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Diagnosis of disseminated microsporidiosis: Detection of circulating *Enterocytozoon bieneusi* DNA in blood of HIV/AIDS patients

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Abstract. Disseminated microsporidiosis among HIV/AIDS patients is life-threatening. The incidence may be actually higher than what has been reported. This is due to non-specific presentations of the disseminated cases and also the insensitivity of routine diagnostic technique which contribute to delay in the treatment of the disease. In the present study, we report the use of blood specimens to detect circulating microsporidia DNA, which has not been reported for diagnosis of disseminated microsporidiosis. Blood samples from HIV/AIDS-positive patients were collected over a period of one year. These samples were subjected to PCR assay using species-specific primer EBIEF1/EBIER1. Out of 100 patients, seven were confirmed positive for *E. bieneusi* by PCR. A fragment of 607 bp was successfully amplified. Identification of circulating microsporidia DNA in blood samples may aid in early diagnosis, thereby allows timely administration of anti-parasitic treatment.

INTRODUCTION

Microsporidia are obligate and opportunistic intracellular parasites known to infect both vertebrates and invertebrates. Out of five genera of human Microsporidia, only two species are associated with humans; *Enterocytozoon bieneusi* (*E. bieneusi*) and *Encephalitozoon intestinalis* (Al-Mekhlafi *et al*., 2011) These parasites cause localised and disseminated disease in immuno-compromised individuals, especially in AIDS patients (Ambrosioni *et al*., 2010). Usually, the clinical manifestations of microsporidia infection in AIDS patients include diarrhoea, nausea, vomiting, malabsorption, and loss of weight (Anuar *et al*., 2013).

There are many reports on disseminated microsporidia infection, which primarily caused by *Encephalitozoon* species. However, disseminated microsporidiosis was only diagnosed after post-mortem or autopsy in most of these cases (Carlson *et al*., 2004). Delay in the diagnosis is mainly due to lack of awareness on the potential of the infection to disseminate. In addition, an invasive procedure is needed to obtain biopsy sample from the patient in order to rule out any infection (Cowley *et al*., 1997). These factors contribute to the increase in mortality rate among the infected patients, who were untreated or received late treatment.

Despite a high frequency of disseminated microsporidiosis caused by *Encephalitozoon* species, *E. bieneusi* rarely disseminates. Normally this parasite is found in the upper gastrointestinal tract. However, there are a few reports of *E. bieneusi* detected in the respiratory system. For instance, a study by Georges *et al.* (1998) reported that *E.*

bieneusi infected various organs in a HIV-infected patient. The researchers found spores in the stools, duodenal biopsy, nasal discharge and sputum. The evidences shown by these studies indicated that *E. bieneusi* has the capability to disseminate to the extra-intestinal organs.

Microsporidiosis in HIV/AIDS patients is commonly present with non-bloody and watery diarrhoea. Routine diagnosis consists of microscopic detection of the spores in stool using Weber's or Ryan's modified trichrome stain or Gram-chromotrope Kinyoun stain (Fedorko *et al*., 1995, Salleh *et al*., 2011). However, in disseminated cases, patients may not present with diarrhoea (Scaglia *et al*., 1998) and this scenario may lead to misdiagnosis of the disease. In suspected disseminated microsporidiosis cases, body fluids or tissue specimens are examined for the presence of spores. Histology using biopsies samples for detection of *E. bieneusi* have several limitations including the observation that developing forms of the parasite do not stain well with standard hematoxylin and eosin stains. In addition, the routine diagnosis of microsporidiosis cannot determine the species of the parasite due to similar morphology and sizes. Transmission electron microscope is an alternative tool for species identification (Velasquez *et al*., 1996). However, the sensitivity and specificity of visualization method by light and electron microscopy are poorly described.

Polymerase chain reaction (PCR) is a sensitive and specific method for diagnosing intestinal microsporidia infections and has been performed by several investigators (Fedorko *et al*., 1995, Velasquez *et al*., 1996, da Silva *et al*., 1996, Katzwinkel-Wladarsch *et al*., 1996, Kock *et al*., 1997, Liguory *et al*., 1997, Ombrouck *et al*., 1997, Verweij *et al*., 2007). However, many of these studies used stool and biopsy samples, only. To the best of our knowledge, there is no single study on the application of PCR using clinical blood samples to diagnose disseminated microsporidiosis. In the present study, we investigate the usefulness of detecting circulating microsporidia DNA, particularly *E. bieneusi* in blood specimens from HIV/AIDS patients using PCR and its association with clinical symptoms.

