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Abstract. Stool samples from 86 immunocompromised patients (51 human immunodeficiency virus (HIV)–infected patients and 35 patients with haematologic malignancies) were systematically screened for intestinal microspordiosis by microscopic examination and polymerase chain reaction (PCR) using universal primer V1/PMP2. Nine samples (10.5%) showed amplification with the predictive size of fragment (6 from HIV–infected patients and 3 from patients with myeloma). Only 5 out of them (all HIV–infected patients) were revealed positive by microscopy. By means of amplicons fragment size, species–specific primers (V1/EB450, V1/IS500) and sequencing, 3 microsporidia species were for the first time identified in Tunisia: *Enterocytozoon bieneusi* (3 isolates), *Encephelitozoon intestinalis* (2 isolates), and *Encephalitozoon hellem* (1 isolate). Systematic use of such sensitive and discriminative molecular tools will contribute to determining the true prevalence of microsporidiosis in Tunisia and to better management of infected immunocompromised subjects.

INTRODUCTION

Intestinal microsporidiosis ranks among the most common causes of diarrhea among immunocompromised individuals.[1] Most cases have been documented in patients infected with the human immunodeficiency virus (HIV) and only a few of them have been related to other immunosupressive etiologies.[2] Diagnostic procedures of intestinal microsporidiosis have been markedly improved by the use of Weber's chromotrope-based stain.[3] Molecular-based techniques, such as polymerase chain reaction (PCR), are mainly used for positive case confirmation and/or species differentiation.[4] Despite the important implications of microsporidia species identification on the clinical and therapeutic management of patients, the corresponding data are still rare.[5] *Enterocytozoon* (*E.*) *bieneusi* and *Encephalitozoon* (*E.*) *intestinalis* are the two species responsible for the majority of infections in man.[6] Published human cases caused by *Encephalitozoon hellem* and *Encephalitozoon cuniculi* are rare.[6,7]

In Tunisia, microsporidia parasites have been found in both immunocompetent and immunocompromised diarrheal subjects.[8,9] However, no species identification has been performed yet. The purpose of this study was to detect and to identify microsporidia species in stools from immunocompromised Tunisian patients.

MATERIALS AND METHODS

Stool samples. Stool samples were collected from 86 immunocompromised Tunisian patients; 51 HIV–infected patients, and 35 suffering from haematologic malignancies (HM) (8 chronic myeloid leukaemia, 8 myeloma, 7 acute lymphoid leukaemia, 6 chronic lymphoid leukaemia, and 6 Hodgkin disease). Fifty-four patients were diarrheic. Collections were done, after individual consent, within the framework of routine screening for opportunistic pathogens. All specimens were systematically investigated for microsporidia infection by light microscopy and PCR.

Weber's modified trichrome stain. Each stool specimen was mixed with 10% formalin (1:3 ratio) then filtered through sieves with pore diameters of 400 μm; 3 mL of ether were added to the filtrate. The mixture was shaken for a minute and centrifuged at 2,000 g for 2 minutes. From pellet a thin smear was prepared on a glass slide, dried, fixed with methanol for 5 minutes, and stained with Weber's modified trichrome stain (MTS), as adapted by Kokoskin and others.[10] Each stained smear was examined using Leitz Laborlux binocular microscopes (Wetzlar, Germany) with built-in illumination and a total magnification of 1000× (10× widefield eyepieces and a 100× oil immersion lens). Negative results were reported after at least 10 minutes of examination.

DNA extraction. The DNA was extracted from frozen samples using the QIAamp DNA Tissue Kit (Qiagen Inc, Germany). Briefly, 200 μL of stool suspension was washed three times in phosphate-buffered-saline (PBS) solution by centrifugation at 12,000 g for 5 minutes. The final pellet was suspended in 180 μL of tissue lysis buffer and incubated with proteinase K for 2 hours at 55°C. The manufacturer's recommendations were followed for purification and elution of DNA, which was then stored at −20°C until analyzed.

PCR amplifications. Two sets of PCR primers were used in this study as described in previous reports. The forward primer V1: 5′-CACCAGGTTGATTCTGCCTGAC-3′ and the reverse primer PMP2: 5′-CCTCTCCGGAACCAAACCCTG-3′) amplify the small-subunit ribosomal DNA (SSU-rDNA) of four human microsporidia, *E. bieneusi*, *E. intestinalis*, *E. cuniculi*, and *E. hellem*.[11] Samples proved positive by V1/PMP2 primers with predictive size fragment about 250 to 279 bp, have been subject to two other PCR, using species–specific primers, V1/EB450 (5′-ACTCAGGTGTTATACTCACGTC-3′) and V1/SI500 (5′-CTCGCTCCTTTACACTCG-3′) to differentiate *E. bieneusi* infection from *E. intestinalis* infection, respectively.[12]

Amplification for all PCR was done in 50 μL reaction mixtures under the following conditions: 1 × PCR buffer, 2.5 mM

