Disseminated Microsporidiosis Caused by *Encephalitozoon cuniculi* III (Dog Type) in an Italian AIDS Patient: a Retrospective Study

Antonella Tosoni, B.Sc., Manuela Nebuloni, M.D., Angelita Ferri, M.D., Sara Bonetto, M.D., Spinello Antinori, M.D., Massimo Scaglia, M.D., Lihua Xiao, D.V.M., Ph.D., Hercules Moura, M.D., Ph.D., Govinda S. Visvesvara, Ph.D., Luca Vago, M.D., Giulio Costanzi, M.D.

Pathology Unit, "L.Sacco" Hospital and Institute of Biomedical Sciences, University of Milan, Italy (AT, AF, SB, LV, GC); Institute of Infectious Diseases and Tropical Medicine, University of Milan, "L. Sacco" Hospital, Milan, Italy (SA); Pathology Unit, Hospital of Vimercate, Milan, Italy (MN); Infectious Diseases Research Laboratories and Department of Infectious Diseases, University of Pavia—IRCCS, San Matteo, Italy (MS); and Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, Atlanta, Georgia (LX, GSV, HM)

We report a case of disseminated microsporidiosis in an Italian woman with AIDS. This study was done retrospectively using formalin-fixed, paraffin-embedded tissue specimens obtained at autopsy. Microsporidia spores were found in the necrotic lesions of the liver, kidney, and adrenal gland and in ovary, brain, heart, spleen, lung, and lymph nodes. The infecting agent was identified as belonging to the genus *Encephalitozoon* based on transmission electron microscopy and indirect immunofluorescence. Additional molecular studies, including sequence of the rDNA internal transcribed spacer region, identified the agent as *E. cuniculi,* Genotype III. We believe that this is the first report of a human case of disseminated microsporidial infection involving the ovary.

KEY WORDS: Adrenal gland, AIDS, Brain, Disseminated infection, *Encephalitozoon cuniculi,* Kidney, Liver, Microsporidia, Ovary, Pathology.

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Microsporidia are spore-forming obligate intracellular protozoan parasites belonging to the phylum Microspora, which includes >100 genera and >1000 species. They infect a wide range of invertebrate and vertebrate hosts (1).

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Address reprint requests to: Antonella Tosoni, B.Sc., Anatomia Patologica Ospedale "L.Sacco," Via G.B. Grassi n°74, 20157 Milan, Italy; e-mail: antonellatosoni@virgilio.it; fax: 39-2-38200385.

The prevalence of opportunistic microsporidial infections in humans greatly increased during the AIDS pandemia, particularly before the advent of HAART. At least 12 species, belonging to seven genera (*Enterocytozoon, Encephalitozoon, Pleistophora, Trachipleistophora, Brachiola, Nosema*, and *Vittaforma*), have been identified. Additionally, a catch-all genus, *Microsporidium*, of uncertain taxonomic status, is also known to infect humans (2, 3).

The most frequently recognized species in humans is *Enterocytozoon bieneusi*. It is mainly found in the upper gastrointestinal tract and associated with diarrheal illness, although a couple of reports have identified *E. bieneusi* in respiratory samples (4). *Encephalitozoon intestinalis*, the second most frequently identified microsporidian, causes disseminated microsporidiosis, including the gastrointestinal tract (4). The two other species of *Encephalitozoon, E. cuniculi* and *E. hellem,* are also known to cause disseminated infections including the urogenital, respiratory, and ocular organs. There are, however, two reports of enteric localization of *E. cuniculi* (2, 5), and very recently, an *E. hellem* was recognized for the first time in stools of two immunocompetent travelers with diarrhea (6).

Encephalitozoonosis has been associated with a wide range of pathological features, including tubulointerstitial nephritis, ureteritis, cystitis, prostatitis, rhino-sinusitis, bronchiolitis, pneumonia, and keratoconjunctivitis (2, 7). Two cases of disseminated infections with brain involvement caused by *E. cuniculi* have also been reported (8, 9). *E. cuniculi* is the prototype species of the *Encephalitozoon* genus and a well-known agent of microsporidiosis in various mammalian hosts (1, 10).

