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# Renal *Encephalitozoon (Septata) intestinalis* infection in a patient with AIDS

## Post-mortem identification by means of transmission electron microscopy and PCR

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**Abstract** We describe the occurrence of renal *Encephalitozoon (Septata) intestinalis* infection in a 35-year-old AIDS patient who died with disseminated tuberculosis. The patient did not complain of specific symptoms involving the kidney or lower urinary tract during life, but at autopsy, light microscopic examination of the kidney revealed numerous small round or oval bodies in the tubules and tubular cell cytoplasm that were interpreted as intracellular protozoa. Transmission electron microscopy of tissue retrieved from paraffin-embedded samples identified these organisms as microsporidia belonging to the Encephalitozoonidae family, but did not allow definitive identification of the species of infecting parasite. This was made possible only by means of Southern blot hybridization after the polymerase chain reaction, which recognized the micro-organism as *E. intestinalis*.

**Key words** AIDS · Microsporidium · Kidney · Transmission electron microscopy · Polymerase chain reaction

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## Introduction

Microsporidia are obligate intracellular spore-forming protozoan parasites causing intestinal (*Enterocytozoon bieneusi* and *Encephalitozoon (Septata) intestinalis* and disseminated (*E. hellem*, *E. cuniculi* and *E. intestinalis*) infections, which are being reported with increasing frequency in patients with AIDS [5, 8, 9, 11]. Because of the small size of micro-organisms and spores, diagnosis by means of light microscopical examination of biopsy and cytology specimens may be difficult when routine histochemical stains are used, although modified Gram, Warthin-Starry and fluorescent chitin stains have made diagnosis considerably easier [12, 14]. Diagnosis at autopsy is even more challenging, because of the presence of post-mortem tissue artifacts. Microsporidia infections are therefore undoubtedly underdiagnosed, especially in clinically unsuspected cases. Furthermore, their morphological speciation is a problem in autopsy specimens even when transmission electron microscopy (TEM) is used, because, as in the case of well-preserved biopsy material, all the *Encephalitozoa* have the same appearance with the exception of *E. intestinalis*, which can be identified by its unique honeycombed parasitophorous vacuole.

We report an autopsy case of renal *E. intestinalis* infection in a symptom-free AIDS patient. The uncertain histological diagnosis was supported by means of TEM and DNA polymerase chain reaction (PCR) investigation

## Clinical history

A 35-year-old, HIV-positive, male intravenous drug user was admitted to the "Ospedale Maggiore della Carità", Novara, in November 1993 because of cervical lymphadenopathy. The results of the laboratory tests were unremarkable with the exception of the CD4 count (180 cells/ml), serum alaninaminotransferase (ALT; 89 U/ml) and aspartatoaminotransferase (AST; 42 U/ml). The lymph node biopsy revealed tuberculous lymphadenitis and the patient was discharged after specific therapy.

In January 1995, the patient was hospitalized again following the onset of fever and lethargy. Laboratory tests revealed hyponatraemia (Na+ 120 mEq/l). His renal function was normal (blood

urea nitrogen 7 mmol/l; creatinine 0.7 mg/dl), and no proteinuria or haematuria was observed. The patient's condition progressively worsened and he died as a result of septic shock.

A complete autopsy was performed 26 h after death.

## Materials and methods

Histopathological examination of the brain, lungs, liver, kidneys and small and large intestine was carried out using tissue fixed in 10% formalin and embedded in paraffin. Sections were stained with haematoxylin and eosin (H&E), Giemsa, periodic acid–Schiff (PAS), Grocott methenamine silver (GMS) and Zihel-Nielsen. Immunohistochemistry was carried out on paraffin-embedded material using the polyclonal antibody anti-*Toxoplasma gondii* (Sera Lab, Crawley Down, Sussex, England, dilution 1:200).

For transmission electron microscopy renal tissue was retrieved from paraffin blocks and embedded in epoxy resin. Semithin sections were stained with toluidine blue; appropriate areas were selected for ultrathin sectioning, stained with uranyl acetate and lead citrate, and examined by means of TEM.

