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Identification of *Enterocytozoon bieneusi* Spores in Respiratory Samples from an AIDS Patient with a 2-Year History of Intestinal Microsporidiosis

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***Enterocytozoon bieneusi*, a microsporidian parasite, has been recognized since 1985 as an agent of intestinal microsporidiosis leading to malabsorption syndrome, diarrhea, and weight loss in AIDS patients. Recently, however, we have identified *E. bieneusi* spores in the sputum, bronchoalveolar lavage, and stool samples of an AIDS patient with a 2-year history of intestinal microsporidiosis. The spores were characterized by Weber's chromotrope-based staining, immunofluorescence tests, and PCR. No microsporidia were detected in urine samples by the same techniques. PCR was performed with DNAs purified from specimens with *E. bieneusi*-, *Encephalitozoon cuniculi*-, *Encephalitozoon hellem*-, and *Encephalitozoon* (*Septata*) *intestinalis*-specific primers. Treatment with albendazole and loperamide resulted in an improvement of intestinal symptoms, without eradication of the parasite. To our knowledge, this is the second report of the identification of *E. bieneusi* spores in respiratory and enteric samples obtained from an AIDS patient. Although no pulmonary pathology could be established in either of these cases, it is now clear that *E. bieneusi* is capable of colonizing the respiratory tract and it is suggested that investigators should be aware of the possibility of finding *E. bieneusi* spores in respiratory secretions.**

Microsporidia are obligate intracellular parasites that lack mitochondria. They are characterized by the production of environmentally resistant spores containing a highly coiled tubular apparatus, the polar tubule, which upon extrusion injects the infective sporoplasm into the host cell. Microsporidia have been recognized as a cause of disease in almost all groups of animals, including humans (28).

Since the first case of human microsporidial infection was reported in 1959 (16), over 400 cases have been documented. Most of these infections have occurred in patients with human immunodeficiency virus (HIV) or AIDS (21, 28). Human microsporidial infections are caused by members of several genera, *Encephalitozoon*, *Enterocytozoon*, *Nosema*, *Septata*, *Pleistophora*, *Trachipleistophora*, and *Vittaforma. Septata*, however, has been reclassified as *Encephalitozoon* (14). Among the above-named organisms, two species, *Enterocytozoon bieneusi* and *Encephalitozoon* (*Septata*) *intestinalis*, have been identified in intestinal infections. The most frequently encountered intestinal microsporidium in AIDS patients is *E. bieneusi* (17, 28), which is thought to be present in 7 to 50% of those with unexplained chronic diarrhea (28). Extraintestinal dissemination of this microsporidium is uncommon; however, it has recently been detected in the biliary tracts of patients with cholangitis and cholecystitis (28). Respiratory tract microsporidiosis has infrequently been reported and is mainly associated with *Encephalitozoon* spp. (7, 28). To date, only one instance of pulmonary involvement of *E. bieneusi* has been documented for a patient with intestinal microsporidiosis (29). Here, we report the detection of *E. bieneusi* spores in the sputum and bronchoalveolar lavage (BAL) fluid of an HIV-infected patient with respiratory symptoms. This patient was previously diagnosed as having intestinal microsporidiosis, and spores were repeatedly identified in his fecal smears for a period of 18 months. The pathologic and/or colonizing features of *E. bieneusi* are discussed, as well as the use of the PCR technique with specific primers for species-specific diagnosis and its implication in treatment effectiveness.

CASE REPORT

A 36-year-old male born in Ghana and living in Spain since 1990 was admitted, for the first time in August 1994, to the Ramon y Cajal Hospital in Madrid (Spain). He had a 3-month history of progressive fever, productive cough, diarrhea with eight bowel movements per day, and weight loss of 20 kg. Physical examination showed malnutrition, oropharyngeal candidiasis, diffuse wheezing involving both lungs, vesicular lesions in the penis, and an anal fissure. Anti-HIV type 1 antibodies were detected, and the CD4 cell count was 11/mm^3; a tuberculin test was negative. The search for sexually transmitted diseases was negative in rectal samples; herpesvirus was isolated from genital samples, and syphilis serology was positive. A chest radiograph showed a diffuse bilateral alveolo-interstitial infiltrate. Giemsa and Ziehl-Neelsen staining and an immunofluorescence test with specific anti-*Pneumocystis carinii* monoclonal antibodies performed on an induced sputum sample were negative. The patient was empirically treated intravenously with cefotaxime and steroids, with no clinical improvement. A computed tomography scan showed no thoracic lymphadenopathies. A fibrobronchoscopy with BAL was then performed, with no diagnosis (including cytomegalovirus cell

