ABSTRACT

Background: Diagnosis of microsporidiosis is difficult due to its small size needing special stains and identification by an expert. Nowadays, application of polymerase chain reaction (PCR) made diagnosis more sensitive, specific and easier.

Objectives: To update the prevalence of intestinal microsporidia and to clarify their genotype patterns in symptomatic and asymptomatic immune compromised and immune competent cases.

Patients and Methods: Totally, 323 stool samples were collected and subdivided as 173 from immune compromised (group I) and 150 from immune competent (group II) individuals. Samples were examined for microsporidiosis by light microscopy smears stained by Weber’s modified trichrome (WMT) and modified Ziehl Neelsen (MZN), as well as by nested and RFLP PCR techniques.

Results: Microsporidial spores were microscopically detected in 45/323 (13.9%) individuals; 25/173 (14.5%) immune compromised and 20/150 (13.3%) immune competent cases. In the two groups, 25/45 (55.6%) were symptomatic complaining of diarrhea, abdominal pain, distension, mal-digestion and weight loss, with statistical significant difference (P<0.001) between infection and presence of symptoms. Nested and RFLP PCR missed only one positive case, thus scoring 97.8% sensitivity and 100% specificity, positive predictive value 100%, negative predictive value 99.6% and diagnostic accuracy 97.8%. In group I, 5 cases were associated with Enterocytozoon (Ent.) bieneusi, 5 with Encephalitozoon (Enc.) species and 15 had mixed infection. In group II, 6 had Ent. bieneusi, 3 had Enc. species and 10 had mixed infection. Sequencing of the internal transcribed spacer of rDNA of five samples demonstrated the Ent. bieneusi anthroponotic genotype B and the zoonotic potential for genotypes D and K, in addition to one Enc. intestinalis sequence.

Conclusion: Prevalence of microsporidiosis was insignificantly higher in immune compromised than immune competent population. Intestinal microsporidiosis can be manifested by different abdominal symptoms or it can be asymptomatic. PCR technique highlighted that mixed infection with Ent. bieneusi and Encephalitozoon species was the commonest finding among the studied groups. Ent. bieneusi genotypes appeared to be related to animal contact and human infection. This, however, could not be accurately defined due to the limited number of available sequences.

Keywords: Enc. intestinalis, Ent. bieneusi, genotypes, immune competent, immune compromised, microsporidia.

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INTRODUCTION

Microsporidia are small, spore forming, obligate intracellular protozoa detected in many hosts. Up till now, 1300 microsporidian species or more, related to 150 genera, were detected[1]. Ent. bieneusi together with three species of genus Encephalitozoon, Enc. hellem, Enc. intestinalis, and Enc. cuniculi, were the main species discovered in HIV-infected patients[2], renal transplants recipients[3] and cancer patients[4]. The diagnosis is often missed as the spores of microsporidia are variably small in size ranging from 1-4 μ. Earlier, transmission electron microscopy was considered the gold standard for diagnosis of intestinal microsporidiosis; however, it was found to be time consuming, expensive and not available in most settings[5]. Presently, laboratory diagnosis depends upon direct visualization of spores in fecal samples by light microscopy using WMT staining and Fluorescent microscopy using Calcoflour White staining technique, but both techniques cannot differentiate between the species[6]. Polymerase chain reaction is being widely used for species and genotype detection of microsporidia in immune compromised and immune competent individuals[6,7]. Observed variation among microsporidia isolates as a result of different internal transcribed spacer (ITS) gene region of rRNA, help in the discrimination between host-adapted and zoonotic genotypes, in addition to geographical distribution of these genotypes[9]. Species differentiation has important therapeutic implications, as Enc. intestinalis and Ent. bieneusi respond variably to the currently used drug albendazole[10]. Although albendazole is highly active against all of the Encephalitozoonidae in vitro[10], it is not very effective against Ent. bieneusi[11].
In the present study, we aimed to investigate the occurrence of intestinal microsporidiosis in immune compromised and immune competent individuals, and determine the most prevalent and pathogenic genotypes in Egypt. Prevalence was determined by examining stool smears stained by WMT and MZN stains. Genotypes in recorded cases were identified by nested and RFLP PCR techniques.