Paraffin-embedded sections of kidney (four) and brain (two) were dewaxed with xylene, followed by centrifugation at 15,000 *g* for 1 min, and DNA was extracted as previously described [3]. DNA from tissue culture-derived microsporidia was used as a control.

In order to amplify the ribosomal genes of the microsporidia, PCR was used with a set of primers that amplify the small subunit rRNA genes (SSUrDNA) of all the microsporidians reported to infect man [10]; the sequences of these pan-microsporidian primers are 5′-CACCAGGTTGATTCTGCCTGACG-3' and 5'-TTATGATCCTGCTAATGG TTCTCC-3'.

PCR gene amplification was performed in 100 µl reactions containing 0.05 µM of each primer, 0.2 mM of each dNTP (Promega, Madison, Wis.), 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Promega), approx. 10 ng of genomic DNA, and 100 µl of overlaid mineral oil. The PCR assays were performed using a Perkin-Elmer thermocycler (Norwalk, Conn.) with a profile of 94°C for 1 min, 55°C for 2 min, 72°C for 2 min for 35 cycles. The PCR products were purified using Magic PCR Prep mini-columns as described by the manufacturer (Promega) and were run on 1.5% agarose gels. The length of PCR products was 1000 bp.

The remaining portions of the PCR products were stored at 4°C until used.

For Southern blot analysis aliquots of PCR products (5 µl and 1 µl) were electrophoresed in 1.2% agarose gels which were stained with ethidium bromide to visualize the DNA [3] and blotted onto Magna Graph nylon transfer mambranes (Micron Separations, Westoro, Mass.) using 2 × SSC buffer. DNA was bound to the nylon membrane by ultraviolet irradiation (Stratolinker; Stratagene, Menasha, Wis.). The blots were then hybridized and stained using an ECL 3'-oligolabelling and detection system as described by the manufacturer (Amersham Life Science, Arlington Heights, Ill.). Briefly, the microsporidian oligonucleotide probes were labelled with fluorescein-11-dUTP and added to the blots at a final concentration of 5–10 ng/ml. The oligonucleotide sequences for the *E. cuniculi*, *E. hellem*, and *E. intestinalis* probes were, respectively, 5'-TAGCGGCTGACGAAGCTGC-3', 5'-TGAGTGTGAGAGTGTTTTTACAT-3', and 5'-CGGGCAGGAGAACGAGGACGG-3' (3).

The blots were incubated at 54°C for 2 h in a shaking water bath when the *E. cuniculi* or *E. hellem* probes were used or at 64°C for 2 h with the *E. intestinalis* probe, after which the blots were washed twice in 5 × SSC containing 0.1% SDS for 5 min each followed by two 2-min washes at 54°C with 1 × SSC containing 0.1% SDS. The blots were then incubated with the blocking solution provided in the ECL kit followed by incubation with horseradish peroxidase-conjugated anti-fluorescein antibody, rinsed with the detection solutions and exposed on film.

## Pathological findings

Post-mortem examination revealed active tuberculosis involving the lungs, spleen and, especially, the meninges and brain. Multiple foci of inflammatory infiltration, mainly consisting of granulocytes with rare lymphocytes and occasional multinucleated giant cells, were found extending from the meninges into the Virchow–Robin spaces and brain parenchyma.