METHODS

Blood specimen collection and control

Specimens were collected at Sungai Buloh Hospital and Universiti Kebangsaan Malaysia Medical Centre (UKMMC). One hundred blood samples from HIV patients were collected in EDTA tubes with the inclusion criteria of CD4 cell count must be below 200 cells/mm^3 and they were required to sign informed consent. Positive control for *E. bieneusi* was extracted from faecal samples that were confirmed positive for microsporidia by microscopy and PCR, followed by sequencing for species confirmation. Distilled water was used as a negative control.

DNA extraction

DNA extraction was carried out on the whole blood using QIAamp DNA Mini kit (Qiagen, USA). The procedure was executed in accordance with the manufacturer's instruction for DNA purification from blood or body fluids. The extracted DNA was used as a template for PCR.

PCR

The identification of *E. bieneusi* was verified using defined species-specific primer pair; EBIEF1 (5'-GAAACTTGTCCACTCCTTACG-3') and EBIER1 (5'-CAATGCACCACTCCT GCCATT-3'). These primers were based on nucleotides 295 to 315 and 881 to 901, respectively, of the *E. bieneusi* SSU-rRNA sequence. The PCR was conducted with HelixAmp *Taq* Polymerase (NanoHelix Co., Ltd, South Korea) and it was performed in a 25 µl volume with 8 µl template DNA extracted from blood, 10X *Taq* buffer, 10 mM of dNTP mix, 0.1 µM of each primer and 1.25 U *Taq* DNA Polymerase. The volume of template used was the same in each assay. Amplification was performed using Mastercycler Pro S (Eppendorf, Germany) with initial denaturation at 94°C for 5 min,

followed by 35 cycles at 94°C for 30 sec, 59°C for 30 sec, and 72° for 90 sec and a final extension at 72°C for 10 min. Amplified DNA was analyzed by electrophoresis in a 2% (w/v) agarose gel and visualized under image analyser. The PCR was repeated twice. Since the present study was the first of its kind to describe the application of PCR using blood specimens to diagnose disseminated microsporidiosis, the above assay was performed based on an earlier study with slight modifications. Annealing temperature was adjusted to 59°C rather than 55°C, which was meant for amplification of microsporidia DNA in stool specimens (da Silva *et al*., 1996). Modification of the previously reported amplification method was required as non-specific bands were obtained, and only resolved when the annealing temperature was increased. The stringency of the primers was also increased with the temperature increase, which leads to higher specificity of the target regions and reproducible amplification (Malhotra *et al*., 1998). According to Hartskeerl *et al*., 1993, there was a difference in base-pair lengths between *E. bieneusi* that infect the small intestine (1250 bp) and *E. bieneusi* infecting extra-intestinal (1294 bp) sites.

Procedures for avoiding contamination were strictly followed. DNA extraction, preparation of reaction mixtures, and amplification and analysis were physically separated and performed in three different rooms. Dual filtered tips were used for all manipulation, and negative controls containing reaction mixtures without DNA were used during all amplifications to avoid cross contamination.

Sequence analysis and phylogenetic analysis

For sequence analysis, all samples which were PCR positive for microsporidia were sent for sequencing together with positive controls for *E. bieneusi*. The sequencing was performed by a local company (MyTACG, Malaysia). Sequences obtained from the samples were then entered into the National Center for Biotechnology Information's World Wide Web site, and BLAST search was performed to identify homologous sequences. The accuracy of the sequence was confirmed by sequencing the same sample with two separate PCR products. Comparison was made between the ITS sequences obtained and the published records in Genbank by using BLAST analysis. Multiple alignment of the nucleotide sequences were performed using the CluxtalX 2 program. Phylogenetic analysis was carried out on the aligned sequences to examine the relationships among the *E. bieneusi* positive samples. In this analysis, the published *Enterocytozoon*-ITS nucleotide sequences were aligned with the ITS sequences obtained from the HIV patients with *E. bieneusi* infection. A maximum likelihood tree was constructed using the Molecular & Evolution Genetic Analysis software version 7 (Mega7) program based on the evolutionary distances calculated by the Jukes and Kantor model. A sequence of *Enterocytozoon* sp. from a dog (GenBank Accession No. AF059610) was used as the outgroup in the phylogenetic analysis.