**PATIENTS AND METHODS**

**Sample collection:** In a case control study, a total of 323 stool samples were examined for intestinal microsporidiosis in the period from June 2017 to May 2018. One hundred and seventy three samples were from immune compromised patients (exposed to chemotherapy and/or radiotherapy, receiving corticosteroids due to organ transplant or autoimmune diseases, malnutrition in children and from elderly patients). They were chosen from patients admitted to the Chemotherapy, Radiotherapy, Hematology, Nephrology, Geriatric and Pediatric Departments, Faculty of Medicine, Ain Shams University. In addition, 150 stool samples were collected and examined from immune competent individuals attending Parasitology Research Unit, Medical Parasitology Department, Faculty of Medicine, Ain Shams University. A full history sheet was completed for each subject including age, sex, diagnosis, disease stage, treatments received as well as contact with animals. The immune compromised patients were selected from isolation sectors in the mentioned departments according to their estimated low CD4+ counts and total leukocyte count of less than 4000/m3, in their investigation file.

**Sample processing:** Three consecutive stool samples were collected from each patient and control in properly labeled screw capped plastic containers and taken directly to the laboratory of Medical Parasitology Department, Faculty of Medicine, Ain shams University. Air dried stool smears were fixed with methanol, stained with WMT[12] and MZN[13] stains, and examined under light microscope (x1000). Only stool samples positive for microsporidia were aliquoted in sterile 2 ml eppendorf tubes and stored in -20°C for DNA extraction.

**Molecular studies:** DNA extraction from stool samples was performed using QIAamp-DNA stool minikit (Qiagen, Valencia, CA, USA) according to Sokolova et al.[14]. DNA was eluted and kept at -20°C. PCR amplification of the internal transcribed spacer (ITS) and flanking regions of the small subunit (SSU) and large subunit (LSU) of the ribosomal DNA (rDNA) were the targets for the nested-PCR procedure[14]. The following table shows the used primers.

| Primers     | Sequence                                      | Identification                  |
|-------------|-----------------------------------------------|---------------------------------|
| Upstream    |                                               |                                 |
| MS-1        | TGA ATG [G/T]GCC TGT                         | Microsporidium spp.             |
| MS-3        | GGA ATT CAC ACC GCC GGT [A/G][C/T] TAT       |                                 |
| Downstream  |                                               |                                 |
| MS-2B       | GTT CAT TCG CAC TAC T                        | Ent. bieneusi                   |
| MS-4B       | CCA AGC TTA TGC TTA AGT CCA GGG AG           |                                 |
| MS-2A       | TCA CTC GCC GCT ACT                          | Encephalitozoon spp.           |
| MS-4A       | CCA AGC TTA TGC TTA AGT [C/T][A/C]A [A/G]G GGT |                                 |

The first PCR mix included 1 µl each of primers MS-1, MS-2A, and MS-2B with 3 µl of the template DNA. The second nested reaction contained a mixture of 1 µl each of MS-3, MS-4A, and MS-4B and 1 µl of the first amplification reaction mixture. On 2% gel electrophoresis the assumed amplicon size for the MS-3 and MS-4B (Ent. bieneusi) product was 500 bp, and nearly 300 bp for the MS-3 and MS-4A (Enc. and other species). Amplifications were done for primary and nested reactions with a Thermo Scientific, Biometra thermocycler, which included at first a denaturation step for 5 min at 95°C and 36 cycles of denaturation for 30 sec at 95°C, primer annealing at 55°C for 1 min, and elongation at 72°C for 2 min. The final elongation step reached to 10 min hold cycle at 4°C. This contained the positive control DNA extract of Ent. bieneusi and Enc. intestinalis both provided by Dr. Spencer Polley from England and by Dr. Karim Aoun from Tunisia.

RFLP-PCR protocol performed according to Katzwinl-Wladarsch et al.[15] for all positive nested PCR samples used Fast Digest MnlI restriction enzyme (Fermentas life sciences #FD1074). The PCR micro tubes were incubated for 5 min in a heat block at 37°C. This was followed by heating the enzyme for 5 min at 65°C for inactivation. Finally, RFLP-PCR products were observed by agarose gel electrophoresis.