Other species of microsporidia have been only sporadically reported in patients with ocular, muscle, and disseminated infections (1, 2).

Some stains, such as modified trichrome, Gram, and fluorochrome-based techniques, used by microscopists, make it possible to detect the microsporidia spores in various biological specimens (1, 11–13). To differentiate the microsporidian genera and most of their species, a transmission electron microscopy study is usually enough, but immunological, biochemical, or molecular analyses are required to distinguish *E. cuniculi* from *E. hellem* (1, 2).

CASE REPORT

A 33-year-old Italian female, an injection drug user who had been HIV seropositive since 1985, was hospitalized in November 1995 because of a 1-month history of fever, myalgia, and abdominal pain and was treated with co-trimoxazole as primary PCP prophylaxis. She had also received antiretroviral therapy with zidovudine from 1990 to 1992 and had recurrent oropharyngeal candidiasis and lower respiratory *Pseudomonas aeruginosa* infection. In April 1995, an atypical disseminated mycobacteriosis was diagnosed and successfully treated with clarithromycin, rifabutin, and ethambutol. A few months later, a human papillomavirus–related high-grade cervical intraepithelial neoplasia was also diagnosed by means of cytological examination of cervical smear. Upon admission to L. Sacco Hospital (in Milan, Italy) her absolute $CD4^+$ lymphocyte count was 4 per microliter. Results of direct microscopic evaluation and blood, stool, and urine cultures for microbial pathogens were repeatedly negative. A stool examination with modified trichrome staining for microsporidia and immunoassay for *Cryptosporidium parvum* were also negative. An abdominal computed tomography scan revealed multiple lesions involving the ovaries, right adrenal gland, kidney, and intraperitoneal lymph nodes. At that time, a solid nodule appeared in the right breast, and cytological evaluation of a fine-needle aspirate showed large immunoblastic cells typical of non-Hodgkin's lymphoma. A subsequent bone marrow examination demonstrated a complete substitution of the parenchyma by the same pathological cells. A cerebral computed tomography scan showed dense brain lesions and cranial erosions, and a blood examination revealed pancytopenia, a high level of lactate dehydrogenase (7850 U/L), and hypercalcemia. Chemotherapy was started with vincristine, prednisone, etoposide, and idarubicin, and the hypercalcemia was treated with clodronate. Despite an initial response and a partial improvement of the general condition, bilateral pleural and peritoneal effusion developed, and the patient died in January 1996. A complete autopsy was performed 19 hours after death.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded tissues including brain, lung, heart, breast, kidney, adrenal gland, liver, pancreas, spleen, small and large bowel, lymph nodes, ovary, uterus, and bone marrow were sectioned and stained. Sections were stained with hematoxylin and eosin, Kinyoun's acid-fast, Giemsa, AFIP-modified Gram, and special stains for microsporidia (aniline blue modified trichrome, Ref. 13; "quick-hot" trichrome Gram-chromotrope, Ref. 12).

Tissues were also examined by means of transmission electron microscopy using a ZEISS EM109 electron microscope. Selected areas from formalin-fixed, paraffin-embedded blocks of kidney, adrenal gland, liver, spleen, brain, lung, and small and large bowel that were positive for microsporidia in the corresponding histological sections were retrieved and deparaffinized with xylene over a 12-hour period. After rehydration and fixation with osmium tetroxide, the samples were reembedded in araldite (Durcupan ACM Fluka). Semithin and thin sections were stained using conventional methods.