Ethical consideration

Ethical approval was obtained from the Ethics Committee UKMMC (Reference Number: 06-01-02-SF0920). This research was approved by National Medical Research Register Committee (13-147-14600) and subjected to MREC's ethics review and approval. A standardised form was used to collect information, which included socio-demographic characteristics (gender, age, and race), and clinical symptoms information. Clinical data such as CD4 cell counts and other information were obtained from the medical records with prior permission from health authorities.

Statistical analysis

For descriptive analysis, rate (percentage) was used to describe the characteristics of the studied population, including the prevalence of *E. bieneusi*. A chi-square test (X^2) was used to test the association between the variables at a significant level of 5%. The relative proportions were calculated with confidence interval of 90%.

RESULTS

Out of 100 blood samples from the HIV/AIDS patients, *E. bieneusi* DNA was detected in 7 (7%) of the patients. PCR assay successfully amplified a 607-bp DNA fragment. Figure 1 shows positive DNA amplification of *E. bieneusi* from blood samples of 7 different HIV/AIDS patients. The sequences of amplified DNA fragments from the blood samples matched the sequence of GenBank database accession numbers, thus indicating the successful amplification of *E. bieneusi* from the blood samples (Table 1).

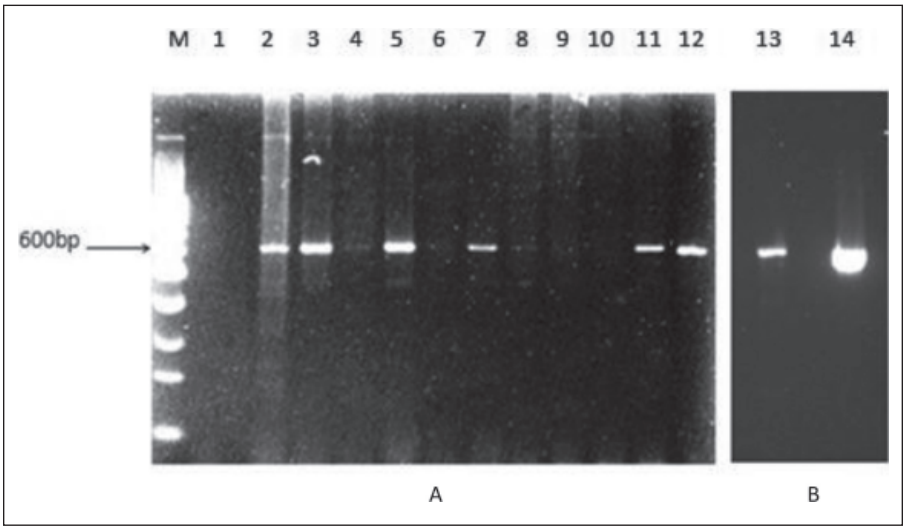

Figure 1. PCR results showing 7 positive samples followed by positive control. Panel A; Lane M: DNA ladder (100 bp). Lane 1: Negative control. Lane 2-5: Positive samples (No 43, 53, 54, 56). Lane 6: Negative sample. Lane 7: Positive sample (No 60). Lane 8-10: Negative samples. Lane 11: Positive sample (No 101). Lane 12: Positive control. Panel B; Lane 13: Positive sample (No 35). Lane 14: Positive control.

Table 1. List of sequences of amplified DNA fragments from positive *E. bieneusi* samples

Sample no.	Sequence ID	Identities	Name
35	gb|KF271507.1	100%	*Enterocytozoon bieneusi* isolate PDEn-22 small subunit ribosomal RNA gene, partial sequence
43	gb|KF271513.1	100%	*Enterocytozoon bieneusi* isolate PDEn-28 small subunit ribosomal RNA gene, partial sequence
53	gb|KF271513.1|	99%	*Enterocytozoon bieneusi* isolate PDEn-28 small subunit ribosomal RNA gene, partial sequence
54	gb|KJ719492.1	77%	*Enterocytozoon bieneusi* isolate Mpl_Eb12 18S ribosomal RNA gene, partial sequence
56	gb|KF271513.1|	99%	*Enterocytozoon bieneusi* isolate PDEn-28 small subunit ribosomal RNA gene, partial sequence
60	gb|KF271510.1	88%	*Enterocytozoon bieneusi* isolate PDEn-25 small subunit ribosomal RNA gene, partial sequence
101	gb|KF305579.1	94%	*Enterocytozoon bieneusi* isolate GD-1 small subunit ribosomal RNA gene, partial sequence
Positive control	gb|KF271518.1	99%	Enterocytozoon bieneusi isolate PDEn-33 small subunit ribosomal RNA gene, partial sequence

