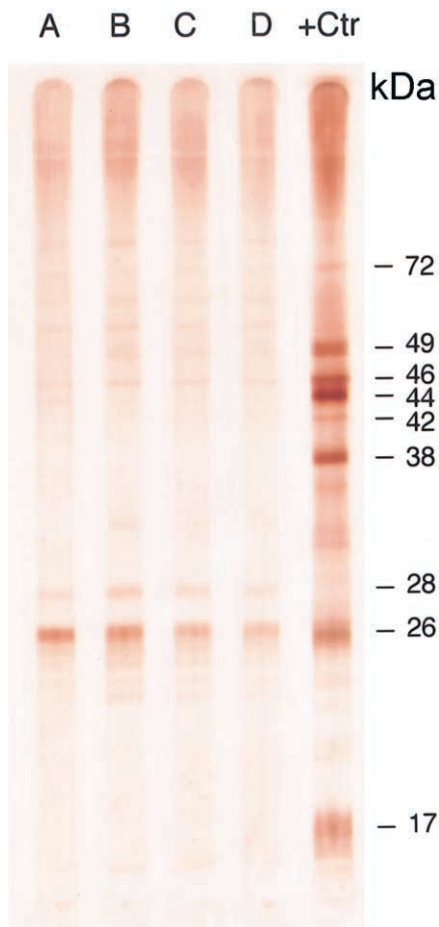
To obtain insight into the serological response after *E. cuniculi* infection and for the development of reliable serological tests, immunocompetent “index” case patients should be studied when both the moment of infection and the infecting species are known and when long-term follow-up is available. Studies of such case patients have until now not been published for microsporidia infections. Bergquist et al. [7] reported the presence of spores of *Encephalitozoon* species in urine samples obtained from a child with convulsive seizures, which was accompanied by strong IgG and IgM antibody responses. Details of the serological tests used in this study were not provided. In an anonymous World Health Organization report, in which most likely the same case was described, a carbon immunoassay

with a mixed antigen preparation of *Toxoplasma gondii* and *E. cuniculi* spores was used as the principal test [15]. Determination of the infecting species, presumably *E. cuniculi*, was based on light-microscopic studies, reactivity with an anti-*E. cuniculi* conjugate, and propagation of infection in mice. At the time of these studies, *E. intestinalis* and *E. hellem* were not known, and molecular and electron microscopic studies were not performed; therefore, doubt remains about the true nature of the infecting species. The moment of infection was unknown, and, because of the presence of abnormal T cell counts, the immune status of the child also was uncertain [16].

The present study, to our knowledge, is the first in which the serological response after proven *E. cuniculi* infection in an immunocompetent person is described in detail. Although no serum samples were available before the infection, the 3-fold increase in IgG antibody titer strongly suggests that the antibody response was a result of the infection. The highest titer was observed 20 months after infection, but it is possible that this titer was obtained earlier. Because no samples were available 1–20 months after infection, titer levels could not be studied in more detail.

Specific antibodies were detected up to 3 years after infection by use of both IFAT and Western blot. In a sample obtained 6 years after the accident, low levels of specific antibodies were still present, which suggests that persistence is probably even longer (data not shown). Data about the persistence of specific antibodies after *Encephalitozoon* species infection in humans are, to our knowledge, not available. In dogs, persistence of specific antibodies has been reported for up to 1 year after infection [11].

One month after infection, specific antibodies were directed only against the spore wall, a reaction that evolved toward both the spore wall and the polar tube in the months after infection. This evolution in antibody production from spore wall to both spore wall and polar tube has not been reported before in humans. In earlier serological studies in humans, in which IFAT and ELISA techniques were used, only the spore wall was used