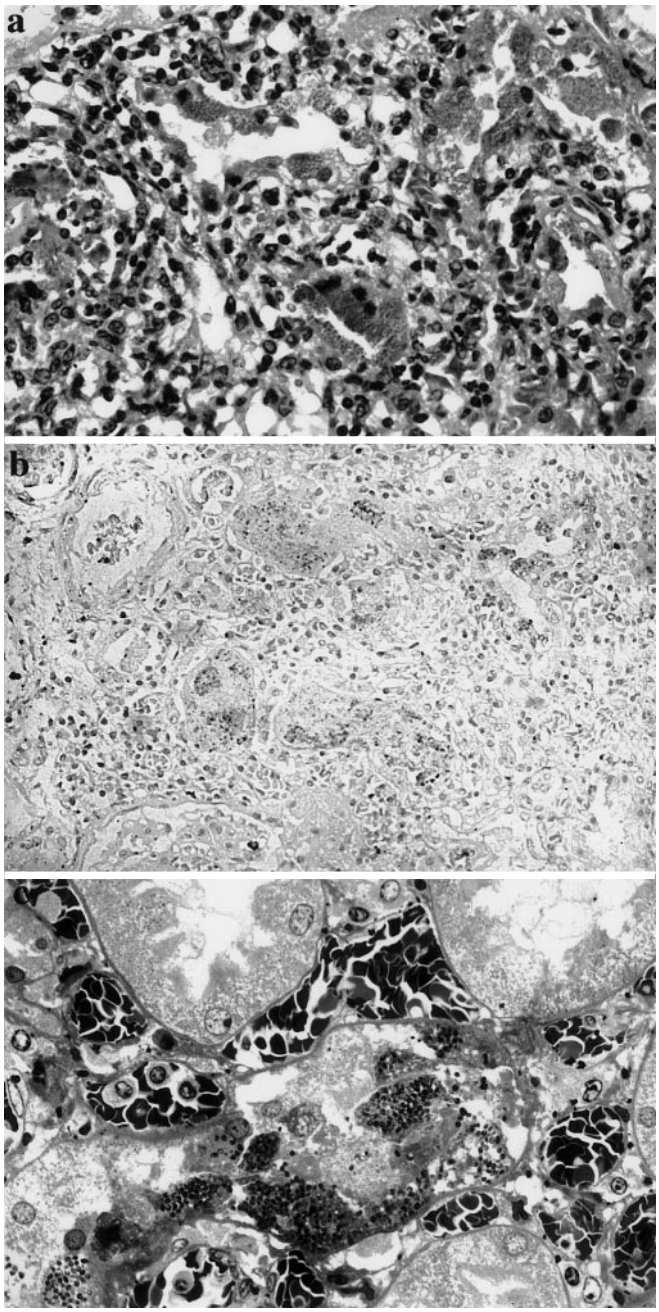


**Fig. 1** Tubular cells of the kidney filled by many roundish bodies (**a**). That were positive for anti-t Gondii antibody (**b**). Semithin section stained with toluidine-blue examined at high magnification, revealed many round dark-blue particles, with a central vacuole, suggestive of microsporidium (**c**). **a**: HE × 400, **b** haematoxylin counter staining × 250; **c** toluidine-blue × 630

**Fig. 2** Fragments of parasitophorous vacuole (*arrows*) incompletely surrounding spores and the proliferative stages of *Encephalitozoon intestinalis*. Note the profiles of six polar tube coils arranged in a single row (*arrowheads*). ×17,500, *bar* 500 nm