To understand the genetic diversity among *E. bieneusi* isolates in HIV-positive patients, a maximum likelihood tree was constructed with the aligned ITS sequences from the 7 HIV-positive patients. Sequences revealed by this study were found to group with all previously reported *E. bieneusi* genotypes. The first major cluster consisted of all published sequences of *E. bieneusi* from humans, and five of the ITS sequences were from the present study, including the positive control. Samples number 54 and 60 formed another group, which were placed at the base of the phylogenetic tree (Fig. 2). Sample 101 was not included in the tree due to its low DNA concentration.

Socio-demographic analysis of the study population is shown in Table 2. The age range of the HIV/AIDS patients were between 23-64 years old, with the number of specimens collected from male outnumbered female. Positivity rate of *E. bieneusi* infection among HIV/AIDS patients was significantly higher in female patients (p=0.003), and there was no significant association between age and race.

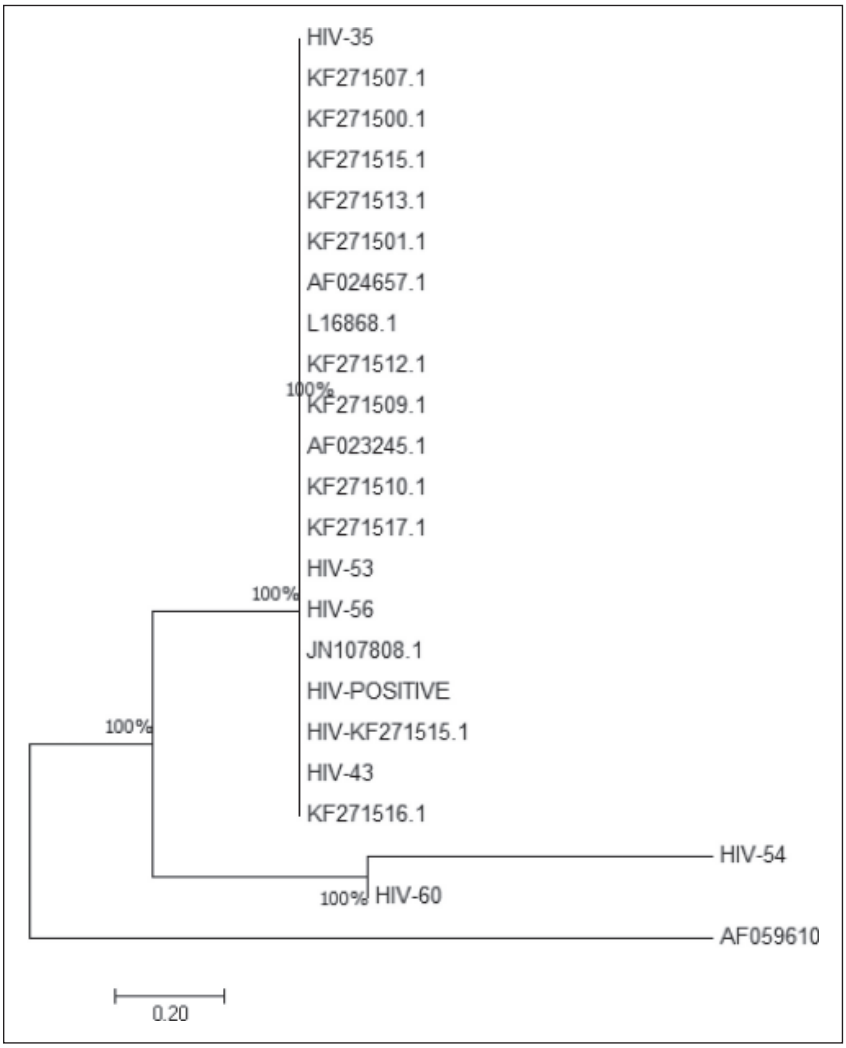

Figure 2. Phylogenetic tree was inferred by the maximum-likelihood analysis based on comparisons of the *E. bieneusi* ITS sequences from the sequences given in Table 1. The ITS sequence of a taxonomically unresolved species related to *E. bieneusi* (Genbank accession number AF059610) was used as the outgroup. Numbers at nodes indicate bootstrap confidence levels.

