FIRST CASES OF MICROSPORIDIOSIS IN TRANSPLANT RECIPIENTS IN SPAIN AND REVIEW

Running Title: Microsporidiosis in transplant recipients

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ABSTRACT

Microsporidia are currently considered as emerging pathogens responsible for life-threatening infections in organ transplant recipients. Here, we describe the first cases of intestinal microsporidiosis by *Enterocytozoon bieneusi* genotype D in two non-HIV-infected renal transplant recipients from Spain. Previously reported cases of microsporidiosis in organ transplant recipients have also been reviewed, highlighting the necessity of considering organ transplant recipients as a risk group for microsporidiosis. A systematic search for these parasites is recommended in cases of persistent diarrhea and in the differential diagnosis of other syndromes, such as chronic fever of unknown etiology.
INTRODUCTION

Microsporidia are ubiquitous, obligate intracellular opportunistic parasites, recently related to fungi, capable of infecting a wide range of vertebrate and invertebrate hosts (2, 12). Within microsporidia, 8 genera and 14 species have been associated with human infections, among which Enterocytozoon bieneusi and Encephalitozoon intestinalis are the most commonly reported (12). These opportunistic pathogens cause a variety of systemic and non-systemic diseases, with chronic diarrhea as the most common clinical manifestation, although the spectrum of diseases caused by them is broad and includes eye, respiratory, renal, and central nervous system infections (12).

Most microsporidial infections have been reported in severely immunocompromised individuals, mainly HIV/AIDS patients, but cases in HIV-negative people, including travelers and elderly people, are constantly increasing (11, 27). Additionally, the number of non-HIV-patients with other forms of immunosuppression is also increasing. Among these, organ transplant recipients (OTR) have recently been considered as a group of patients at risk for these pathogens (22). To date, only 21 cases of microsporidiosis in solid organ transplant (SOT) and bone marrow transplant (BMT) HIV-negative recipients have been described (4, 13-17, 19, 20, 22, 23, 28-31, 34, 40, 44, 49). In these patients, diarrhea is the most frequent clinical manifestation, and E. bieneusi, the species mainly involved (Table 1). Moreover, there are three retrospective studies in which microsporidia have been reported in transplant patients (25, 35, 46). Liguory and collaborators studied microsporidial infection...
in stool specimens from 100 patients obtained over a 6-year period (1994-2000), and they found 8 organ transplant recipients (6 renal, 1 liver, and 1 heart-lung) that were positive for *E. bieneusi* (25). Rabodonirina and collaborators, in a retrospective study carried out in France, found 23 cases of microsporidiosis in transplant patients, including 3 who had already been described in the literature between 1993 and 2001 (35). Recently, ten Hove and collaborators performed a retrospective phylogenetic analysis on isolates of *E. bieneusi* collected between 2003 and 2004 and they included five kidney transplant recipients that were positive for this microsporidia (46, 47).

We describe the first findings of intestinal infection caused by *E. bieneusi* in two renal transplant recipients from Gran Canaria, Spain, and we review the published cases of microsporidiosis in SOT and BMT recipients.

### CASE REPORTS

**PATIENT 1:** A 66-year-old male who received a renal transplant in April 2009 was admitted to the Nephrology unit of The Hospital Universitario Insular de Gran Canaria (Spain) for severe leucopenia (1000 cells/mm$^3$) and abundant liquid diarrhea in July of the same year. He had a history of chronic renal failure secondary to a nephroangiosclerosis, arterial hypertension and a heart attack. Immunosuppressive therapy consisted of steroids, tacrolimus, and mycophenolate mofetil (MMF). This treatment, as well as valganciclovir and septrim, was suspended after admittance to the hospital, due to the suspicion of pharmacologic toxicity. No bacterial or viral pathogens were found in the stool samples. The colonoscopy was normal. Cytomegalovirus (CMV) antigen in...
colonic mucosa was positive. Calcofluor white (48) and modified trichrome (53) stains showed structures evocative of microsporidia spores in stool samples (figure 1). The patient was treated with three doses of filgrastrim, fluid therapy, and diet control. Clinical symptoms disappeared and the patient was discharged afebrile, with a normal white blood count and normal bowel movements.