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culture), but *Mycobacterium avium* complex (MAC) was isolated from the respiratory samples. At that time, the patient developed a progressive pancytopenia. A bone-marrow biopsy was performed, with no evidence of lymphoma or *Leishmania* and *Cryptosporidium* spp.

Stool cultures for pathogenic bacteria and a search for ova and parasites in stained permanent smears (including Kinyoun staining and a specific monoclonal antibody test for *Cryptosporidium*) were negative. Weber's chromotrope-based staining method detected the presence of microsporidia spores. The urine sample was negative for parasites, including microsporidia.

Therapy with clarithromycin, ethambutol, penicillin-benzatine, acyclovir, and fluconazole was begun. The patient received albendazole (400 mg twice a day per os) and loperamide (8 mg/day) for microsporidiosis. The treatment led to general clinical improvement, including improved respiratory symptoms and diarrhea (one to two liquid bowel movements per day). Microsporidial spores, however, continued to be shed in feces. After 40 days, the patient was discharged with clarithromycin, ethambutol, and albendazole, as well as trimethoprim-sulfamethoxazole prophylaxis.

Fecal examinations carried out throughout 1995 confirmed the absence of parasites other than microsporidial spores.

In February 1996, 16 months after discharge from the hospital, the patient was readmitted with fever, productive cough, diffuse abdominal pain, and diarrhea (seven liquid stools per day). The patient had followed the prescribed treatment but had stopped taking albendazole 2 months after he was discharged from the hospital. Chest radiography revealed new diffuse bilateral interstitial infiltrates. A body computed tomography scan showed multiple retroperitoneal and mesenteric adenopathies, as well as multiple ill-defined amorphous lesions of about 3 to 4 cm in length in both kidneys. A BAL was performed because of the clinical progression of pulmonary symptoms. MAC and *Aspergillus* sp. were cultured from an induced sputum sample. Microsporidia were readily identified in stool samples, BAL, and induced sputum but not in a urine sediment collected 24 h after admission. MAC was also cultured from computerized axial tomography scan-guided renal puncture samples, and the patient was treated with amikacin, ofloxacin, rifabutin, ethambutol, and clarithromycin. He also received albendazole (400 mg twice a day per os) and loperamide for the control of microsporidial infection. After a clear clinical improvement (disappearance of fever and respiratory symptoms but two liquid depositions per day), the patient was discharged from the hospital. A repeat examination 4 months later found the patient to be stable with anti-MAC and albendazole treatment.

MATERIALS AND METHODS

Patient samples. A diarrheic stool sample, a 24-h collection of urine, and an induced sputum sample were obtained at the time of hospitalization. Subsequently, 10 additional stool samples were obtained at months 8, 13, 17, 18, 21, and 24 of the study. In addition, two urine samples at 8 and 18 months, three sputum samples at 18, 19, and 20 months, and a BAL sample at 20 months were also obtained. The entire first stool sample was fixed in formalin; a portion of the others was frozen for use in PCR analysis. Thin smears were prepared directly from the stool and the sputum samples by dropping 20 μl of the appropriate sample on a microscope slide and spreading it with an applicator stick. Urine and BAL samples were centrifuged at 1,500 × *g* for 20 min, the sediment was washed three times in distilled water or saline solution, and thin smears were prepared as described above. The smears were stained by using trichrome or Weber's chromotrope techniques (27). Smears were also prepared for study by immunofluorescence (22).

Microorganisms. *Encephalitozoon cuniculi* (ECLD), *Encephalitozoon hellem* (CDC:V257), and *Encephalitozoon intestinalis* (CDC:V297) were cultured on E6 monolayers (22, 24) to be used as controls.