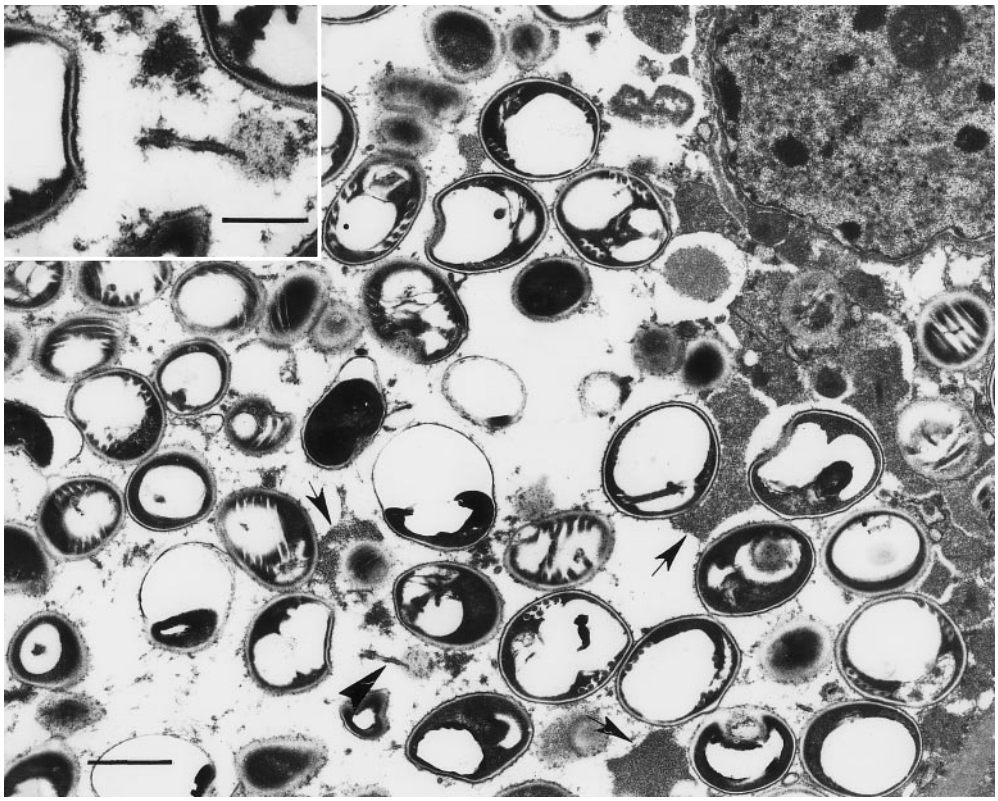

**Fig. 3** Remnants of the fibrillar septa of the parasitophorous vacuole (*arrows*) and "tubular appendages" (*arrowhead*) are also evident in poorly preserved material. These ultrastructural findings are considered highly suggestive of *E. intestinalis* ×11,000, *bar* 1 mm. *Insert*: a clear illustration of a tubular appendage, showing its characteristic bulb-like ending. ×22,000 *bar* 500 nm

The kidneys were unremarkable on gross examination, but light microscopy of H&E-stained sections revealed multiple small aggregates of lymphocytes, plasma cells and macrophages surrounding the convoluted tubules; the epithelium was often necrotic and detached from the basal membranes. On oil immersion, many small round or oval bodies discharged from necrotic cells (Fig. 1a) were seen in the tubules but not in the glomeruli. They stained slightly with Giemsa and PAS, but were negative to GMS staining. Despite a careful search, they were not found in the brain, lungs or liver, or the small or large intestine. These structures were tentatively considered to be intracellular protozoa, a hypothesis that was further confirmed by their high degree of positivity to specific antibodies for *T. gondii* (Fig. 1b).

Semithin, toluidine-blue stained sections of the cytoplasm of the better preserved tubular cells showed that it was filled with many round dark blue particles with a clear central vacuole measuring about 1.5–2.0 μm (Fig. 1c). These findings were not consistent with *T. gon-*