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Patient no.	Clinical and biologic status*	Results of		Results of PCR using primer pair		Sequencing result
		MTS	PCR†	V1/EB450	V1/SI500	
1	HIV (CD4‡ = 23)	−	+	+	−	*E. bieneusi*
2	HIV (CD4 = ND§)	+	+	−	+	*E. intestinalis*
3	HIV (CD4 = 46)	+	+	−	−	ND
4	HIV (CD4 = 21)	+	+	−	−	ND
5	HIV (CD4 = 22)	+	+	+	−	*E. bieneusi*
6	HIV (CD4 = 44)	+	+	+	−	*E. bieneusi*
7	Myeloma	−	+	−	−	*E. hellem*
8	Myeloma	−	+	−	+	*E. intestinalis*
9	Myeloma	−	+	−	−	ND

* HIV, human immunodeficiency virus infection.
† Result of polymerase chain reaction (PCR) using primer pair V1/PMP2.
‡ CD4+ T cells count per µL.
§ ND = not determined.

of MgCl$_2$, 20 pmol of each primer, 200 µM concentrations of each dNTP, 1.25 U of Goldstar Taq DNA polymerase (Applied biosystem, Roche, Switzerland), and 10 µL of the DNA. Amplification consisted of 5 minutes at 94°C followed by 35 cycles of 45 seconds at 94°C, 30 seconds at 55°C, and 45 seconds at 72°C; a final phase of extension at 72°C for 10 minutes. The amplified products were separated by electrophoresis on a 2% agarose gel and visualized after staining with ethidium bromide.

Each set of experiments included a negative (distilled water) and two positive PCR controls. The positive controls were DNA template extracted from positive stool specimens infected with *E. bieneusi* and *E. intestinalis* kindly provided by Marc Thellier from Pitié-Salpetrière Hospital, Paris-France.

DNA sequencing analysis. All DNA fragments obtained by universal primers V1/PMP2 were sequenced. The PCR products were purified using GenElute PCR Clean-Up kit (Sigma, St. Louis, MO). Sequencing reactions were performed directly on the amplification products using ABI Prism 377 DNA Sequencer (Applied Bio System, Foster City, CA). The sequences were compared with those available in the GenBank Database with the BlastN program located at (http://www.ncbi.nlm.nih.gov).

RESULTS

Examination of the 86 stool samples by microscopy showed microsporidian spores in 5 samples (5.8%), all 5 out of 51 (9.8%) HIV–infected patients. Using universal primers V1/PMP2, 9 out of 86 (10.5%) stool samples showed a positive amplification. Considering that technique, the prevalence rate of microsporidia infection were 11.8% (6 out of 51) and 8.6% (3, all with myeloma, out of 35) for, respectively, HIV and HM groups (Table 1). Of the nine 7 were diarrheic.

Among the 9 samples that proved positive by PCR, microsporidia species identification was successfully achieved in 6 isolates. A 250 bp DNA fragment, suggesting infection with *E. bieneusi*, was obtained from stools of 3 HIV–infected patients (Figure 1). In those samples, amplification of 353 bp DNA fragment was achieved with *E. bieneusi*–specific primers and no amplification was obtained with *E. intestinalis*–specific primers. The 3 sequences had 98–99% sequence identity with *E. bieneusi* (Genbank accession no. AY257180). A 270 bp DNA fragment, suggesting infection with *E. intestinalis*, was amplified from stool specimens from 2 patients (1 HIV patient, 1 patient with myeloma) (Figure 1). A 375 bp DNA fragment was obtained

from these samples after PCR with *E. intestinalis*–specific primers (Figure 2). The 2 amplicons had 96% and 99% sequence identity with *E. intestinalis* (Genbank accession no. EU436735). A 279 bp DNA fragment was amplified in one sample from a myeloma patient, suggesting infection with *E. hellem*. This sample, negative by both *E. intestinalis* and *E. bieneusi*–specific primers, had 98% sequence identity with *E. hellem* (Genbank accession no. L19070). In the remaining 3 positive samples (2 HIV and 1 myeloma patients), the length of the obtained fragment was too weak for further analysis. These 3 samples revealed negative by both species–specific primers (Table 1).