For DNA sequencing, PCR products of the second nested PCR reaction mixture were refined using
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QiAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and the eluted DNA was stored at -20°C. Nucleotide sequencing was done using BigDye Terminator v3.1 Cycle Sequencing Kit (Cat. NO.: 4337455, USA). The BigDye X Terminator TM purification kit (Cat. NO.: 4376486, USA) was used for purification of labeled extension products from excess dye terminators. Finally, establishment of DNA sequence was done by DNA analyzer (Applied Biasystems, California 94404, USA). The primers for sequencing were MSP-3, MSP-4A, and MSP-4B and each amplicon was sequenced in the two directions.

Statistical analysis: Data analyzing was performed by Chi-square test; a P value <0.05 was considered statistically significant. For comparative evaluation of the three chosen techniques; staining, nested PCR and RFLP PCR, presence of microsporidia by two or more techniques was considered as true positive. Absence of microsporidia by all the three techniques was considered true negative. Sensitivity, specificity, positive predictive value, negative predictive values as well as diagnostic accuracy were calculated as per standard formulae.

Ethical consideration: The study protocol was approved in August 2016 by the Institutional Ethics Committee of Faculty of Medicine, Ain Shams University, Egypt, together with other ethical statements for human consents and treatment of infected patients.

RESULTS

By microscopic examination of WMT and MZN stained smears, microsporidial spores appeared as pink or reddish oval bodies against a light green background by both stains. Spores ranged from 0.97–1.34 µm in width, and 2.19–2.56 µm in length (Fig. 1).

The age range for the selected 323 subjects was between 2-68 years with mean age of 37.7±15.1; 149 (46.1%) were males and 174 (53.9%) were females. There was no statistical significance between microsporidiosis and age and sex (P=0.12).

The recorded general prevalence of microsporidiosis as determined by examination of stained smears, was 45/323 (13.9%); where 25/173 (14.5%) were immune compromised, and 20/150 (13.3%) were immune competent. Out of the 45 positive cases in the two groups the percentage was 25/45 (55.6%) in immune compromised, and 20/45 (44.4%) in immune competent groups, with no statistical difference between the two groups (Table 1). Positivity in subgroups I and II recorded 15/173 (8.7%) in symptomatic group Ia, 10/173 (5.8%) in asymptomatic group Ib. In symptomatic and asymptomatic groups Ia and Ib, positivity was 10/150 (6.7%) for both. Of the total 45 positive cases, 15/52 (60%) of group I, and 10/20 (50%) of group II were symptomatic with no statistical significance (Table 1). Complaints of symptomatic cases in the 45 positive cases were shared in some cases and included diarrhea 23/45 (51.1%), abdominal pain 15/45 (33.3%), distension 14/45 (31.1%), malnutrition 10/45 (22.2%) and weight loss 10/45 (22.2%). By exclusion, 20 cases (44.4%) were asymptomatic with statistically significance difference (P<0.001) (Table 1). History of animal contact was noted in 9/45 (20%) of positive cases in groups I and II; 1/25 (4%) immune compromised (insignificant, P=0.16) and 8/20 (40%) immune competent positive cases (significant, P<0.01) (Table 1).

Etiological distribution in the 25 positive immune compromised patients in group I showed that 3 (12%) had autoimmune diseases, 4 (16%) had renal failure, 2 (8%) had leukemia, 5 (20%) were subjected to chemotherapy and 4 (16%) to radiotherapy, 4 (16%) were elderly, 3 (12%) had lymphoma, and 2 (2%) had undergone renal transplantation. Some patients had more than one of these causes for their compromised immune state (blended etiology). As shown from table (2), the relation between microsporidiosis and the immune status of the individuals or etiology of immune compromised condition was insignificant (P>0.05).