Indirect immunofluorescence analyses were performed on smears made from the two reference strains, *E. cuniculi* CDC:V282 (14) and *E. hellem* CDC:V213 (15), as well as on sections from formalin-fixed, paraffin-embedded tissue samples. Culture-derived spores of the two reference strains were harvested from culture supernatants and purified as described elsewhere (15, 16). Rabbit polyclonal antibodies against the reference strains were used in the indirect immunofluorescence test, as described elsewhere (17).

Molecular analyses were performed on formalin-fixed and paraffin-embedded tissue blocks of kidney. Paraffin-embedded renal sections were dewaxed with xylene, and DNA was extracted and amplified from the retrieved tissue as described elsewhere (18, 26). The *E. cuniculi* parasite present was genotyped by sequence analysis of the polymerase chain reaction (PCR) product of the internal transcribed spacer (ITS) of the rRNA gene (26) and confirmed by direct PCR analysis of the polar tube protein gene (18).

RESULTS

Light Microscopy

Large necrotic lesions with a ring of macrophages, fibrosis, and a few lymphocytes and granulocytes were found in the hematoxylin and eosin–

stained sections of liver (Fig. 1), cortical kidney, and the adrenal medulla. The last two organs also showed tubulointerstitial nephritis and small granulomatous reactions. At high magnification, oval, pinkish, and occasionally slightly refractile bodies of about 2 μm in diameter were seen in the necrotic areas and surrounding cells, thus suggesting microsporidial infection. Paraffin sections stained with Kinyoun's acid-fast, Giemsa, Gram, Gram chromotrope, and modified trichrome stain confirmed the presence of several microsporidian spores in the necrotic lesions and surrounding cells in the kidney, liver (Fig. 2), and adrenal medulla (Fig. 3). Despite the poor preservation of the autopsy tissues, the morphology and location of the infected cells suggested macrophages and endothelial or fibroblastic cell types. Gram and modified trichrome staining also identified the parasite in necrotic renal tubules and the epithelial cells of the adrenal gland. Microsporidia spores were focally associated with granulomatous reactions. Furthermore, microsporidia were occasionally detected in the liver sinusoidal cells and myocardial myocytes, as well as in scattered macrophages and fibroblast-like cells in the kidney, adrenal gland, periadrenal connective tissue, subpleural fibrotic areas, lymph nodes, and the spleen capsule.

The brain and ovary were also infected by microsporidia. The infection of the ovarian medullary and cortical stromal cells was focal, without any sign of specific tissue damage (Fig. 4), whereas the brain showed a more diffuse parasitic involvement, mainly in the gray matter, with infected glial cells scattered throughout the parenchyma (Fig. 5) and gathered in a micronodule (Fig. 6A). Other histopathological findings included mild pulmonary edema, follicular atrophy of lymph nodes, micronodular encephalitis, ovarian atrophy, human papillomavirus–related cervical squamous carci-

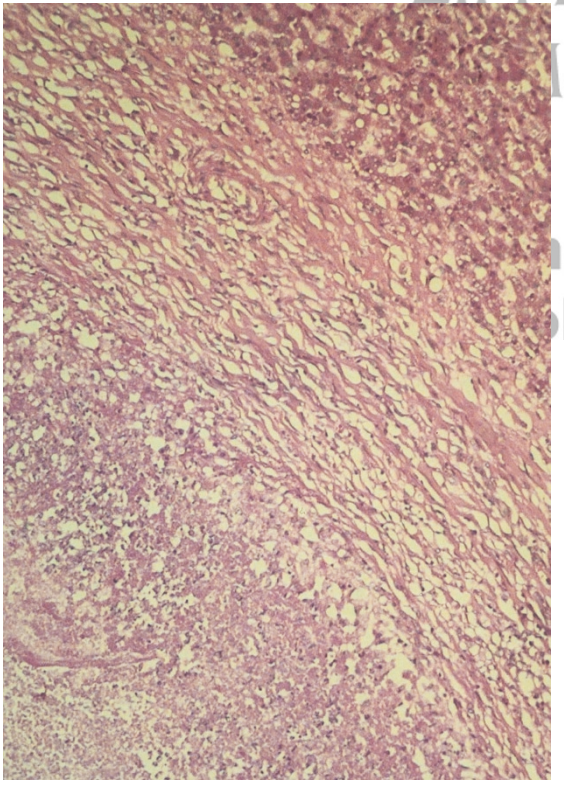

FIGURE 1. The image of the liver lesion shows a wide fibrotic reaction surrounding necrotic areas (at the bottom). (Hematoxylin–eosin staining, original magnification, 200×.)