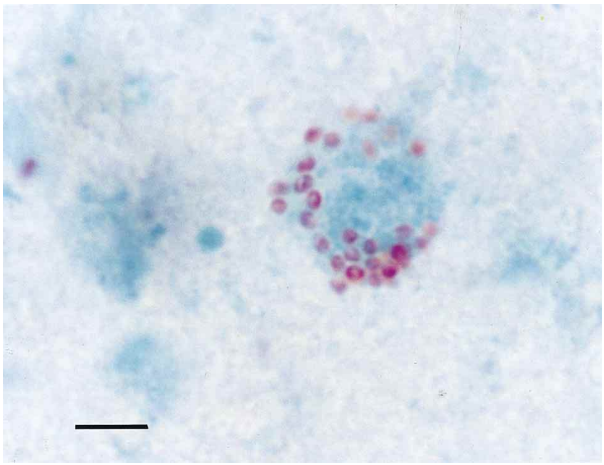

FIG. 1. Sputum smear stained with the Weber's chromotrope stain showing the pink-stained spores of *E. bieneusi*. Bar, 5 μm.

IIF test. Spores of *Encephalitozoon cuniculi* (ECLD), *Encephalitozoon hellem* (CDC:V257), and *Encephalitozoon intestinalis* (CDC:V297) were harvested from culture supernatants and washed three times in Hank's balanced salt solution before being counted in a hemocytometer. They were then suspended in saline solution containing 1% formalin to obtain 10^7 spores/ml and processed for the indirect immunofluorescence (IIF) test as described previously by Visvesvara et al. (22, 24). IIF was also performed on smears of the patient's fecal, sputum, BAL sediment, and urine sediment samples, as well as on control feces known to contain *Encephalitozoon intestinalis* or *E. bieneusi.*

DNA extraction and purification. DNAs from unpreserved stools, sputum, BAL sediment, and urine sediment were extracted by following the methods described earlier (5, 19). An aliquot of DNA extracted from the BAL sample was concentrated with the QIAquick PCR kit (Qiagen, Chatsworth, Calif.) and used per the manufacturer's instructions.

PCR amplification. Microsporidial small-subunit (SSU)-rRNA-coding regions were amplified with the following species-specific primers: EBIEF1/EBIER1 for *E. bieneusi* (5), SINTF/SINTR for *Encephalitozoon intestinalis* (6), and finally the PCR primers described by Visvesvara et al. (23) for *Encephalitozoon cuniculi* and *Encephalitozoon hellem*. PCR amplification was done with a GenAmp kit (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's directions. The concentration of each primer was 0.1 μg per 50-μl final PCR mixture volume containing 1 or 0.1 μl of the purified stool, urine, or sputum sample extract or 5 or 1 μl of unconcentrated or concentrated BAL extract. The positive controls used included 0.03 ng per 50-μl PCR mixture of the corresponding cloned SSU-rRNA-coding region. Conditions for PCRs were denaturation at 94°C for 30 s in all cases, annealing at 45°C for 30 s for *Encephalitozoon intestinalis* primers and at 55°C for 30 s for the rest of primers, and extension at 72°C for 90 s. Thirty-five cycles were done in all cases. Amplification products were analyzed after electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide.

Test for PCR inhibitors in purified samples. Purified samples were tested for the presence of PCR inhibitors by spiking the samples with 0.03 ng of the corresponding cloned SSU rRNA. Amplification of a band of the correct size indicated the removal of PCR inhibitors in the DNA purification process.