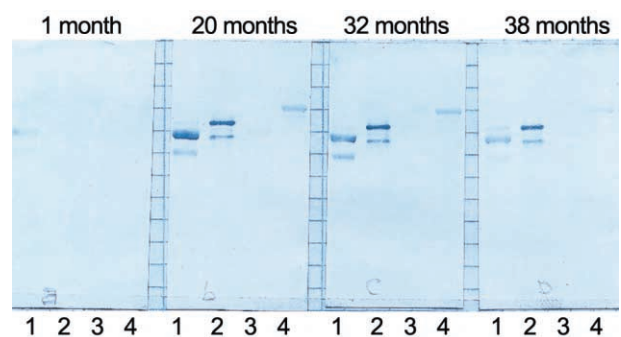
**Figure 2.** Western blot analysis with *Encephalitozoon intestinalis* antigen with serum samples obtained from the index case patient infected with *Encephalitozoon cuniculi* (A–D) and a sample of an HIV-infected case patient with *E. intestinalis* (+Ctr). “A,” “B,” “C,” and “D” correspond to serum samples taken 1, 20, 32, and 38 months, respectively, after infection.

as the principal antigen [8–11, 17]. The advantage of IFAT used in the present study is that it allows for both antigens of the spore wall and polar tube located within and outside the host cell to be studied. Also, in animals experimentally infected with *Encephalitozoon* species, specific antibodies against the polar tube have been observed [18, 19]. These data suggest that, apart from spore-wall antigens, polar-tube antigens are important targets for specific antibody responses after *Encephalitozoon* species infection.

In the present study, we cloned and expressed in *E. coli* 4 recombinant antigens from *E. cuniculi*: 3 polar-tube proteins, PTP1, PTP2, and PTP3, and a spore-wall protein, SWP1. The serum samples from our case patient did show strong reactivity with PTP1 and SWP1, which suggests that these recombinant antigens could be useful for application in serological tests. The absence of reactivity against PTP1 after 1 month of infection is in agreement with IFAT results, after which, at that time, no reaction was observed against the polar tube.

By use of Western blot, only 1 strong band at 28 kDa was clearly recognized 1 month after infection. However, after 20 months, a profile was observed with multiple strong bands (17, 20, 28, 30, 32, 34–38, 42, and 47 kDa) and some weaker bands (27, 44, 55, 70, and 150 kDa), which were still visible 3 years after infection. Prominent recognition of the 28-kDa band at 1 month after infection, which was the time when only immunofluorescence of the spore wall was observed by use of IFAT, suggests that this antigen is most likely located on the spore wall. The antigens recognized by use of Western blot after 20 months could represent additional spore-wall or polar-tube proteins. The 150-kDa band could be related to the recently identified PTP3 [20]. However, it is unlikely that PTP1, PTP2, and SWP1 are visible, because these proteins can only be efficiently extracted by use of high concentration of detergents and prolonged incubation with reducing agents [21–23]. With *E. cuniculi* type III strain, a similar profile was observed with only small differences at 55–65 kDa.

A major difficulty with earlier serological studies was the possibility of cross-reactivity with other microsporidia [16]. In the present study, by use of IFAT, significant cross-reactivity was observed with spore-wall antigens of *E. intestinalis* and *E. hellem*, but not with *Vittaforma corneae*. Although this suggests genus specificity of the antibody reaction, cross-reactivity with other microsporidia genera cannot be fully excluded. Several studies have demonstrated the existence of multiple cross-reacting antigens on the spore wall of microsporidia of different genera [24–26]. In contrast to spore-wall antibodies, we observed little cross-reactivity with polar-tube antigens of *E. intestinalis* and *E. hellem*, with a titer of 1:20 being the borderline



**Figure 3.** Reactivity of different serum samples in time against 4 recombinant antigens of *Encephalitozoon cuniculi*. Lane 1, spore-wall protein 1 (SWP1); lane 2, polar-tube protein 1 (PTP1); lane 3, polar tube protein 2 (PTP2); lane 4, polar tube protein 3 (PTP3). The different serum samples were tested at a 1:200 dilution. Secondary antibodies were anti-human IgG alkaline phosphatase conjugate at a 1:10,000 dilution. The analysis was done in the presence of 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium, according to the manufacturer’s instructions (Promega).

result, which suggests a high specificity of the anti-polar-tube antibodies.