Table 2. Prevalence of *E. bieneusi* infection in HIV patients based on age, gender, race, CD4 count, and symptoms

Categories	*n* positive	*n* examined	% positive	X^2	*P*
Age group					
20-40	4	67	5.97	0.331	NS
>40	3	33	9.1		
Gender					
Male	3	83	3.6	8.5962	0.003369
Female	4	17	23.5		
Race					
Malay	5	58	8.62	0.56	NS
Non-malay	2	42	4.76		
CD4 count					
<100	5	79	6.33	0.26	NS
<200	2	21	9.52		
Symptoms					
Diarrheic	1	20	5	0.15	NS
Non-diarrheic	6	80	7.5		
Symptoms					
Fever	3	37	8.1	0.11	NS
No fever	4	63	6.35		
Symptoms					
Cough	3	25	12	1.28	NS
No cough	4	75	5.33		
Total	12	100	12		

*NS = not significant.

Microsporidia was detected in 8.62% (5/58) of Malays, whom comprised the largest ethnic group in the sample, but the difference between Malays and non-Malays was insignificant. Our study showed that microsporidiosis occurs in patients with CD4 cell counts ranging from 26 to 180 cells/µl. Based on CD4 classification (Table 2), *E. bieneusi* was mostly found in HIV-infected individuals with CD4 counts of <100 cells/mm^3 (26.6%). However, the number of *E. bieneusi*-infected patients was insignificantly associated with the CD4 cell counts. The clinical symptoms observed in the patients are shown in Figure 2. Fever and cough were symptoms most frequently correlated with dissemination of *E. bieneusi* infection. Only 14.3% (1/7) of the positive specimens were diarrheic. This suggested that diarrhea was a non-specific symptom in patients with microsporidiosis.

DISCUSSION

Most of the disseminated microsporidiosis cases were diagnosed at autopsy (Hartskeerl *et al*., 1993, Schwartz *et al*., 1992). Delay in the diagnosis is due to the lack of appropriate diagnostic protocols to rule out the infection as well as negligence of physicians is not suspecting disseminated microsporidiosis.

Table 3 . Clinical symptoms among HIV patients infected with *E. bieneusi*

Clinical symptoms	n positive	n examined	% positive
Cough	3	25	12
Fever	3	37	8.1
Diarrhea	1	20	5
Seizure	1	2	50
Rash	1	4	25
Epigastric pain	1	2	50

Detection of the small size of this parasite in tissue biopsies by light microscopy is difficult and may contribute to misdiagnosis. Electron microscopy is therefore needed to confirm the diagnosis, but the method is costly, time consuming, and not available in most laboratories in the hospitals (Lamps *et al.*, 1998). Thus, direct detection of microsporidia DNA in blood circulation is useful for prompt diagnosis of the disease. Detection of circulating microsporidia DNA by PCR assay for the diagnosis of disseminated microsporidiosis is crucial, as treatment for the infection is genus-specific. Furthermore, the duration of therapy also depends on the host immune status and whether the infection is localized or disseminated (Costa & Weiss, 2000).

PCR has been acclaimed as an outstanding sensitive and specific molecular diagnostic tool for many diseases, including microsporidiosis. PCR assay for detection of microsporidia has been reported by various researchers using clinical stool samples or stool samples spiked with microsporidia spores. In the present study, out of 100 blood samples obtained from HIV-infected patients, 7 tested positive for microsporidia (*E. bieneusi*) by PCR. This denoted the prevalence of microsporidia among the HIV patients is 7%. Our findings differed from previous local reports which stated a prevalence of 8.5% in HIV/AIDS patients (Lono *et al.*, 2011). The sample size was smaller (n=100) compared to the study carried out by Lono *et al.*, 2011 (n=247) (Lono *et al.*, 2011). In addition, their study used stool specimens instead of blood for detection of microsporidia DNA (Lono *et al.*, 2011). The present finding shows that clinical blood samples can be used as a template for the molecular diagnosis of disseminated microsporidia. The sequencing results showed that there were different variations of ssuRNA of *E. bieneusi* in the positive samples. This suggests that there could be more than 1 strain of *E. bieneusi* in the community. Further study is needed to determine the different strains of *E. bieneusi* in HIV patients since different isolates can belong to the same strain.