RESULTS

Weber's chromotrope-based stain. Stool, BAL, and sputum samples contained, throughout the study, various numbers of spores that stained pinkish red with the Weber's stain and measured 0.9 to 1.2 μm in length (Fig. 1). Each of the 11 stool samples collected during the study contained a moderate number (1 to 100) of microsporidial spores per high-power field. The spores were easily located because of their pinkish color, which facilitated their final identification with an oil immersion lens. The three sputum samples studied were positive for microsporidia, with a lower number of spores; 0 to 10 spores per field (high power) were found. The BAL sediment showed the lowest number of microsporidial spores, with 0 to 10 spores per field (high power) widely scattered on the slides. However, it was found that some of these spores were observed as intra-

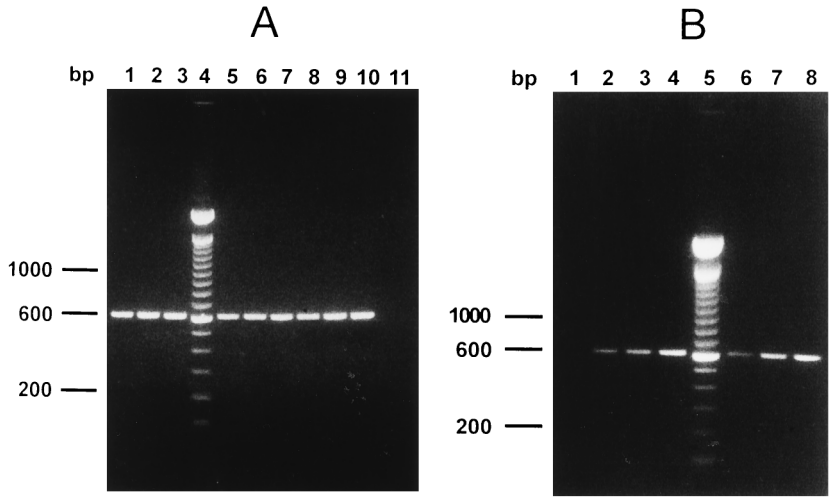

FIG. 2. Agarose gel analysis of PCR-amplified products. (A) PCR performed with DNAs from stool samples from months 8 (lanes 1 to 3), 17 (lanes 5 to 7), and 21 (lanes 8 to 10). Lanes 1, 5, and 8, 0.1 μl of sample extracts; lanes 2, 6, and 9, 1 μl of sample extracts; lanes 3, 7, and 10, 0.1 μl of sample extracts, including a positive control; lane 4, 100-bp-ladder standard; lane 11, negative control. (B) PCR performed with DNAs from sputum samples from months 18 (lanes 2 to 4) and 20 (lanes 6 to 8). Lane 1, negative control; lanes 2 and 6, 0.1 μl of sample extracts; lanes 3 and 7, 1 μl of sample extracts; lanes 4 and 8, 0.1 μl of sample extracts, including a positive control; lane 5, 100-bp-ladder standard.

cellular microsporidial spores. No microsporidia spores were detected in the two 24-h urine sediments.

IIF. Microsporidial spores present in the smears made from stools, BAL, and sputum samples of the patient did not react with the rabbit anti-*Encephalitozoon intestinalis*, anti-*Encephalitozoon hellem*, or anti-*Encephalitozoon cuniculi* serum by IIF. Fecal samples known to contain *E. bieneusi* spores showed no fluorescence activity with any of the tested rabbit sera. The two urine samples also did not react with any of the tested rabbit sera. The control preparations of *Encephalitozoon hellem*, *Encephalitozoon cuniculi*, and *Encephalitozoon intestinalis*, however, reacted with their respective homologous antisera and produced bright-apple-green fluorescence.

PCR. PCR was performed with unfixed stool, sputum, BAL sediment, and urine sediment specimens from this patient. The DNAs isolated from the stool, BAL, and sputum samples, when they were amplified by PCR with *E. bieneusi*-specific primers, showed a diagnostic band of 607 bp in the agarose gels (Fig. 2 and 3). BAL DNA extract showed the lowest concentration of the *E. bieneusi* target (Fig. 3), and the QIAquick PCR-concentrated BAL sample generated stronger bands under the same PCR conditions. No such diagnostic band was seen when the amplification was performed with the same primers and the DNAs from the two 24-h urine sediments. When the templates obtained from the three types of specimens from different dates were assayed with the *Encephalitozoon cuniculi*-, *Encephalitozoon hellem*-, or *Encephalitozoon intestinalis*-specific primers, no amplification was obtained in any of the samples studied. No PCR inhibitors were detected in templates obtained from DNA purification of any patient samples under study.