PATIENT 2: A 54-year-old man with a history of kidney transplant in 1994 was admitted to the Nephrology unit of The Hospital Universitario Insular de Gran Canaria, for persistent liquid diarrhea on 9th June 2009. His immunosuppression regimen consisted of tacrolimus and MMF. The patient did not improve after MMF suspension and dietary treatment. Laboratory tests showed a slight deterioration of renal function. Cytomegalovirus antigen was negative. *Clostridium difficile* toxin was detected in fecal samples. Fecal smears showed microsporidia spores stained by Calcofluor white (48) and modified trichrome (53). Clinical symptoms disappeared after initiation of fluid therapy, diet control, and metronidazole treatment. Normal bowel habits and renal function were recovered. Two weeks later, the patient showed episodes of diarrhea with epigastric pain, microsporidia spores were observed in stool samples, and *Clostridium difficile* toxin, virus, bacteria and parasites were negative. Treated with albendazole, he became asymptomatic but continued seeding a lower amount of microsporidia spores, detected only by PCR.

MATERIALS AND METHODS
Staining Methods. Thin smears from one diarrheic stool sample from patient 1 and three from patient 2 were prepared and stained, using Calcofluor white (48) and Weber’s chromotrope-based stain (53).

DNA extraction and purification. DNA from unpreserved stools was extracted by following the methods described earlier (5). DNA from fecal samples was extracted by bead disruption of spores using the Fast-DNA-Spin kit, according to the manufacturer’s instructions (Bio 101, Carlsbad, Calif.). PCR inhibitors were removed using the QIAquick PCR kit (QIAGEN, Chatsworth, CA).

PCR amplification. Microsporidial small subunit rRNA (SSU-rRNA) coding regions were amplified using the following species-specific primers: EBIEF1/EBIER1 for *E. bieneusi* (5), SINTF/SINTR for *E. intestinalis* (6), EHELF/EHELR for *E. hellem* (50), and ECUNF/ECUNR for *E. cuniculi* (7). The PCR amplification was done with the GenAmp kit (Perkin-Elmer Cetus, Norwalk, CT) according to manufacturer’s procedures and the conditions for the reaction described previously (8). Purified samples were tested for the presence of PCR inhibitors, as described previously (8). Amplification products were analyzed, electrophoresis was performed in 2% agarose gel, and they were visualized by ethidium bromide staining (8).

DNA sequencing analysis. Genotyping of *E. bieneusi* was performed by sequence analysis of the ITS region of rDNA. For this purpose, primers that amplified a fragment of 536 bp containing the 243 bp of the ITS were designed (primer F: 5'CTTCGGCTCTGAATATCTAT3' and Primer R: 5'GCCACTACTAACGGAATCTA3'). PCR amplifications were performed following the cycling conditions: denaturing at 94°C for 30 seconds, alignment at 55°C for 30 seconds and extension at 72°C for 90 seconds. Each PCR product
was sequenced in both directions using the BigDye terminator sequencing kit in an ABI PrismR 3130 genetic analyzer (Applied Biosystems). The resulting sequences were analyzed by the Bioedit program and compared with reference sequences from the GeneBank.

RESULTS

Staining Methods. The two patients studied showed structures evocative of microsporidia spores in the sample analyzed from patient 1 and in two samples from patient 2, obtained before albendazol treatment when analyzed by Calcofluor white (48) and Weber’s chromotrope-based stain (53). Spores in the chromotrope-stained smears appeared pinkish red and measured 0.9 to 1.2 µm in length. Many spores exhibited the characteristic posterior vacuole and beltlike stripe in the middle (figure 1). However, in the second stool sample from patient 2, analyzed two and a half months later and after albendazol treatment, no microsporidia spores were observed by the staining methods.