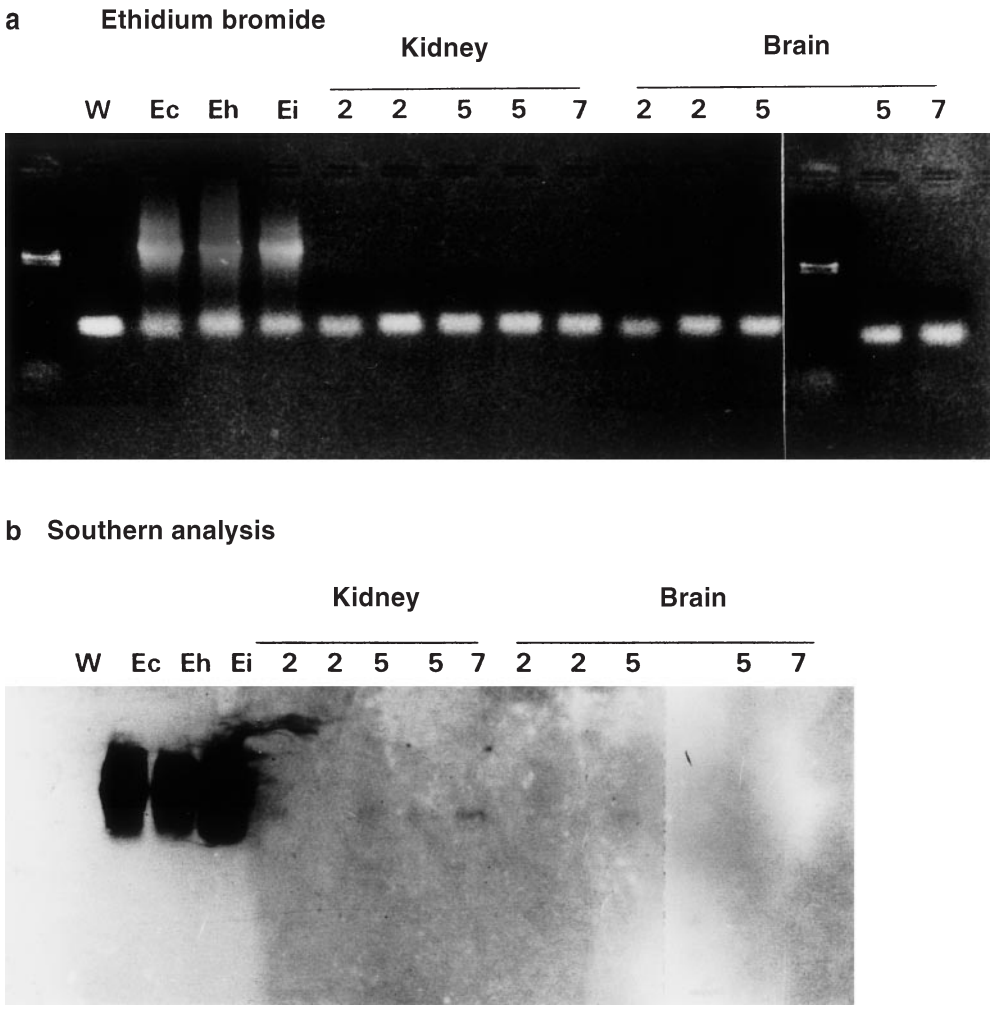


**Fig. 4a, b** PCR amplification of microsporidian SSUrDNA and Southern blot analysis. The DNA of tissue-culture derived *Encephalitozoon cuniculi* (*Ec*), *Encephalitozoon hellem* (*Eh*), and *Encephalitozoon intestinalis* (*Ei*), and also that of paraffin-embedded kidney and brain tissue, was extracted and amplified by means of PCR. **a** PCR products stained with ethidium bromide. Two microliters of each tissue culture-derived microsporidian rDNA PCR product were loaded per lane, together with the number of microliters of the tissue-derived PCR products listed for each *lane*. The lane marked "*W*" is the PCR water control. *Lanes 1*, *14* phage X174 *Hae*III digest marker (872 bp). **b** Southern blot analysis of the PCR products probed using the *E. intestinalis* oligonucleotide. Weak signals are seen in the 7-ml kidney and the 5-ml brain *lanes*

*dii* infection, but suggested the presence of microsporidia.

TEM examination revealed microsporidia at different stages of development: a large number of spores were identified within the tubular cells and free inside the lumen, and were occasionally seen in macrophages (where they were undergoing lysosomal digestion), as well as within fibroblasts or free in the interstitium. The spores measured up to about 1.4 × 2.1 μm. The polar tube showed single rows of between four and seven coils (most often five), a feature that is consistent with the genus *Encephalitozoon*. Even in this poorly preserved autopsy material retrieved from paraffin, there was a suggestion of septation of the parasitophorous vacuole and, thus, of the species *E. intestinalis* (Fig. 2). Fragments of the polar tube measuring about 70–90 nm in diameter were seen free among the spores. Tubular structures 40–50 nm in diameter and ending in a bulblike process (corresponding to the "tubular appendages" described for *E. intestinalis)*, were found near a few parasites (Fig. 3).