Phylogenetic analysis was performed to show the evolutionary relationships among the various *E. bieneusi* strains obtained. Small Subunit ribosomal RNA (SSU rRNA) is useful in phylogenetic analysis of Microsporidia spp. (Tay *et al.*, 2005 and Dong *et al.*, 2010). According to the result in Figure 2, near relationship was shown between all of *E. bieneusi* strains obtained in this study. This is due to the host similarity of *E. bieneusi* for all samples used, i.e. human. Furthermore, it is interesting to note that although sample no 54 and 60 were positioned differently from other samples, they were grouped nearer than the outgroup (AF059610). This reflects the diversity across the ITS region in our positive samples from HIV/AIDS patients.

The present study did not focus on patients with diarrhoea as only blood samples were collected. Although persistent diarrhoea is the most common clinical manifestation for microsporidiosis in immunocompromised patients, a study by Sarfati *et al.*, 2006 showed that *E. bieneusi* infection in HIV-positive patients may or may not cause diarrhoea. Co-infections with other microorganism in immunocompromised patients may also contribute to diarrhoea. A study by Rabeneck *et al.*, 1993, also showed

that *E. bieneusi* did not necessarily cause gastrointestinal symptoms in HIV patients. In our study, only one patient positive for *E. bieneusi* infection had diarrhoea. Fournier *et al.*, 2000 did a study on disseminated microsporidia in 12 cases and also observed diarrhea in a single case. According to Ambrosioni *et al.*, 2010, clinical manifestations of microsporidia are non-specific. This is supported by a case report by Loignon *et al.*, in 2014 in a patient who died due to disseminated microsporidiosis even though no symptoms were related to microsporidia. Fever and cough are the most common symptoms presented in these patients. This finding is in accordance with a report by Talabani *et al.*, 2010, which reported that fever and cough were among the commonest clinical manifestations of disseminated microsporidia infection. From the clinical manifestations, we postulate that those microsporidiosis-positive HIV/AIDS patients without gastrointestinal symptoms might acquire the infection by inhalation of spores from contaminated aerosols rather than fecal-oral ingestion. However, it is still possible that the infection might be from the gastrointestinal (GI) infection because we did not know the status of the GI track of the patients as no stool was examined in this study.

Microsporidia have been detected nearly in all organs and may elicit symptoms associated to their specific location. Dissemination of microsporidia should be considered in diagnosing HIV patients as it can infect any sites, resulting a broad range of symptoms (Talabani *et al.*, 2010). To the best of our knowledge, only a few cases of pulmonary and respiratory involvements with *E. bieneusi* have been reported in AIDS patients (del Aguila *et al.*, 1997). There were few reports on *E. bieneusi* associated with pulmonary and sinonasal symptoms of the patients (del Aguila *et al.*, 1997). Infections with *E. bieneusi* were commonly limited to gastrointestinal sites. It was not clearly understood how the parasite could be found in other parts of the body (del Aguila *et al.*, 1997). According to Hartskeerl *et al.*, 1993, the sizes for *E. bieneusi* gene in intestinal and extraintestinal sites were different, but their srRNA gene had a 99% similarity. This indicates that this species may consist of different strains which might contribute to the capability of the parasite to disseminate to other parts of the body. It is likely that virulence strains of microsporidia causing disseminated microsporidiosis exist, but there are no reports or data yet showing that microsporidia strains vary in their virulence.

The dissemination of *E. bieneusi* may be suspected a patient does not respond to treatment. It should also be suspected in severely immunocompromised patients with CD4 count less than 200 cells/ul who present with multi-organ involvement, including fever, renal failure, conjunctivitis, sinusitis, respiratory and central nervous system symptoms. A study by Carlson *et al.*, 2004 showed that a patient died because of disseminated microsporidia (*Encephalitozoon cuniculi*). The autopsy of the patients indicated that the microsporidia infected the peritoneal cavity perhaps through leakage at his duodenal-vesicular anastomoses. The tendency of *E. bieneusi* to disseminate from a breach in the gut epithelium is yet to be proven. However, the dissemination of *E. bieneusi* may be considered as a simple carriage associated with an intestinal infection (Botterel *et al.*, 2002). Since this parasite lives in intestine, the best explanation is that this parasite is disseminated by macrophages or other inflammatory cells into the blood circulation.

In conclusion, the findings of the present study revealed the presence of circulating microsporidia DNA in blood of HIV/AIDS patients with non-symptomatic clinical manifestations. We strongly suggest the application of blood PCR for early diagnosis of disseminated microsporidiosis since this approach may help the physician to improve patient management.

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