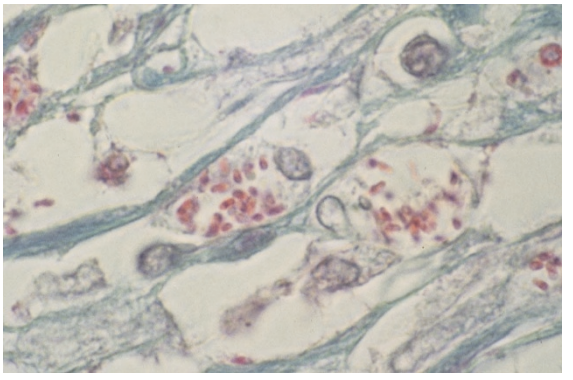

FIGURE 2. High magnification of a section from hepatic fibrotic areas showing infected macrophage-like cells containing red-stained spores. Note the presence of a characteristic central stripe within some spores. (Modified trichrome, original magnification, 1000×.)

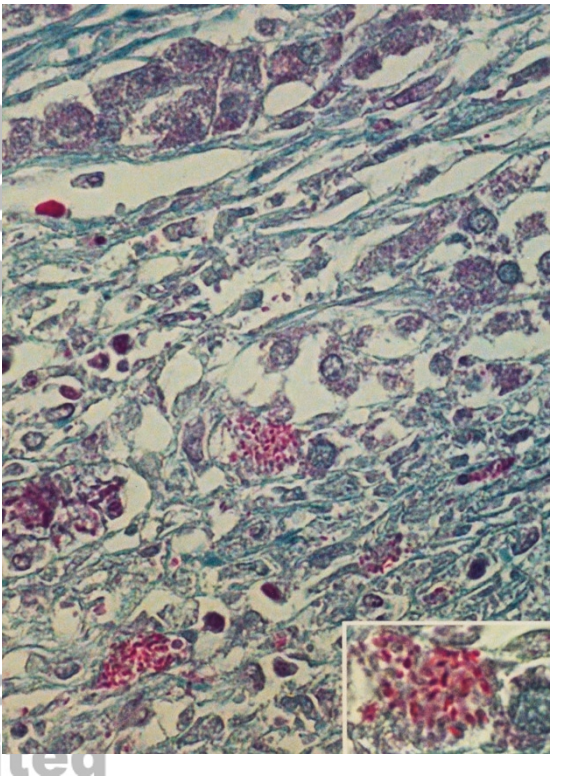

FIGURE 3. Adrenal gland section showing groups of specifically red-stained spores among some necrotic glandular epithelial cells (**inset**) and in spindle cells whose morphology and location suggest an endothelial or fibroblastic cell type. (Modified trichrome, original magnification, 400×. **Inset,** original magnification, 1000×.)