The Western blot profile presented in the present study most likely is specific for *E. cuniculi* infection, which also was confirmed by minor reactivity with the *E. intestinalis* antigen. As a positive control for the Western blot study with *E. intestinalis*, samples from an HIV-infected patient with proven *E. intestinalis* infection were used. The profile observed in Western blot with this case patient was quite distinct from our index case patient with *E. cuniculi* infection. Bands at 44–49 kDa and the single band at 38 kDa especially were discriminative. An identical profile was observed with serum samples of another HIV-infected case patient with proven *E. intestinalis* infection (data not shown), which suggests that this profile could be characteristic for *E. intestinalis* infection. However, because of severe immunodeficiency in these case patients, antibody reaction could be incomplete. Therefore, Western blot profiles might differ from immunocompetent persons with *E. intestinalis* infection [27]. Studies with serum samples from immunocompetent patients with parasitological proven *E. intestinalis* infection are needed to confirm the usefulness of the profile presented in the present study.

Western blot analysis, with knowledge of specific profiles of different *Encephalitozoon* species, seems of major importance for discrimination between frequent occurring cross-reacting antibodies and antibody responses caused by true *Encephalitozoon* species infection. In a serological survey with the IFAT from the present study, of 490 French pregnant women, 38 had antibodies against the spore wall of *E. cuniculi*. In 3 of these women, a Western blot profile was observed that was identical to the profile of the index case patient in the present study. By use of IFAT, these case patients also had high antibody titers against the polar tube (data not shown).

Hollister et al. [11] also used Western blot as a confirmatory technique for case patients found to be positive with an ELISA technique. Because Hollister et al. [11] were not aware of specific Western blot profiles of humans infected with *E. cuniculi*, profiles from serum samples of mice and rabbits infected with *E. cuniculi* were used. Some patients with high ELISA values had Western blot profiles that closely resembled the values obtained from rabbits and mice serum samples, which suggested true *Encephalitozoon* species infection. Some of the bands observed in these studies also were present in the Western blot profile of our case patient, for example, at ~17, 34, 42, and 47 kDa. However, good comparison with our data is difficult, because Hollister et al. [11] used mercaptoethanol as a reducing agent, whereas we obtained most information without the use of this reagent. In the study conducted by Hollister et al. [11], many case patients were observed with high ELISA values, but without any reactivity in Western blot. Because whole spores were used as the antigen source in their ELISAs, it is likely that many

of these positive ELISA results were caused by cross-reacting antibodies not related to *Encephalitozoon* species infection.

In our opinion, results obtained with serological tests with only the spore wall as the antigen source should be regarded with caution. Positive samples should be examined by use of confirmatory assays, such as Western blot or, alternatively, by use of recombinant antigens.

Although infection in our case patient was caused by a laboratory accident, microsporidial infections of eyes in humans are not uncommon under natural conditions. Keratoconjunctivitis due to *Encephalitozoon* species infection is frequently observed in HIV-infected persons with systemic infection [2]. Most patients present with bilateral conjunctival inflammation and also exhibit bilateral punctate epithelial keratopathy that leads to decreased visual acuity [2], which resembles the clinical picture observed in the case patient in the present study. Also, microsporidial keratoconjunctivitis is described more frequently in healthy, nonimmunocompromised individuals [28]. Ocular infection with *Encephalitozoon* species might occur by direct inoculation of spores by contaminated fingers or by the aerosol route [29].

The results reported in the present study provide significant support for the development and interpretation of the serodiagnosis of *Encephalitozoon* species infection in human. It will especially be of help for discrimination between frequent occurring cross-reacting antibodies and antibodies resulting from true *E. cuniculi* infection. Recent reports of severe *Encephalitozoon* infection in renal transplantantation patients [30–32] suggest that, in HIV-uninfected patients, *Encephalitozoon* species infection is of major clinical importance and that this infection is probably more prevalent than originally assumed. Therefore, reliable serological tests could be important tools for providing information regarding the prevalence and clinical significance of *Encephalitozoon* species infections in humans.

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