**INTRODUCTION**

Microsporidia are obligate intracellular pathogens that emerged in humans during AIDS epidemic. The most identified Microsporidia species is *Enterocytozoon bieneusi* (Didier, 2005). It is often associated with chronic diarrhea and wasting in immunocompromised patients especially those infected with human immunodeficiency virus (HIV) (Endeshaw et al., 2006). Despite its isolation in animals and environmental samples, sources and transmission modes to human of *E. bieneusi* are still not well established (Coupe et al., 2006; Mathis et al., 2005). Molecular tools based on analysis of internal transcribed spacer (ITS) of rRNA gene, notably those developed by Rinder and Liguory have been employed to delineate the transmission of *E. bieneusi* (Rinder et al., 1997; Liguory et al., 2001). The method of Rinder based on sequencing of the 243 bp internal ITS region has highlighted a considerable genetic diversity within *E. bieneusi* isolates with more than 50 distinct genotypes reported to date in humans and animals. Some of these genotypes are host-specific and other have zoonotic potential (Thellier & Breton, 2008; Santin et al., 2008). In Tunisia, the prevalence of *E. bieneusi* infection in patients with HIV was determined at 5.9 % (Chabchoub et al., 2009), but genotypic data are not available. The purpose of this study was to identify genotypes of *E. bieneusi* strains isolated from stool samples of HIV-infected Tunisian patients using sequence analysis of the ITS region located in rRNA genes.

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**MATERIALS AND METHODS**

**STOOL SAMPLES AND CLINICAL INFORMATION**

Seven *E. bieneusi* positive stool specimens from HIV-infected patients were collected during the period of 2005-2008 at the infectious diseases department of Rabta hospital, Tunis. The patients...
mean age was 28.9 years and the sex ratio was 0.4 (male/female). Five patients had CD4 cell counts below 50 cell/mm$^3$ and four patients presented with diarrhea (Table I).

SCREENING FOR MICROSPORIDIAN SPORES

All stool samples were analyzed by both light microscopy (Weber’s modified trichrome stain) and PCR using universal primers V1/PMP2 as previously described (Chabchoub et al., 2009; Fedoroko et al., 1995). PCR using species-specific primers V1/EB450 as previously described allowed the identification of E. bieneusi (Coyle et al., 1996).

DNA EXTRACTION

The DNA was extracted from frozen samples using the QIAamp DNA Tissue Kit (Qiagen Inc, Germany). Briefly, 200 μL of stool suspension were washed three times in phosphate-buffred-saline (PBS) solution by centrifugation at 12,000 g for five minutes. The pellet was then resuspended in 50 μL of cetyltrimethylammonium bromide at 2 % (CTAB) and 180 μl of tissue lysis buffer and incubated with proteinase K during two hours at 55 °C. The manufacturer’s recommendations were followed for DNA purification and elution. Extracted products were stored at -20 °C.

PCR AND SEQUENCING OF THE ITS REGION

PCR reactions were done using MSP3A (5'-GGAATT-CACACCGCCGTGMYTAT-3') and MSP4B (5'-CCAA-GCTTATGCTTAAGTCCAGGGAG-3') primers, which amplify a 508 bp fragment containing 122 bp of the 3:end of the small-subunit rRNA gene, 243 bp of the ITS, and 143 pb of the 5' region of the large-subunit rRNA gene (Rinder et al., 1997). PCR amplification was performed in 50 μL reaction mixtures containing 1X PCR buffer, 2.5 mM of MgCl$^2$, 25 pmol of each primer, 200 μM of each dNTP, 2 U of Goldstar Taq DNA polymerase (Applied biosystems, Roche, Switzerland), and 5 μL of DNA. PCR conditions were 94 °C for 2 min, followed by 40 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; with a final extension phase at 72 °C for 4 min. Amplified products were separated by electrophoresis on a 2 % agarose gel and visualized after staining by ethidium bromide. Negative (distilled water) and positive (DNA extracted from E. bieneusi positive sample) controls were systematically included.

PCR products were sequenced in both directions with a Big Dye Terminator Cycle sequencing kit on ABI PRISM 377 DNA sequencer (Applied Biosystem, USA). The genotype of E. bieneusi from each specimen was classified by comparing sequence to those available in the GenBank database (http://www.ncbi.nlm.nih.gov) and by alignments performed by Clustal X 1.83 for windows (Thompson et al., 1994).

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The GenBank accession numbers for 3 distinct obtained sequences Tn14, Tn106 and Tn42 are JF797332, JF797333 and JF797334, respectively.

RESULTS

PCR products of 508 bp in size were successfully amplified for the seven stool samples. Sequencing of these DNA fragments indicated that all the sequences corresponded to E. bieneusi. The analysis of ITS sequences of the seven isolates revealed three E. bieneusi genotypes previously described as B, D and Peru 8 (GenBank accession nos AF101198, AF101200, AY371283). Polymorphic sites were revealed by comparison to previously reported ITS sequences (genotypes B, D and Peru 8) as shown in Fig. 1. Four isolates (Tn44, Tn106, Tn110, Tn124) were identical to the published E. bieneusi ITS sequences from genotype D (also identified as genotype pigEBITS9, WL8, Peru9, coch112 and Falco5) isolated in humans and animals. Genotype B, also characterized as genotype I, was found in two isolates (Tn14, Tn15). The last sequence (Tn42) displayed identity to Peru 8 genotype.

| Patient | Sex | Age (years) | Habitat | Stool aspect | CD4 count (cells/μl) | ITS (Genotype) |
|---------|-----|-------------|---------|--------------|----------------------|---------------|
| Tn14    | F   | 39          | Urban   | Molded       | 23                   | B             |
| Tn15    | F   | 1 month     | Urban   | Molded       | 991                  | B             |
| Tn42    | M   | 40          | Urban   | Diarrheal    | 22                   | Peru 8        |
| Tn44    | M   | 32          | Urban   | Molded       | 44                   | D             |
| Tn106   | F   | 29          | Urban   | Diarrheal    | 17                   | D             |
| Tn110   | F   | 24          | Rural   | Diarrheal    | NA                   | D             |
| Tn124   | F   | 34          | Rural   | Diarrheal    | 4                    | D             |

F: female, M: male, NA: not available.