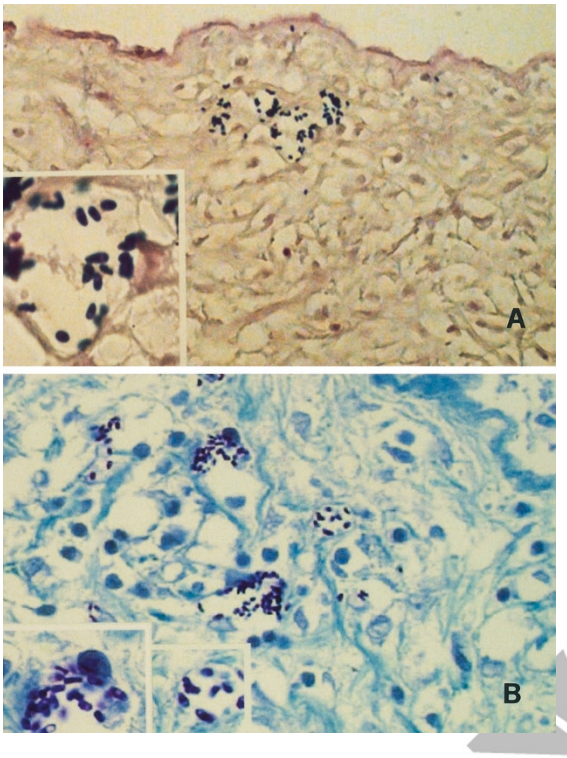

FIGURE 4. Ovary sections show groups of spores both free and within some stromal cells (**insets**). The microsporidian spores are Gram positive (**A**) and stain dark violet (**B**) to red-violet with quick-hot Gram-chromotrope. (**A,** AFIP-modified Brown Brenn Gram, original magnification, 400×. **Inset,** original magnification, 1000×. **B,** Quick-hot Gram-chromotrope, original magnification, 400×. **Inset,** original magnification, 1000×.)

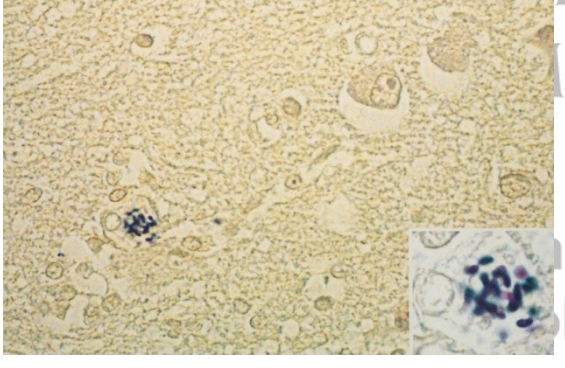

FIGURE 5. A glial cell containing Gram-positive spores in a cortical brain section. Note the absence of any sign of associated lesions. (AFIP modified Brown Brenn Gram, original magnification, 400×. **Inset**, original magnification, 1000×.)

noma, widely diffused non-Hodgkin's lymphoma, and ileal mycobacterial infection.

Transmission Electron Microscopy

The ultrastructural studies of all the examined tissues confirmed microsporidial infection with the recovery of protozoal merogonic and sporogonic intracytoplasmic stages. The presence of the parasites within a nonseptated parasitophorous vacuole (Fig. 6B) and the ultrastructural morphology of the