PCR. PCR was performed with unfixed stools from the two patients. Amplification of DNA isolated with specific primers for the most common microsporidia infecting humans showed positive results with E. bieneusi-specific primers (5) in both cases in all samples. However, E. intestinalis-specific PCR (6), E. hellem-specific PCR (50), and E. cuniculi-specific PCR (7) were negative (figure 2) in the two patients studied.

Genotyping. Genotyping of the E. bieneusi isolates was performed by sequence analysis of the ITS region of rDNA. The sequence analysis of PCR amplified products showed 100% homology with genotype D in both cases (GeneBank accession number AF101200.1) (38).
DISCUSSION

Enterocytozoon bieneusi is the most common microsporidian associated with human disease, particularly in severely immunosuppressed individuals with CD4+ counts <100/mm³ (12). In the presence of HIV infection, it is associated with diarrhea and wasting syndrome, and cellular immunoresponse has been considered essential for the control and elimination of this microsporidia (12). In SOT and BMT recipients, an immunosuppressive therapy is always prescribed, leading to a profound cellular immunodeficiency (22). However, few cases of microsporidiosis have been reported in transplant patients (Table 1). Chronic diarrhea is the main clinical manifestation in most infections, and *E. bieneusi*, the most common species encountered in more than half of the cases in OTR, followed by *E. cuniculi* (Table 1) (22, 35). This agrees with the observations of the two patients in our study; persistent diarrhea with *E. bieneusi* detected in stool samples by PCR and modified trichrome stain. However, microsporidiosis occurred in reported cases from 19 days up to 7 years after transplantation (22). In our patient 1, it appeared 3 months later, but in patient 2, it appeared 15 years after transplantation.

In both patients, we detected other microorganisms that have been associated with gastrointestinal symptoms in transplant recipients, including diarrhea. Patient 1 was positive for CMV antigen in colonic mucosa, which is a common finding, since CMV infection is one of the major infectious complications in transplant recipients with non-systemic symptoms that include fever, diarrhea, myalgias, malaise, and in severe cases hepatitis, pneumonia,
and colitis (18). However, the gastrointestinal tract is one of the least common sites of CMV disease. Taking into account that this patient showed no other symptoms besides diarrhea and received prophylaxis with an antiviral, we believe that *E. bieneusi* would play an important role in the diarrhea observed. It should be noted that in one of the renal transplant recipients reviewed, the authors suggest that CMV infection may further enhance the susceptibility to microsporidial infection (30). Patient 2 showed a test positive for *Clostridium difficile*, which is a significant pathogen leading to diarrhea and colitis in transplant recipients (32). However, the majority of *C. difficile* infections concern patients in the early post-transplant period, with a prior long-lasting treatment with antibiotics, which was not the case of patient 2. Nevertheless, the patient was treated with metronidazol, a first line therapy for *C. difficile* (21), and the *C. difficile* test was negative from then on.

Regarding the patients’ outcomes: patient 1 showed a clinical course similar to that described in previously reported cases in SOT recipients (31) in which suspension of the immunosuppressive treatment led to recovery. Therefore, we suggest that restored immune balance after MMF withdrawal and filgrastim treatment allowed the recovery of the patient. Patient 2 was only capable of resolving the symptoms after a sequential therapy with metronidazole and albendazole: metronidazol treatment initially allowed the elimination of *C. difficile* and afterwards albendazol treatment allowed a patent decrease in microsporidia spore seeding and the resolution of diarrheal symptoms. To date, no curative therapy for *E. bieneusi* infection exists. Metronidazol, which is indicated for *C. difficile* treatment (21), has been occasionally reported to cause transient improvement of the symptoms of
microsporidiosis (17, 41, 54) However, albendazole, which is effective against
microsporidia other than *E. bieneusi*, seems to alleviate diarrhea in *E. bieneusi-
infected patients without clearing the infection, but with a notable decrease in
spore seeding (8, 41, 51, 52).