Table I. – Clinical and epidemiological data of patients with Enterocytozoon bieneusi infection.
Fig. 1. – DNA sequence alignment of the rRNA genes sequences of *E. bieneusi*.

Our sequences (Tn44, Tn106, Tn110, Tn124, Tn14, Tn15 and Tn42) are compared with sequences available in Genbank Genotypes B (accession no. AF101198), D (accession no. AF101200) and Peru 8 (accession no. AY371283). Dots denote sequence identity. The 243 bp of ITS region is underlined. The beginning of the sequence corresponds to the last 106 bp of small-subunit rRNA gene and the end corresponds to the first 135 bp of large-subunit rRNA gene.
The ITS flanking regions (122 bp of the small-subunit rRNA and 143 bp of the large-subunit rRNA) of the seven sequences showed 100 % identity to the corresponding regions of E. bieneusi rRNA sequences from genotype B, D and Peru 8 (Fig. 1). Genotype Peru 8 is shorter (395 bp) than the others because Sulaiman et al. used different primers (Sulaiman et al., 2003). Nucleotide A (position 404) located out 243 bp of the ITS sequence is likely an error in the GenBank sequence of Peru 8 (Sulaiman et al., 2003).

The two genotype B strains were isolated from patients with molded stool living in urban area. Genotype D strains were detected in patients with both urban and rural origin and presented both molded and diarrheic aspects of stool. Genotype Peru 8 was isolated from a diarrheic patient living in an urban area (Table I).

**DISCUSSION**

The genotyping of E. bieneusi isolates is a valuable tool for epidemiological investigation (Breton et al., 2007). The present study demonstrates that ITS sequence analysis is applicable to typing and classification of E. bieneusi strains isolated from HIV Tunisian patients stools. Despite its high cost and technical complexity, ITS sequencing is the most commonly used method for epidemiological studies (Breton et al., 2007; Touzeau et al., 2000). It has a high discrimination power and permits the identification of multiple and new genotypes (Espern et al., 2007; Leelayoova et al., 2006). In fact, ITS sequencing allows the detection of very subtle differences (one base pair) between sequences. To date, at least 34 different genotypes of E. bieneusi have been reported in humans (Thellier & Breton, 2008). Several studies indicate that the genotypes of E. bieneusi probably differ according to their geographical distributions (Breton et al., 2007; Touzeau et al., 2000; Espern et al., 2007). Besides, it has been recently proposed that predominant genotypes could be related in different sites to distinct transmission sources (Breton et al., 2007). The current study reports for the first time identification of E. bieneusi genotypes in North Africa. The results showed three distinct genotypes in E. bieneusi strain isolated from HIV infected Tunisian patients with intestinal microsporidiosis.

All identified genotypes were previously described elsewhere. The most frequently observed genotype D (n = 4) has a big variety of hosts and a large geographic range. It was first demonstrated in human in Germany then in American, Asian and African countries (Rinder et al., 1997; Breton et al., 2007; Leelayoova et al., 2006; Sulaiman et al., 2003). It was also observed in a wide variety of isolates from domestic (cattle and cat) and wild animals (macaque, muskrat, beaver and fox) supporting a zoonotic route of transmission (Breton et al., 2007; Dengjel et al., 2001). Genotype B, observed in two patients, is considered host specific and was recovered exclusively from humans (Rinder et al., 1997; Liguory et al., 2001 Sulaiman et al., 2003; Breitenmoser et al., 1999). It is the most common genotype reported in HIV-positive patients in Europe and has been recently isolated from three HIV positive patients in Cameroon (Breton et al., 2007). Genotype B is also the dominant strain in France, Germany, Switzerland and United Kingdom, making up 50 % to 85 % of the isolates (Rinder et al., 1997; Liguory et al., 2001; Breitenmoser et al., 1999; Sadler et al., 2002). The last identified genotype Peru 8 was described only in HIV patients in Peru, where genotype D has been also reported but not genotype B (Sulaiman et al., 2003). To our knowledge, this is the second report of Peru 8 genotype in the world.

According to such results, different transmission modes of E. bieneusi could exist in Tunisia. The predominance of genotype D in our patients (57.1 %) suggests a frequent transmission from animals to humans after exposure to animals or to contaminated surface water. On the other hand, the presence of anthroponotic genotypes B and Peru 8 underlines also the person to person transmission, facilitated probably by enteric carriage of E. bieneusi spores as supported by previous studies (Bretagne et al., 1993; Turnwine et al., 2005). Further studies including larger numbers of human and animal isolates are needed to confirm both hypotheses.

**ACKNOWLEDGEMENTS**

Financial support: this study was supported by the Ministry of Higher Education, Research and Technology, Tunisia in the setting of the Research Lab “Parasites emergentes” LR 05-SP-03.

The authors thank Pr Taoufik Ben Chaabane and all the physicians in charge of the patients for their collaboration in the collection of stools and clinical data. They also thank Dr Imen Ben Abda for her precious help.

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