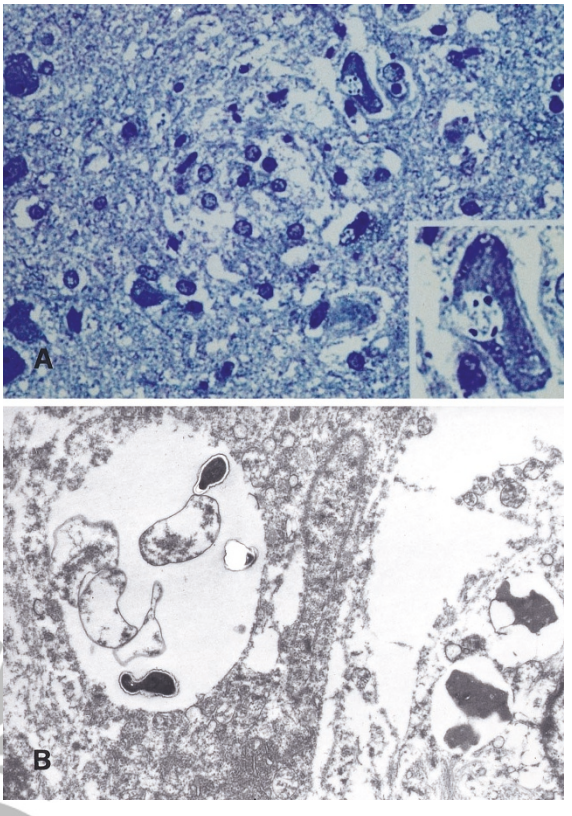

FIGURE 6. (**A**) In a microglial nodule, two cells contain intensely blue-stained spores within single clear vacuole that is more detailed at higher magnification in the **inset.** (**B**) Ultrastructural study of the same area shows the presence of two spores and proliferative forms of microsporidia within a nonseptated vacuole in a glial cell. (**A,** toluidine blue; original magnification, 200×. **Inset,** original magnification, 400×. **B,** lead citrate, uranile acetate; original magnification, 3000×.)

spores (Fig. 7), identified the species as either *E. hellem* or *E. cuniculi*. No other microorganisms were identified, other than mycobacteria in a few ileal macrophages.

Indirect Immunofluorescence Assay

Sections of liver, kidney, ovary, and brain as well as the culture-derived spores of *E. hellem* and *E.*

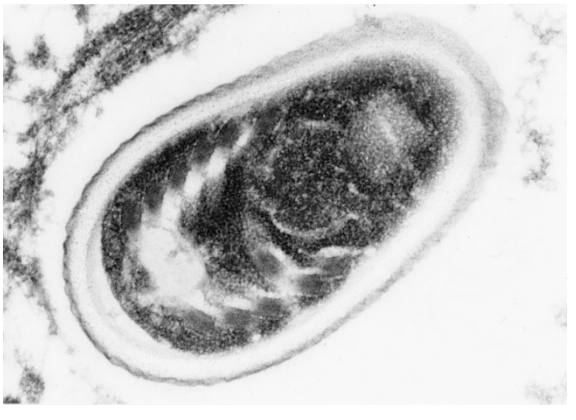

FIGURE 7. Transmission electron micrograph of a section from a necrotic area of adrenal gland showing an *Encephalitozoon cuniculi* spore containing a polar tube arranged in a single row with six coils. (Lead citrate, uranile acetate; original magnification, 30000×)

cuniculi reacted brightly with both the anti–*E. cuniculi* and anti–*E. hellem* sera and produced bright apple-green fluorescence at the 1:500 dilution. At higher dilutions, for example at 1:750 and 1:1000, the spores of *E. cuniculi* and *E. hellem* reacted, however, with the homologous serum only and showed no reactivity with the heterologous sera. On the other hand, the tissue sections reacted with both sera at the 1:1000 dilution. But the spores in the sections did not react with the anti–*E. hellem* serum absorbed with the *E. cuniculi* spores. However, they reacted with the anti–*E. cuniculi* serum absorbed with *E. hellem* spores (Fig. 8), although the fluorescence had decreased quite considerably. These tests indicated that the spores belonged to *E. cuniculi*.

Molecular Analyses

The autopsy specimens were genotyped by sequence analysis of the ITS. Nucleotide sequences obtained from the specimens had four 5'-GTTT-3' repeats, which is typical of *E. cuniculi* Genotype III. In contrast, reference isolates of Genotypes I and II had three and two 5'-GTTT-3' repeats, respectively. The identification of *E. cuniculi* Genotype III was further confirmed by PCR analysis of the polar tube protein gene. PCR amplification of DNA from the autopsy specimens with primers 5'-GCAGTTCC-AGGCTACTAC-3' and 5'-AGGAACTCCGGATGTT-CC-3' produced a 285-bp fragment, which was also obtained from a positive-control Genotype III. In contrast, positive controls of Genotypes I and II produced PCR fragments of 363 bp. Sequence analysis of the PCR products showed that the product from the autopsy specimen had three copies of 78-bp repeats, whereas Genotypes I and II from reference isolates had four copies of the repeats (data not shown).