For the molecular assays, nested-PCR confirmed microsporidiosis in all of the 25 (100%) microscopically positive samples of group I. Out of the total 45 microscopically positive patients in the

Fig. 1: Microsporidial spores stained by (A): modified trichrome stain, x1000 magnification, (B): modified Ziehl-Neelsen stain, x1000 magnification.
two groups 44/323 (13.6%) were confirmed positive using PCR (Table 3). Ent. bieneusi reacted at 500 bp and Enc. intestinalis at 300 bp molecular weight (MW), as compared to reference strains from England and Tunisia (positive control) (Fig. 2). In group I patients, Ent. bieneusi, Encephalitozoon spp. and mixed infection with both were identified in 5 (20%), 5 (20%) and 15 (60%) samples respectively. In group II, 19/20 (95%) microscopically positive samples included 6 (31.6%) infected with Ent. bieneusi only, 3 (15.8%) infected with Encephalitozoon species only and 10 (52.6%) individuals had mixed infection with both. There was no statistical significance for presence of certain species among the immune compromised group I patients and the immune competent group II individuals (Table 4). In RFLP PCR procedure, MnlI restriction enzyme cut the Ent. bieneusi DNA into bands with different MWs of 20, 30, 50, 60, 80, 90 and 180 bp. The Enc. intestinalis DNA was cut into bands with MWs of 20, 30, 60 and

| Table 1: Relations between microsporidiosis and different variables in the studied groups. |
|-------------------------------|---------------------------------|----------------|-----------------|
| Variable                      | Microsporidiosis                | Statistical analysis |
|                               | Negative (278) No. (%) | Positive (45) No. (%) | X² Square test | P value* |
| Groups                        |                                |                  |                |
| Group I (No.=173)             | 148 (53.2)                     | 25 (55.6)        | 0.08           | 0.77 (NS) |
| Group II (No.=150)            | 130 (46.8)                     | 20 (44.4)        |                |           |
| Symptomatic/asymptomatic      |                                |                  |                |
| Group I (No.=173)             | 141 (95.3)                     | 10 (40)          | 58.86          | <0.01 (S) |
| Group II (No.=150)            | 7 (4.7)                        | 15 (60)          |                |           |
| Absent                        | 125 (96.2)                     | 5 (3.8)          | 41.3           | <0.01 (S) |
| Present                       |                                |                  |                |           |
| Symptoms*                     |                                |                  |                |
| Absent                        | 12 (4.3)                       | 25 (55.6)        | 100.25         | <0.01 (S) |
| Present                       | 6 (22)                         | 23 (51.1)        | 113.57         | <0.01 (S) |
| Absent                        | 6 (2.5)                        | 15 (33.3)        | 57.94          | <0.01 (S) |
| Present                       | 5 (1.8)                        | 14 (31.1)        | 60.11          | <0.01 (S) |
| Absent                        | 3 (1.1)                        | 10 (22.2)        | 44.82          | <0.01 (S) |
| Present                       | 3 (1.1)                        | 10 (22.2)        | 44.82          | <0.01 (S) |
| Contact with animals          |                                |                  |                |
| Group I (No.=173)             | 127 (85.8)                     | 24 (96)          | 2.00           | 0.16 (NS) |
| Group II (No.=150)            | 21 (14.2)                      | 1 (4)            |                |           |
| Absent                        | 116 (89.2)                     | 12 (60)          | 11.8           | <0.01 (S) |
| Present                       | 14 (10.8)                      | 8 (40)           |                |           |

*Symptoms overlapped in some cases, NS: non-significant, S: significant.

| Table 2: Relation between microsporidiosis and etiology among immune compromised patients in group I. |
|--------------------------------|---------------------------------|----------------|----------------|
| Etiology*                     | Microsporidiosis                | Statistical analysis |
|                               | Negative (148) No. (%) | Positive (25) No. (%) | P value* |
| Autoimmune disease (45)       | 42 (28.4)                     | 3 (12.0)         | 0.08 (NS) |
| Renal failure (33)            | 29 (19.6)                     | 4 (16.0)         | 0.67 (NS) |
| Leukemia (32)                 | 30 (20.3)                     | 2 (8.0)          | 0.14 (NS) |
| Chemotherapy (30)             | 25 (16.9)                     | 5 (20.0)         | 0.70 (NS) |
| Radiotherapy (23)             | 19 (12.8)                     | 4 (16)           | 0.67 (NS) |
| Elderly (19)                  | 15 (10.1)                     | 4 (16.0)         | 0.39 (NS) |
| Lymphoma (15)                 | 12 (8.1)                      | 3 (12.0)         | 0.52 (NS) |
| Renal transplant recipient (6) | 4 (2.7)                       | 2 (8.0)          | 0.18 (NS) |