DISCUSSION

Microsporidia are emerging pathogens and are being increasingly recognized as a cause of disease among AIDS patients. Members belonging to six genera, *Enterocytozoon*, *Encephalitozoon*, *Nosema*, *Pleistophora*, *Trachipleistophora*, and *Vittaforma*, are responsible for human microsporidiosis. Although the clinical spectrum of microsporidiosis has expanded from the original description of chronic diarrhea to a wider variety of disease patterns, the most prevalent microsporidium-associated disease continues to be chronic diarrhea with wasting syndrome, and *E. bieneusi* is the most commonly reported microsporidium associated with the gastrointestinal syndrome (28). Microsporidium-associated pulmonary disease principally due to *Encephalitozoon* spp. is also being reported, but less frequently (28). We report the diagnosis of a microsporidial infection in an AIDS patient in whom microsporidial spores were found, over a 24-month period, in sputum, BAL, and stool samples but not in urine sediment. The infection was later identified as due to *E. bieneusi* based on PCR analysis.

Diagnosis of microsporidial infections has traditionally depended on microscopic demonstration of the organisms. Until recently, the "gold standard" method for demonstrating the presence of these parasites was electron microscopy (18, 19). However, for diagnostic purposes, electron microscopy is time-

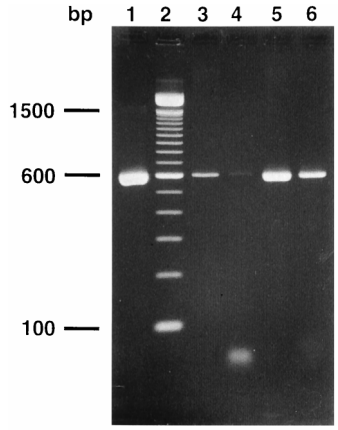

FIG. 3. Agarose gel analysis of PCR-amplified products from a BAL sample. Lane 1, cloned *E. bieneusi* SSU-rRNA-coding region used as a positive control; lane 2, 100-bp-ladder standard; lane 3, 5 μl of sample extract; lane 4, 1 μl of sample extract; lane 5, 5 μl of the QIAquick concentrated sample; lane 6, 1 μl of the QIAquick concentrated sample.

consuming and relatively insensitive and requires considerable expertise (8). The development of reliable differential staining techniques, such as that with Weber's chromotrope-based stain, has facilitated the widespread diagnosis of microsporidia at the light-microscope level, and electron microscopy is mainly applied to species differentiation. Our patient was initially diagnosed as having intestinal microsporidiosis on the basis of Weber's chromotrope-based staining technique, which stains spores a pinkish red (27). Since both *E. bieneusi* and *Encephalitozoon intestinalis* are known to cause intestinal disease, identification would be possible if PCR or a specific immunologic test was used. PCR confirmation could not be made in our case, as the only available sample was limited and had been formalin fixed. Formalin fixation not only interferes with electron microscopy analysis but also eliminates the possibility of PCR analysis when the sample has been fixed for longer than 10 days (5). However, immunological techniques such as immunofluorescence have proven to be useful and reliable in diagnosing human microsporidiosis (1, 20, 23, 24). In the IIF test, no spore reactivity was observed with anti-*Encephalitozoon intestinalis* serum, so that the other microsporidium implicated to date in intestinal disease was not responsible for the process. Further analysis with anti-*Encephalitozoon hellem* and -*Encephalitozoon cuniculi* serum was performed to eliminate any possibility of the presence of other human microsporidia belonging to the genus *Encephalitozoon*. Finally, as no other intestinal pathogens, including other parasites or bacteria, were found and because of the relatively small size (~1.0 μm) of the spores, it was assumed that the disease was caused by *E. bieneusi* and the patient was started on a course of albendazole (800 mg/day) and loperamide (8 mg/day). Though intestinal symptoms improved, the patient nevertheless had one to two diarrheic stools per day and microsporidial spores continued to be voided in stools. To date, albendazole appears to be the most effective drug, being capable of total elimination of *Encephalitozoon intestinalis* from the human host (12, 17, 26, 28, 30). However, it is only partially effective against *E. bieneusi* as it produces improvement in symptoms and a decrease, but not elimination, of parasite load (2, 11, 26).