The SSUrDNA-amplified PCR products from the tissue sections and the control PCR products amplified from the tissue culture-derived microsporidia were electrophoresed as shown in Fig. 4a. Since no PCR products could be seen from the tissue sections, Southern blot analysis was performed using oligonucleotides specific for *E. cuniculi*, *E. hellem*, and *E. intestinalis*. Figure 4b shows an overexposed Southern blot demonstrating weak hybridization signals between the *E. intestinalis* oligonucleotide and the PCR products amplified from kidney and brain. No hybridization signals could be detected with either the *E. cuniculi* or *E. hellem* oligonucleotides (not shown). Under optimal exposure conditions [3], the oligonucleotides have been found to be specific for the homologous *Encephalitozoon* species, but no signal can be seen for the brain and kidney PCR products unless the blots are overexposed. However, under conditions of overexposure, each oligonucleotide generates heterologous signals as a result of the high degree of homology existing between *E. cuniculi*, *E. hellem*, and *E. intestinalis* [6]. Since we found a signal only when the *E. intestinalis* oligonucleotide probe was used, and no signal was detected with the *E. cuniculi* or *E. hellem* probes in the overexposed blots, the microsporidian rDNA amplified from the kidney and brain tissues appeared to be most closely related to *E. intestinalis*.

## Discussion

Microsporidia belonging to the family of Encephalitozoonidae are known to infect the urinary tract, where they parasitize the transitional cells of the ureters and urinary bladder, and the tubular cells of the nephron [9, 11, 12]. Urinary infections can be revealed by specific symptoms relating to the kidney (proteinuria, renal failure or flank pain) [1] or the lower urinary tract (micro- or macrohaematuria) [5, 13] and may be preceded by symptoms related to a specific infecting agent (i.e. chronic diarrhoea and malabsorption in *E. intestinalis* infections and upper respiratory involvement in *E. hellem* infections) [9].

The few autopsy reports available in the literature describe the presence of *E. hellem* and *E. intestinalis* in the epithelial cells of the proximal and distal convoluted tubules [9, 11] of patients in whom microsporidiosis had been diagnosed during life because of the onset of specific symptoms and the presence of infected cells in body fluids or surgical specimens. However, tubulointerstitial microsporidiosis was an incidental finding in our patient, who had not complained of symptoms attributable to this infection during life and for whom no remarkable laboratory data had been observed.

Light microscopy gave a false impression of *T. gondii* infection, despite its confirmation by immunohistochemical studies using anti-*T. gondii* antiserum. This may have been due to the low sensitivity of the polyclonal antibody against the parasitic structures and/or the previously reported cross-reactivity of microsporidia [2]. The examination of semithin sections led to a suspicion of microsporidiosis, but the final diagnosis was dependent on the TEM and PCR analyses. The ultrastructural diagnostic features of Encephalitozoonidae include the presence of mononucleated spores of about 1.4 × 2.1 μm developed in a parasitophorous vacuole, a single row of four to seven coils of its polar tube, and the occasional presence of free interstitial spores and spores in macrophages. These findings are common to of all three species of *Encephalitozoa* that infect humans (*E. hellem, E. cuniculi,* and *E. intestinalis*). The first two of these are morphologically indistinguishable, whereas *E. intestinalis* has the distinctive ultrastructural hallmark of a honeycomb-like parasitophorous vacuole consisting of a fibrillar network secreted by the parasite and "tubular appendages" [14]. Because of the post-mortem changes, the honeycomb-like structure of the parasitophorous vacuole could not be visualized with certainty, but its presence was suggested by the finding of residual fragments of fibrillar matrix among the developing spores. Although tubular appendages were occasionally identified, it has been our recent impression that these are not unique to *E. intestinalis* but can be seen in all *Encephalitozoa,* which means the accurate speciation of an infecting parasite is possible only by means of molecular biology techniques. In the present case, post-PCR Southern blot hybridization of kidney and brain tissues retrieved from paraffin allowed us to identify the microrganism as *E. intestinalis*.

Despite the PCR results, we were unable to find any micro-organisms in the brain by means of LM. This may have been because the massive tuberculous meningoencephalitis masked the presence of *E. intestinalis* or because its presence in cerebral blood vessels was a result of haematogenous dissemination [4] rather than localization inside the nervous tissue. This latter hypothesis was supported by the absence of microglial nodules, which have recently been found to be an expression of microsporidian brain involvement [7].

Our data show that unusual and therefore unexpected forms of parasitic infections may occur; their diagnosis may require the use of multiple procedures, including electron microscopy and molecular biology.

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