* Some patients had more than one etiology for their immune compromised state. NS: statistically non-significant.

| Table 3: Sensitivity, specificity, PPV, NPV and diagnostic accuracy of nested and RFLP PCR in diagnosis of microsporidiosis. |
|--------------------------------|---------------------------------|----------------|----------------|
| PCR                           | MTC                             | Statistical analysis |
| Positive                      | Negative Total                  | P value* |
| Positive                      | 44                              | 0 | 44 | 1.00 |
| Negative                      | 1                               | 278 | 279  |
| Total                         | 45                              | 278 | 323  |

Sensitivity = 97.8%  
Specificity = 100%  
PPV = 100%  
NPV = 99.6%  
Diagnostic accuracy = 97.8%
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To obtain the different genotypes and their related importance in pathogenicity and symptom production. Accordingly, Ent. bieneusi genotype D was detected in a renal transplant recipient patient in group Ia with 100% identity with the published blast sequence of Ent. bieneusi genotype D under accession number (AF101200) (Fig. 4A). Ent. bieneusi genotype K was detected in two patients; one in group Ib, and the other in group IIb. Blast alignment gave 98% and 100% identity with the published genotype K blast sequence under accession number (AF267141) respectively (Fig. 4B, 4C). Ent. bieneusi sequence for genotype B was detected in group Ia with no history of animal contact, suffering from diarrhea, abdominal pain, distension, mal digestion and weight loss, with 100% identity to the published Ent. bieneusi genotype B blast sequence under accession number (AF101198) (Fig. 4D). A sequence of Enc. intestinalis belonging to a 26 years old symptomatic patient on corticosteroid therapy due to autoimmune disease, with no history of animal contact, suffering from abdominal pain and distension, gave 99% identity with the published blast sequences under accession number (AB897500) (Fig. 4E).

Table 4: Percentages of infection with Ent. bieneusi alone, Encephalitozoon species alone and mixed infection with both species by nested PCR in studied groups

|                     | Group I (No.=25) | Group II (No.=20) | Statistical analysis |
|---------------------|------------------|-------------------|---------------------|
|                     | Immunocompromised| Immunocompetent    | Test value | P value |
| Ent. bieneusi only  | 5 (20)           | 6 (31.6)          | 0.77**     | 0.38 (NS) |
| Encephalitozoon species only | 5 (20) | 3 (15.8) | 0.13** | 1.0 (NS) |
| Mixed infection     | 15 (60)          | 10 (52.6)         | 0.24*      | 0.63 (NS) |

**Chi square test, ***Fisher exact test. P value > 0.05 is statistically non-significant (NS).

Fig. 2. Nested PCR showed Ent. bieneusi bands at 500 bp and Enc. intestinalis bands at 300 bp molecular weight in comparison to reference strains from England and Tunisia (Positive control).

Lane M: DNA molecular marker (100 - 1000 bp). Lane 1: Positive control for Ent. bieneusi. Lane 2: Positive control for Enc. intestinalis. (Positive control in lanes 1 and 2 were provided from England). Lane 3: Positive control for Ent. bieneusi. Lane 4: Positive control for Enc. intestinalis. (Positive control in lanes 3 and 4 were provided from Tunisia). Group Ia: lanes 8, 10, 14: Ent. bieneusi; lanes 7, 12, 18, 19: Enc. intestinalis. lanes 5, 6, 9, 11, 13, 15, 16, 17: mixed infection (Ent. bieneusi and Enc. intestinalis). Group IIa: lanes 9, 15: Ent. bieneusi; lanes 7, 13: Enc. intestinalis. lanes 6, 8, 10, 11, 12, 14: mixed infection. Group Ib: lane 6: Ent. bieneusi; lane 9: Enc. intestinalis. lanes 7, 8, 10, 11, 12, 13, 14, 15: mixed infection. Group IIb: lanes 6,9,10,14: Ent. bieneusi; lane 15: Enc. intestinalis. lanes 7,8,11,12: mixed infection.