Both patients received immunosuppressive therapy with tacrolimus and
MMF at the time of diagnosis. Other immunosuppressive therapies described in
OTR diagnosed with microsporidiosis included cyclosporine, prednisone,
azathioprine, rapamycin, antilymphocyte globulin or methotrexate (22, 35). It
has been suggested that the lack of γ-IFN resulting from the Th cell depletion
induced by MMF may be responsible -at least in part- for the onset of
microsporidiosis (16) and the triggering of the intestinal symptomatology by the
parasite. Since patient 1 recovered from symptoms after treatment suspension,
it is very likely that this recovery was associated with the immune reconstitution
balance. The same situation was described in previously published reports of
microsporidiosis in renal transplant recipients (16). In relation to patient 2, the
discontinuation of the immunosuppressive therapy helped by metronidazol and
albendazol treatment improved the patient’s health conditions, including normal
bowel movements. The same outcome has been described in two of the 4 OTR
with *E. bieneusi* infection who were treated with albendazol (34, 41). However,
in the other two cases, a complete clearance of spores was achieved (16, 29),
suggesting that the discontinuation of the immunosuppressive therapy was
probably what mainly improved the patients’ health conditions (16, 29, 41)

In reference to the presence of microsporidia in Spain, it has mainly been
reported in HIV/AIDS patients (8-10) but also in HIV-negative individuals,
including travelers (26), elderly people (27), and the immunocompetent
population (1). In most of these studies, *E. bieneusi* was the microsporidia responsible for clinical symptoms. However, to date, no cases of microsporidiosis in OTR have been described. This report recognizes the implication of microsporidia in OTR pathology for the first time in Spain.

In most cases, microsporidia detected in OTR were characterized only to the species level (22, 35). *Encephalitozoon cuniculi* is the best characterized microsporidia in OTR isolates at genotype level (30, 33, 44). In 3 of 5 *E. cuniculi* infections described in SOT and BMT recipients, the genotype was investigated: two of them showed the ITS-related genotype III, known as “dog strain” (30), and in the third one, a new genotype IV was recently described (44). In reference to the genetic variation among isolates of *E. bieneusi* (18, 47), analyses of ribosomal DNA internal transcribed spacer (ITS) sequences have identified more than 70 genotypes of *E. bieneusi* (18, 47). Some of these genotypes have been recognized as host-specific, while others have been found to infect humans and animals, supporting the likelihood of zoonotic transmission (18, 47). In our 2 patients, genotype D was identified. It belongs to Group 1, which includes numerous genotypes from various origins: human, both HIV-positive and negative, but also domestic and wild animals. Genotype D is widespread in nature (47). It was first found in man in Germany, and afterwards in other countries of America, Asia, and Africa. It was also found in numerous diverse animals (swine, cattle, macaque, muskrat, raccoon, beaver, fox, dog, and falcon) (47). Type D genotype was commonly reported in HIV-positive patients in Thailand (24) and Peru (42) and in two isolated cases in Europe (36, 37) and it was recently isolated from 3 HIV-negative individuals in Cameroon (3), which confirms the wide spread of this genotype. Genotype D represents 15
% of isolates from four species of wildlife animals in North America (43) and 26% of isolates found in cats in Colombia (39), supporting a zoonotic route of transmission for this strain.

Information about molecular epidemiological data of *E. bieneusi* isolates from transplant recipients is limited. Several studies have described the predominance of genotype C in this population (25, 41, 46). Liguory and collaborators genotyped 100 *E. bieneusi* isolates from both HIV and non-HIV patients, including eight transplant recipients. In the latter, they found genotype II (genotype C) in seven of these patients and genotype IV (genotype D) in one (25). They also described that the distribution of genotypes was significantly different among HIV-infected patients compared to non-HIV, and they suggested differences in the epidemiology of the infection according to HIV infection status or differences in the virulence of the microsporidia strain (25).

