Detection of *Enterocytozoon bieneusi* (Microsporidia) by polymerase chain reaction (PCR) using species-specific primer in stool samples of HIV patients

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Accepted September 22, 2004

**Background & objectives**: Microsporidia are obligate intracellular parasites causing infections predominantly in immunocompromised patients. *Enterocytozoon bieneusi* is the most important microsporidian causing chronic diarrhoea in AIDS patients. The current method used for diagnosing the microsporidia spores is based on light microscopy using stained smears, which do not differentiate spores at species level. The present study was undertaken to detect microsporidia and confirm at species level (*E. bieneusi*) by PCR from stool samples of HIV positive patients.

**Methods**: During September 2002 to April 2003, stool samples from 153 HIV-positive patients (with chronic diarrhoea n=105; without diarrhoea n=48) were collected and examined microscopically for microsporidia spores using modified Weber's chromotrope stain. Stool samples were subjected to PCR assay using species-specific primer EBIEFI/EBIER1, which amplifies small subunit ribosomal RNA (SSU rRNA) of this microsporidian.

**Results**: A total of 10 HIV positive patients with chronic diarrhoea were positive for microsporidia by microscopic analysis and confirmed as *Enterocytozoon bieneusi* by PCR. No false positive results were observed. A diagnostic DNA fragment of 607 bp of the unique SSU rRNA was amplified from all samples infected with *E. bieneusi*.

**Interpretation & conclusion**: The study revealed that polymerase chain reaction is a useful tool for accurate species identification of microsporidia in stool samples, which serves the benefit of treatment to the patients.

**Keywords** Diarrhoea - *Enterocytozoon bieneusi* - HIV - Microsporidia - PCR

Microsporidia are protozoan parasites responsible for significant gastrointestinal disease in patients with human immunodeficiency virus (HIV) infection. To date, up to six genera of microsporidia have been reported to infect humans. Microsporidia of the genera, *Enterocytozoon*, *Encephalitozoon*, *Nosema*, *Pleistophora*, *Trachipleistophora*, *Vittaforma* and unclassified microsporidia were primarily detected in immunocompromised hosts with a broad variety of clinical presentations. Among microsporidian species infecting humans, *Enterocytozoon bieneusi* is increasingly recognized and most commonly identified agent of chronic diarrhoea in patients with acquired immunodeficiency syndrome (AIDS). *E. bieneusi* was first described in 1985 and found associated with malabsorption and...
diarrhoea in patients with AIDS. Prevalence of *E. bieneusi* infections among AIDS patients with chronic diarrhoea is estimated to be between 7 and 50 per cent, but it may cause diarrhoea in immunocompetent individuals also.

The clinical diagnosis of microsporidiosis has traditionally depended on direct visualization of the parasites by light and electron microscopy but the sensitivity and specificity of these techniques are not known and also dependent on the expertise of the microscopist. Electron microscopy, which can provide species-specific diagnosis in faecal specimens, is a suboptimal method for coprodiagnosis of microsporidian infections.

Further application of antigenic techniques is limited because specific antibodies to all microsporidia are not available. Studies using specific PCR primers based on the region coding for the small subunit ribosomal RNA (SSU rRNA) have shown the usefulness of such tools for identification of microsporidia at species level.

Identification of *E. bieneusi* by PCR in clinical samples necessitates the importance of treatment assessment, prognosis and to reduce dissemination risks among patients. We carried out the present study to identify the microsporidian *E. bieneusi* using species-specific PCR primers in stool samples obtained from HIV seropositive patients.

**Material & Methods**

During September 2002 to April 2003, stool samples were collected from 153 HIV-positive patients admitted to the HIV wards at Government Hospital of Thoracic Medicine, Chennai. These included 105 patients who presented with persistent chronic diarrhoea and 48 patients without diarrhoea. All stool samples were routinely examined for ova and cyst by light microscopy and concentrated by water-ether sedimentation to perform DNA extraction. Microsporidia infections were diagnosed by light microscopy using modified Weber’s chromotrope stain. Stool samples were evaluated for the presence of other enteric coccidian parasites such as *Isospora belli*, *Cryptosporidium parvum*, and *Cyclospora cayetanensis* using modified Kinyoun acid fast stain.

For DNA extraction, 500 µl of concentrated stool samples were used. Samples were pelleted by centrifugation at 18,000 g for 5 min and washed three times in phosphate-buffered saline (0.01M, pH 7.2). The washed pellets were suspended in 250 µl of the digestion buffer containing 50mM Tris-HCl (pH 8.5), 1 mM EDTA and 2 per cent sodium dodecyl sulphate. Proteinase K (AB gene, U.K) was added in the concentration of 1mg/ml, followed by the addition of 100mg of glass beads (trimethyl-silanized 140/270 mesh; Sigma, USA) per µl of total sample volume. Samples were vortexed at full speed at every 10 min for 3 min. Samples were centrifuged at 14,000 g for 30 seconds to pack the beads, and supernatants were transferred to sterile tubes. Again proteinase K was added in the same concentration (1mg/ml) and incubated overnight at 55°C. Finally, samples were incubated for 10 min at 95°C to inactivate proteinase K, allowed to cool at room temperature. DNA was further purified by ethanol – sodium acetate precipitation after phenol-chloroform extraction. DNA was resuspended in 25 µl of TE buffer (10mM Tris HCl, 1mM EDTA (pH7.4)) and stored at 4°C until PCR amplification.