160 bp. The Enc. cuniculi DNA was cut into bands with MWs of 90 and 210 bp (Fig. 3). RFLP-PCR confirmed all of the nested PCR 44/45 (97.8%) positive samples in the two groups. The sensitivity and specificity of both nested and RFLP PCR were 97.8% and 100%, respectively, with positive predictive value (PPV) 100%, negative predictive value (NPV) 99.6%, and diagnostic accuracy 97.8% (Table 4).

Relation between animal contact and different species of microsporidia detected by RFLP PCR showed statistically significant (P=0.03) result of 36.4% only with Ent. bieneusi infection. A significant difference was observed between presence of symptoms and different species of microsporidia except for Enc. cuniculi spp. detected by RFLP PCR (Table 5).

Five nested PCR products (MSP-1,-3,-4, and MSP-2B, MSP-4B) with single species infection were purified and subjected to sequencing and compared to the sequences published on the Blast website to obtain the different genotypes and their related importance in pathogenicity and symptom production. Accordingly, Ent. bieneusi genotype D was detected in a renal transplant recipient patient in group Ia with 100% identity with the published blast sequence of Ent. bieneusi genotype D under accession number (AF101200) (Fig. 4A). Ent. bieneusi genotype K was detected in two patients; one in group Ib, and the other in group IIb. Blast alignment gave 98% and 100% identity with the published genotype K blast sequence under accession number (AF267141) respectively (Fig. 4B, 4C). Ent. bieneusi sequence for genotype B was detected in group Ia with nor history of animal contact, suffering from diarrhea, abdominal pain, distension, mal digestion and weight loss, with 100% identity to the published Ent. bieneusi genotype B blast sequence under accession number (AF101198) (Fig. 4D). A sequence of Enc. intestinalis belonging to a 26 years old symptomatic patient on corticosteroid therapy due to autoimmune disease, with no history of animal contact, suffering from abdominal pain and distension, gave 99% identity with the published blast sequences under accession number (AB897500) (Fig. 4E).
Fig. 3. RFLP PCR showed *Ent. bieneusi* bands at 20, 30, 50, 60, 80, 90, 180 bp, *Enc. intestinalis* bands at 20, 30, 60, 160 bp, and *Enc. caniculi* at 90, 210 bp molecular weight, in comparison to positive controls. *Lane M*: DNA molecular marker (50-1000 bp). *Lane 1*: Positive control for *Ent. bieneusi*. *Lane 2*: Positive control for *Enc. intestinalis* (Positive control in lanes 1 and 2 were provided from England). *Lane 3*: Positive control for *Ent. bieneusi*. *Lane 4*: Positive control for *Enc. intestinalis*. (Positive control in lanes 3 and 4 were provided from Tunisia). *Group Ia*: lanes 8, 10, 14: *Ent. bieneusi*; lanes 7, 12, 18, 19: *Enc. intestinalis*; lanes 5, 6, 9, 11, 13, 15, 16, 17: mixed infection (*Ent. bieneusi* and *Enc. intestinalis*). *Group IIa*: lanes 9, 15: *Ent. bieneusi*; lanes 7, 13: *Enc. intestinalis*; lanes 6, 8, 10, 11, 12, 14: mixed infection. *Group Ib*: lane 6: *Ent. bieneusi*; lane 9: *Enc. intestinalis*; lanes 7, 8, 10, 11, 12, 13, 14, 15: mixed infection. *Group Ilb*: lanes 6, 9, 10, 14: *Ent. bieneusi*; lane 15: *Enc. intestinalis*; lanes 7, 8, 11, 12: mixed infection.

Fig. 4. DNA sequencing. A) Alignment on blast of *Ent. bieneusi* genotype D with 100% identity. B) Alignment on blast of *Ent. bieneusi* genotype K with 98% identity. C) Alignment on blast of *Ent. bieneusi* genotype K with 100% identity. D) Alignment on blast of *Ent. bieneusi* genotype B with 100% identity. E) Alignment on blast of *Enc. intestinalis* with 99% identity.
The present work showed no statistical significant difference of microsporidiosis between immune compromised (55.6%) and immune competent (44.4%) positive patients (P = 0.77). In agreement, microsporidial parasites were found in both immune competent and immune compromised diarrheal subjects in Tunisia[15], while a higher prevalence was previously reported among immune compromised children with malignant diseases (25.56%) than in the healthy control group (3.33%).[28]. Concerning the different etiologies predisposing for microsporidiosis in immune compromised positive cases, there was no
statistical significant difference between the infection and the underlying etiology for compromised immunity. Also, Chabchoub et al.\textsuperscript{10} reported microsporidial infection in 11.8% and in 8.6% of HIV-infected patients and patients with myeloma, respectively.