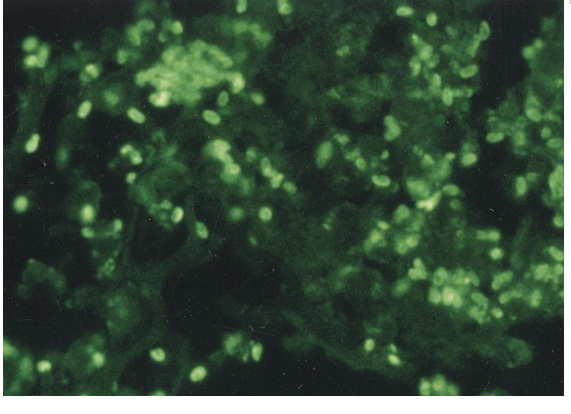

FIGURE 8. *Encephalitozoon cuniculi* spores stained apple-green by indirect immunofluorescence with anti–*E. cuniculi* serum on a liver section. (Rabbit polyclonal anti–*E. cuniculi* serum absorbed with *E. hellem* spores, FITC-conjugated goat anti-rabbit IgG; original magnification 1000×.)

DISCUSSION

Encephalitozoon spp. are the most common cause of disseminated microsporidiosis in humans (1, 2). Published reports on the infections caused by the *Encephalitozoon spp.* are based mainly on light- and electron-microscopic identification of the protozoa in body fluids or cytological samples. Histological studies are rarely performed, and little is known about the systemic pathology (7). We report here results of a retrospective autopsy study of an Italian woman with AIDS in whom disseminated *E. cuniculi* infection was diagnosed. Pathologic features included large necrotic lesions with histiocytic and fibrotic reactions in the adrenal gland, kidney, and liver. The infection also involved a series of other organs and tissue, including heart, spleen, lymph nodes, cerebral cortex, and ovary, but with no or only mild histological lesions. Molecular methods applied to tissues retrieved from paraffin characterized the species as *E. cuniculi* Genotype III ("dog strain").

Although *E. cuniculi* infection has been described in humans since 1959, the initial reports concentrated on histological and ultrastructural studies, which cannot distinguish *E. cuniculi* from *E. hellem* (19). However, more recent reports of *E. cuniculi* infection in both HIV-positive and -negative patients with renal, respiratory, ocular, oral, and maxillary sinus involvement have been based on more precise molecular methods (14, 20–22). An additional report of a possible *E. cuniculi* infection involving the gastrointestinal tract in an asymptomatic patient has also been reported (23). *E. cuniculi* is the prototype species of the *Encephalitozoon* genus and a well-known agent of microsporidiosis in various mammalian hosts (1, 10).

Three genotypes of *E. cuniculi* have been described on the basis of differences in their rDNA ITS region (24). The three genotypes differ from one another by a small repetitive sequence consisting of 5'-GTTT-3'. In Genotype I, as seen in rabbits, the 5'-GTTT-3' sequence is repeated three times, and this Genotype I has also been seen in some human isolates, for example, from some Swiss patients as well as in an Italian patient (24, 25). The mouse genotype or Genotype II, described principally in rodents, has two repeats of this sequence (24). In the Genotype III, or the dog genotype, this sequence is repeated four times, and this genotype has also been seen in *E. cuniculi* isolates characterized from a few patients in the United States, Mexico, Spain, and United Kingdom (2, 9, 24, 26). Genotyping of the isolate from our patient identified four repeats in the ITS region, which classifies it as *E. cuniculi* Genotype III. Altogether, 14 cases of confirmed (by either immunofluorescent test or PCR-based methods) human *E. cuniculi* infection have

been reported so far. Out of these 14 cases, 1 each has occurred in Germany, Italy, Mexico, the United Kingdom, and Spain; 2 have occurred in the United States; and 7 have occurred in Switzerland (2, 9, 24–26). In all of these cases, organisms were either isolated or identified in various body fluids or tissues, including urine, BAL, sputum, nasal epithelium, feces, cerebrospinal fluid, and brain; only rarely, however, have microsporidie been identified and studied in formalin-fixed and paraffin-embedded tissue (9).