DISCUSSION

As previously reported, PCR using universal primers V1/PMP2 detected more intestinal microsporidia infections (9 cases) than did light microscopy (5 cases).[13] The difference is probably a result of the limited sensitivity of light microscopy
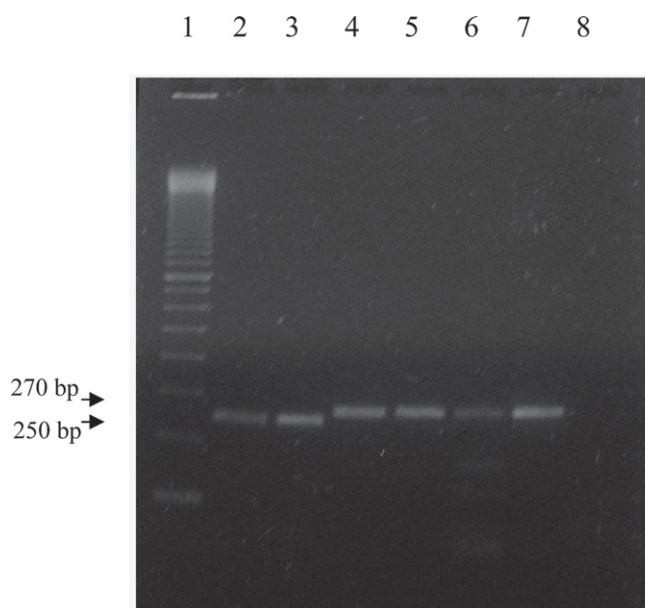

FIGURE 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) products with V1/PMP2 primers stained with ethidium bromide. Lane 1, 100-basepair DNA ladder (Amersham, UK); lane 2, positive sample of *E. bieneusi*; lane 3, positive control of *E. bieneusi*; lane 4, positive control of *E. intestinalis*; lane 5, 6, positive sample of *E. intestinalis*; lane 7, positive sample of *E. hellem;* lane 8, negative control.
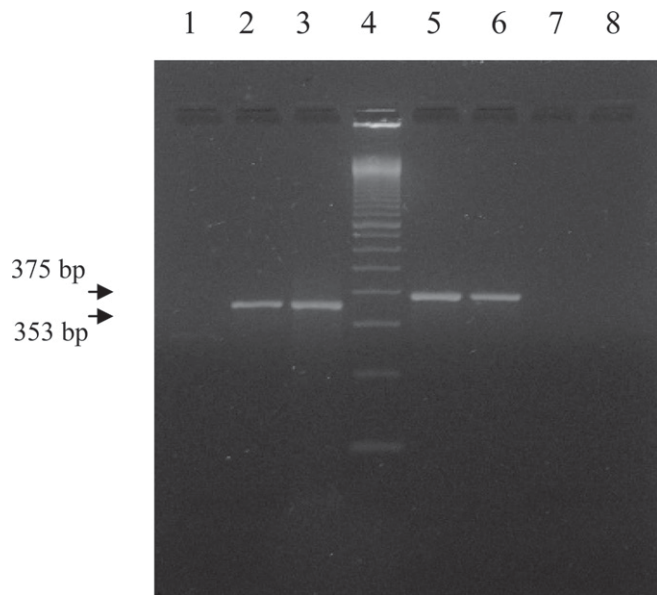
FIGURE 2.   Agarose gel electrophoresis of polymerase chain reaction (PCR) products obtained using species–specific primers V1/EB450 and V1/SI500. Lane 1, negative samples of *E. bieneusi*; lane 2, positive sample of *E. bieneusi*; lane 3, positive control of *E. bieneusi*; lane M, molecular size marker of 100 pb (Amersham, UK); lane 5, positive control of *E. intestinalis*; lane 6, positive samples of *E. intestinalis*; lane 7, negative samples of *E. intestinalis;* lane 8, negative control.

estimated to be between 10^4 and 10^6 spores per g of stool, whereas PCR can detect parasitic loads of 10^2 spores per g of stool.[14] This better sensitivity of PCR allows us to establish a higher prevalence rate (10.5%) of intestinal microsporidiosis in Tunisia in comparison to those reported in previous studies.[8]

The distinction between *Encephalitozoon* and *Enterocytozoon* in light microscopy is often impossible because of the very small size of the microsporidia spores[10]; In fact, spores of *E. bieneusi* average 1.5×1.0 μm and those of *E. intestinalis* 2.2×1.2 μm.[1,6] The PCR and sequencing, which represent the reference tools for species identification,[13,15] are here used for the first time in North Africa to identify the Microsporidia species found in stool samples from a group of 86 immunocompromised Tunisian patients. Such parasites characterization is now necessary for an appropriate therapeutic management of patients. In fact, if albendazole is effective against *Encephalitozoon* spp., only fumagillin have shown to be effective against *E. bieneusi*.[5]

The species identification was successfully performed in 6 isolates, 3 corresponded to *E. bieneusi*, 2 to *E. intestinalis*, and the last to *E. hellem* (Table 1, Figures 1 and 2). As largely described, *E. bieneusi* was predominant in HIV–infected patients (3 out of 4 identifications).[6,12] *Encephalitozzon hellem* and *E. intestinalis* were identified in 2 patients with HM, it is to our knowledge among the rare reports of *Encephalitozoon* spp. in stools from non-HIV infected patients.[6] In fact, *E. intestinalis* has been mainly documented in HIV–infected patients,[16] whereas *E. hellem* has been more described as infecting the epithelial surfaces of ocular and respiratory tissues.[17,18]

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