In the present study diarrhea appeared to be the main complaint in positive symptomatic patients. Out of the 25 immune compromised positive cases in group I, 15 (60%) were symptomatic with complaints of: diarrhea (51.1%), abdominal pain (33.3%), abdominal distension (31.1%), maldigestion and weight loss (22.2%); where all symptoms were statistically significant ($P < 0.01$). Ojuromi et al.\textsuperscript{24} reported a clear association of 95% between microsporidia and diarrhea. Moreover, El-Sobky and El-Nahas\textsuperscript{20} reported microsporidia in a diarrheic group (23%) more than in a non-diarrheic group (5.7%). Concerning the other symptoms higher positive results of 80% were reported by Aikawa et al.\textsuperscript{29} among rheumatic patients who complained of diarrhea (29%), abdominal pain (32%) and weight loss (6%). Out of the 20 immune competent individuals in our study, 50% were symptomatic with various complaints, and the other 50% had no apparent gastrointestinal complaints. It is noteworthy that microsporidial spores were often identified in formed stool samples (10.9%)\textsuperscript{26}. Using PCR a higher incidence was previously reported where diarrhea was recorded in 92.3% of HIV microsporidia positive patients together with weight loss in 94.5%\textsuperscript{130}. Statistical significant lower incidences of diarrhea were also reported in HIV-positive (21.6%) and HIV-negative patients (9%)\textsuperscript{312}. Other authors stated that although diarrhea and malabsorption seemed to be the most common clinical problems associated with microsporidial infections, namely, \textit{Ent. bieneusi}, there were no statistically significant associations between the presence of microsporidial spores in fecal specimens and patients with diarrhea\textsuperscript{130}.

In the current work, there were no statistically significant differences for occurrence of \textit{Ent. bieneusi}, \textit{Encephalitozoon} species and mixed infection between patients of group I and II with $P$ values 0.38, 1 and 0.63, respectively. Similarly, it was reported that \textit{Ent. bieneusi}, \textit{Enc. intestinalis}, and both species were insignificantly identified in immune compromised cases including malignancy, patients receiving cytotoxic drug therapy or not, patients under corticosteroid therapy for more than 6 months, end stage renal failure on dialysis, splenectomized patients and diabetics\textsuperscript{130}.

The prevalence of \textit{Ent. bieneusi}, \textit{Enc. intestinalis} and mixed infection by both species in the 25 immune compromised group I patients were 20%, 20% and 60%, respectively. A study done on HIV-infected patients showed a higher prevalence of \textit{Enc. intestinalis} 12.8%, than that of \textit{Ent. bieneusi} 1.2%\textsuperscript{14}. Moreover, reported prevalence of \textit{Ent. bieneusi}, \textit{Vittaforma}-like species and co-infection with both species was 7%, 8.2% and 1.2% respectively, in fecal specimens of HIV positive patients\textsuperscript{211}.

Our study did not include any HIV patients, simply because they were not available. We recorded a statistically insignificant ($P > 0.05$) association of different microsporidia species with specific disease or state of compromised immunity, except for infection with \textit{Enc. intestinalis} which showed statistical significance ($P < 0.01$) in association with renal transplant recipient patients. On the other hand there was no significant difference for presence of certain microsporidial species in leukemic patients. A previous study stated that the percentage of microsporidiosis using PCR among leukemic patients (28/100) was higher than that in other groups of cancer patients, where \textit{Enc. intestinalis} was the only species detected in positive stool samples\textsuperscript{130}. Regarding \textit{Ent. bieneusi} genotype B, it was detected in the present study with 100% identity with that found in blast sequence in a symptomatic immune competent patient suffering from diarrhea, abdominal pain, distension, maldigestion and weight loss, and with no history of animal contact. Genotype B is considered as an anthropogenic genotype specially found in HIV positive patients\textsuperscript{314-316} and as the dominant anthropogenic genotype in developed countries\textsuperscript{223}. On the contrary, another study reported genotype Bin both HIV-seropositive and HIV-seronegative populations\textsuperscript{311}.