Primers for PCR chosen were highly specific for diagnosis of *E. bieneusi* that would amplify a conserved region of small subunit rRNA. The forward primer, EBIEF1, 5’ - GAAA C T T TT G CCC ACT CCT AC G - 3’ , and reverse primer EBIER1, 5’ - CCAT GC AC C AC TCT G CC AT T - 3’ (Alpha DNA, Canada) were based on nucleotides 295 to 315 and 881 to 901, respectively of the *E. bieneusi* SSU-rRNA (Gen Bank accession no: L16868). Amplification of *E. bieneusi* templates with this primer results in a 607 bp diagnostic DNA fragment. The PCR was performed in 50 µl reaction mixture containing the following : 50pM of each primer, 10X NH₄ PCR buffer; 200 µM of each dNTP, 2mM MgCl₂ and 2 units of Taq DNA polymerase (Bioline, U.K). DNA amplification was performed on thermocycler (MJ Research, Inc, PTC - 100, Massachusetts, USA) and each set of reactions included a negative control (reagent mixture without template DNA) and a positive control (cloned *E.bieneusi* SSU-rRNA provided by Dr. A.J. Da Silva, CDC, Atlanta, USA). The amplification procedure included 5 min of initial denaturation at 95°C, followed by 30 seconds of
denaturation at 94°C, 30 seconds for annealing at 55°C and 90 seconds of extension at 72°C for 35 cycles. A 10 min extension at 72°C was used after 35 cycles.

Amplified products were electrophoretically resolved on a 2 per cent agarose gel (AB gene, U.K) and stained with ethidium bromide to visualize the amplified-PCR products under UV illumination.

**Results & Discussion**

Of the 153 HIV positive patients evaluated in this study; 10 patients with persistent chronic diarrhoea (n=105) were found to be positive for microsporidia by microscopic analysis. In positive stool samples, microsporidia spores were found to be oval or round measuring 1 to 1.5 µm in size (Fig. 1). *E. bieneusi* DNA was amplified in all 10 cases by PCR using the species-specific primer EBIEF1/EBIER1. Positive diagnostic amplification of *E. bieneusi* DNA fragment of 607bp was amplified in all 10 samples (Fig. 2). *E. bieneusi* was detected only as a sole pathogen in all ten positive cases and co-infection with other enteric parasites was not observed.

No microscopic detection or PCR amplification was obtained from HIV positive patients without diarrhoea that were negative for microsporidia (n=48). Other enteric pathogens detected by light microscopy were, *Cryptosporidium parvum* (11.7%), *Isospora belli* (9.1%), *Giardia lamblia* (5.9%) and *Cyclospora cayetanensis* (3.3%), *Strongyloides stercoralis* (3.3%), *Entamoeba histolytica* (1.3%) and *Ascaris lumbricoides* (0.6%) (Table I).

Until recently, microsporidia have rarely been considered in differential diagnosis of opportunistic infections in patients with AIDS and have frequently remained been undiagnosed because of their small size. Prevalence of individual microsporidial species has not been properly estimated, as careful identification at species level was not done for all described cases. *E. bieneusi* is the most prevalent

<table>
<thead>
<tr>
<th>Paraspites</th>
<th>Chronic diarrhoea (n = 105)</th>
<th>No diarrhoea (n = 48)</th>
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<tr>
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<td><em>Isospora belli</em></td>
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<td><em>Entamoeba histolytica</em></td>
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<tr>
<td><em>Ascaris lumbricoides</em></td>
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*Confirmed as *Enterocytozoon bieneusi* by PCR using species-specific primer
microsporidian infecting AIDS patients. The current standard light microscopic techniques used for the detection of microsporidia in stool samples are effective as screening test, but do not provide the species-specific identification necessary for proper clinical management and therapy. Transmission electron microscopy (TEM) cannot discriminate between certain species. The sensitivity of TEM is unknown, but because of its complexity and cost, this technique cannot be used for routine detection of these parasites in clinical specimens.

In this study, we used the species-specific PCR primers for the detection of *E. bieneusi* in stool samples. Only 6.5 per cent (10 of 153) patients were positive for *E. bieneusi* by PCR, which corroborated with the report of another study where different sets of primers were used. False-positive results and cross amplification was not observed. The DNA extraction method in combination with a modified water-ether sedimentation procedure may result in increasing sensitivity raising the number of spores. Increase in spore concentration by water-ether sedimentation has also been reported in another study.

Studies from different parts of the world have demonstrated the detection of microsporidia in clinical stool samples and also compared light microscopy and PCR. This is probably the first report of detection of microsporidia at species level using PCR in this part of the country. The main advantage of employing PCR procedure in facial samples is the differentiation of species of microsporidia, which is helpful in instituting appropriate therapy because dissemination risks and treatment response are not identical for *Encephalitozoon (Septata) intestinalis* and *E. bieneusi*.

In conclusion, the findings of the present study revealed that species-specific PCR will be an efficient tool for the identification of microsporidia in stool samples of HIV-positive patients with diarrhoea which in form will help in instituting appropriate treatment.

**Acknowledgment**

The authors thank Dr. Alexandre J. Da Silva, Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, USA, for providing cloned *Enterocytozoon bieneusi* as a positive control, and Dr Rajsekhar, Government Hospital of Thoracic Medicine, Chennai for providing stool specimens from HIV-positive patients. The financial assistance by University Grant Commission under special assistance programme is gratefully acknowledged.

**References**


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