Eight months later, fresh stool and urine samples were obtained from the patient with the same intestinal symptoms. Weber's chromotrope stain revealed that the fecal samples were positive for microsporidial spores but that the urine samples were negative as before. PCR analysis was performed on unfixed fecal and urine samples with primers specific for *E. bieneusi*, *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, and *Encephalitozoon intestinalis*. A positive result was obtained only with *E. bieneusi* primers in the fecal sample, and a 607-bp band was amplified. These facts confirmed our suspicions that the pathogen responsible for this patient's chronic diarrhea and wasting syndrome was, unequivocally, *E. bieneusi.*

In recent years, the PCR technique has proven useful for microsporidial detection in patient samples (5, 7–10, 13, 14, 22, 23, 31, 33). The SSU-rRNA-coding regions of various species and strains have been sequenced by different groups (22, 23, 25, 32, 33), and species-specific primers are available in specialized laboratories. The recent development of an accurate and reproducible technique for isolation and purification of DNAs from stool samples has also facilitated this analysis (5). It is well known that stool samples may contain a large variety of components, including PCR inhibitors. The method that we used has proven useful in their elimination, and the precaution of spiking a sample with positive control DNA eliminates any doubt of a possible false negative when the result shows no amplified band.

The analysis carried out by these techniques throughout the study period (24 months) showed that *E. bieneusi* continued to be shed in feces but not in urine. During this period, the patient also had pulmonary symptoms. Sputum samples were obtained at 18, 19, and 20 months, and a BAL sample was obtained at 20 months, all of which were analyzed for microsporidia by Weber's chromotrope staining and IIF. The samples were positive for microsporidial spores with the chromotrope stain but negative in the IIF analysis when the three antisera against *Encephalitozoon* spp. were used. It is interesting that intracellular microsporidial spores were detected in the BAL, reinforcing the suspicion of pulmonary colonization by *E. bieneusi* suggested by the spores found in the sputum samples. PCR analysis showed a 607-bp band specific to *E. bieneusi*. The poor PCR results obtained with the unconcentrated BAL DNA extract correlated to the low number of spores detected with the Weber's stain. Although the 607-bp band could be clearly detected with the undiluted sample, the stronger bands obtained after QIAquick concentration only corroborated this result. Species-specific identification of microsporidia in clinical samples is becoming increasingly important due to previously described variable responses to some drugs (8).

To our knowledge, only one instance of pulmonary involvement with *E. bieneusi* has been reported so far for a patient with chronic diarrhea who developed a persistent cough, with scant, nonpurulent sputum, dyspnea, and wheezing (29). In this case, spores were detected in BAL fluid, and a substantial number of alveolar macrophages with intracellular spores were detected in transbronchial biopsy specimens of the lower left lobe, stools, and ileal biopsy specimens (29). Apart from this, individual instances of sinonasal involvement of *E. bieneusi* have also been documented (15, 28). However, most sinonasal or pulmonary symptoms have been associated with the *Encephalitozoon* species (3, 15, 23).

MAC, a recognized pulmonary pathogen, was also detected in our patient's lung samples. An association between the presence of *E. bieneusi* in his respiratory tract and his clinical symptoms could not be established. This was also the case with a previously described pulmonary microsporidiosis (7, 29) in which, although no other pulmonary pathogens were found, a correlation between microsporidiosis and pulmonary symptoms was not established. In any case, this report reinforces the idea that *E. bieneusi* has the capability for pulmonary colonization. Future epidemiological studies will have to determine its pathogenic potential in the lungs. As for the route of acquisition of pulmonary microsporidiosis, it is unclear whether *E. bieneusi* was acquired by inhalation, regurgitation, or direct oro-fecal contamination or disseminated by the hematogenous route from the intestine. The lack of animal models for this parasite complicates these kinds of studies; however, with other human microsporidia for which animal models exist, the oral and the inhalation routes have been demonstrated (4).

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