As regards infection with \textit{Ent. bieneusi} and animal contact we recorded a significant statistical difference ($P = 0.03$). Researchers stated that molecular analysis of the 243-bp internal transcribed spacer of the rRNA gene showed significant genetic variability among \textit{Ent. bieneusi} isolates, which supports the zoonotic transmission of some of its genotypes from animals\textsuperscript{317}. Others revealed that animals may represent a major source for spore transmission of opportunistic infection in humans\textsuperscript{310}.

Genotype D was detected in the present study in a symptomatic immune compromised case with history of animal contact. In Tunisia, four isolates were found identical to the published \textit{Ent. bieneusi} ITS sequences of genotype D\textsuperscript{375}. In China, genotype D was the most prevalent genotype discovered in 17 samples from symptomatic children, HIV-positive patients, and HIV-negative individuals. Furthermore, genotype D was observed in not less than 15 animal species, river water and wastewater\textsuperscript{369}. Our results agree with other studies that reported the zoonotic potential of genotype D\textsuperscript{314,316,411}, and that it is a non host specific genotype with a broad host and geographic range\textsuperscript{42,43}. Genotype K of \textit{Ent. bieneusi} was also recorded in our study in 2 cases; an immune compromised asymptomatic patient with negative animal contact and in an immune competent asymptomatic individual with positive animal contact. Similar results were reported where genotypes D and K were the most prevalent genotypes and indicating that
genotype K was significantly associated with diarrhea among HIV-positive patients\(^4\)\(^,\)\(^5\)\(^,\)\(^6\). Consequently the broad host and geographic range of genotype K was validated\(^6\).  

Regarding \textit{Enc. intestinalis}, the present study detected only one sequence with 99% identity to \textit{Enc. intestinalis} published blast sequences in a symptomatic immune compromised patient on corticosteroid therapy complaining of abdominal pain and distension with negative history for animal contact. This same species was reported in a patient with an unconfirmed HIV status who presented with diarrhea\(^4\). It was also reported in stool specimens from 2 patients (1 HIV patient, 1 patient with myeloma), where the two amplicons had 96% and 99% sequence identity with \textit{Enc. intestinalis} (Genbank accession no. EU436735)\(^4\).  

**Conclusion:** Prevalence of intestinal microsporidiosis was slightly higher in symptomatic immune compromised than immune competent populations, in whom diarrhea was the provoking symptom. Both staining and PCR techniques detected nearly the same result. As regards microsporidia species, a slightly higher prevalence of \textit{Ent. bieneusi} than of \textit{Enc. intestinalis} was observed among studied groups. No relation was observed between etiology of compromised immunity and microsporidiosis. History of animal contact was statistically significant among immune competent group suggesting the zoonotic source for microsporidiosis. As regards microsporidia species, a slightly higher prevalence of \textit{Ent. bieneusi} than of \textit{Enc. intestinalis} was observed among studied groups. The recent application of PCR-based molecular methods helped reveal the distribution of genotypes of \textit{Ent. bieneusi}. All genotyping studies performed until now were based on the analysis of the ITS sequences. Although some \textit{Ent. bieneusi} genotypes in the present study were found to be related to animal contact and human infection, this can’t be accurately defined due to the limited number of available sequences.  

**Limitations and recommendations:** Diagnosis of microsporidiosis requires more than one stool examination by an experienced parasitologist because of its small size and difficulty in recognition by microscopic examination. The identification of the risk factors as immune incompetence and animal contact associated with microsporidiosis, in addition to molecular diagnosis of microsporidiosis, will help in determining the distribution of \textit{Ent. bieneusi} genotypes by geographical location, to improve source tracking, and to calculate the host range and pathogenic potential of an isolate. To determine the zoonotic nature of microsporidiosis, further studies should be done especially for patients who are in direct contact with animals as veterinarians, pet owners, shepherds, as well as workers in poultry breeding and slaughter houses to determine what type of animal is responsible for microsporidiosis.  

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