The only previous complete autopsy study of disseminated human *E. cuniculi* (Genotype III) infection was reported by Mertens *et al.* (9) in an American woman with AIDS. Diffuse necrotizing lesions were seen in the adrenal glands and kidney. A milder infection was present in brain, heart, lymph nodes, spleen, trachea, and urinary bladder. Microsporidial infection was also associated in some organs with CMV or *P. carinii*. In our patient, *E. cuniculi* was also identified in liver and ovary, but no other microorganisms were identified other than mycobacteria in a few ileal macrophages.

Previously, reports of hepatitis caused by *Encephalitozoon*, described in a few patients with granulomatous and suppurative or necrotic portal and parenchymal microsporidial lesions (19, 27), have been based purely on morphological studies, and hence the species involved has been questionable. However, the hepatic lesions caused by *E. cuniculi* infection in our patient were confirmed by precise molecular methods.

Microsporidial ovary infection has never been described before. Our patient showed only mild pathological damage of this organ, and this finding confirms the hypothesis that microsporidia can potentially spread to all organ systems, especially in those cases in which there is profound immunodeficiency, such as in terminal-stage AIDS (28).

Particular attention has been given by some authors to brain involvement during disseminated microsporidiosis because of its significance in animals (2, 29). The first detection in humans of *Encephalitozoon* spores was in the cerebrospinal fluid of patients with neurological symptoms (19). Weber *et al.* (8) have more recently reported another case of disseminated *E. cuniculi* Genotype I infection, in which the presence of protozoa in the cerebrospinal fluid was associated with diffuse cerebral lesions revealed by magnetic resonance imaging and computed tomography. The cerebrospinal fluid analyses were also positive for CMV, but no histological evaluation of the brain lesions was made because autopsy was not performed. In our case, as in that reported by Mertens *et al.* (9), *E. cuniculi* Genotype III was responsible for a diffuse infection of isolated microglial cells, and the organisms were only sporadically localized within microglial nodules. In both cases, clinical symptoms were not easily interpretable because of concomitant cerebral lymphoma and CMV encephalitis, respectively. Significant cerebral lesions have been observed in rabbits infected with *E. cuniculi* Genotype I, but only mild brain lesions have been associated with *E. cuniculi* Genotype III in the few human cases reported so far. This may suggest a difference in the pathogenic potentials of different *E. cuniculi* genotypes. However, more cases with cerebral lesions caused by *E. cuniculi* strains need to be studied before such conclusions can be drawn. Cases of disseminated microsporidiosis with brain infections caused by *E. intestinalis* (30) and by *T. anthropophthera* (31, 32) have been recently described, but only the latter species was associated with a histological cerebral lesion in a single AIDS patient (31).

In conclusion, our study shows that in immunocompromised hosts, *E. cuniculi* can infect a wide range of organs and tissues and cause widespread lesions. Only one autopsy case report of *E. cuniculi* Genotype III–disseminated infection has been previously published.

Disseminated microsporidiosis have been rarely reported in the literature, but its frequency may be higher than previously believed. This infection may be unsuspected during life because of aspecific presentation or the lack of clinical signs. Moreover, specific searches for microsporidia are rarely included in screening analyses. In autopsy studies, routinely applied hematoxylin and eosin staining reveals microspores only with a low degree of sensitivity and requires experience. The limited number of cases reported so far would increase if more autopsy studies were made using specific histological staining that enhance the detection of microsporidia. We believe that a taxonomic designation of the species and strain should always be sought, and even in retrospective studies, this can be achieved by means of molecular analyses.

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