Sing and collaborators also found human genotype C in a liver transplant recipient, based on analysis of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequence (41). Ten Hove and colleagues performed a molecular characterization of *E. bieneusi* isolates from immunosuppressed and immunocompetent patient groups in Malawi and the Netherlands (46). They identified 16 genotypes, nine of which had not previously been described. Genotypes B, K, and D were most prevalent among HIV patients, whereas genotype C was identified in five isolates from kidney transplant recipients and was not seen in any of the other groups of patients (46).

In contrast to the microsporidiosis caused by *E. bieneusi*, which is generally confined to the digestive tract, as shown in reported cases (15-17, 29, 31, 34) and in the 2 cases described here, *Encephalitozoon* infections were
frequently disseminated in OTR (4, 14, 30, 44, 45). In these cases, microsporidia were most frequently identified in urine samples but were also isolated from various tissues or body fluids including stools, sputum, conjunctival scraping, brain, and kidney biopsy. The most commonly reported clinical symptoms in disseminated microsporidiosis in OTR were keratoconjunctivitis, fever, abdominal pain, and respiratory symptoms (cough, thoracic pain) (44). Diagnosis of microsporidiosis in these patients was conducted mainly by trichrome stain and PCR. Most of the cases were described in Europe, but there were also seven in America, two in India, and one in Africa. Antiparasitic treatment used included albendazol, metronidazole, and fumagillin, independent of the microsporidian species found. In most cases of infection by *E. bieneusi*, the recovery was related to immune reconstitution and/or immunosuppressive therapy suspension (22).

In conclusion: transplant recipients undergoing immunosuppressive therapy should be considered as a risk group for acquisition of microsporidiosis. Therefore, in all countries (including those in which microsporidia have not yet been recognized), microsporidia should be considered in cases of persistent diarrhea and also in the differential diagnosis of other syndromes, such as chronic fever of unknown etiology, after ruling out more common causes of diarrheal disease. The search should be performed not only in stool samples, but also, at least, in urine samples. A molecular characterization of the parasite isolates should be considered to convey information about the frequency and distribution of microsporidia species and genotypes in this group of patients.

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REFERENCES


**FIGURE LEGENDS**

Figure 1. A: Microsporidia spores stained with modified trichrome stain from patient 1. B: PCR amplification of rDNA coding region containing the 243 bp of the ITS of *E.bieneusi*, M: Molecular marker (100 bp ladder), lane 2: patient 1, lane 4: patient 2, lane 6: positive control, lane 8: negative control.
TABLE 1. Microsporidiosis in transplant recipients

<table>
<thead>
<tr>
<th>Case No/Reference</th>
<th>Type of transplant</th>
<th>Age*</th>
<th>Gender</th>
<th>Specie/Genotype</th>
<th>Immunosuppressive treatment</th>
<th>Clinical manifestation</th>
<th>Laboratory diagnosis</th>
<th>Treatment/outcome</th>
<th>Country / Publication year</th>
</tr>
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<tr>
<td>1** (25)</td>
<td>Kidney (6), Liver (1), Heart-lung (1)</td>
<td>25</td>
<td></td>
<td>E. bieneusi/C genotype (7) and D genotype (1)</td>
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<td>Diarrhea</td>
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</tr>
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<td>2** (35)</td>
<td>Kidney (14), Liver (5), Heart and/or Lung (4)</td>
<td>F: 7 M:16</td>
<td></td>
<td>ATG, CS, AZ, MMF, Tacrolimus</td>
<td>Asymptomatic, diarrhea, weight loss</td>
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<td>Albendazole, Fumagillin</td>
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<td>Metronidazole/cured</td>
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<td>M</td>
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<td>MTS, PCR</td>
<td></td>
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<td>Intestinal</td>
<td>MTS</td>
<td></td>
<td>USA / 1999</td>
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<td>Intestinal</td>
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<td>MTS, PCR, PCR-RFLP</td>
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<td>Immunosuppressant(s)</td>
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</tr>
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</table>
