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ABSTRACT

The HIV-positive population, due to their immuno-compromised nature, is considered more susceptible to parasitic infections than other populations. However despite the reports of other opportunistic pathogens such as Cryptosporidium and tuberculosis reported in vulnerable communities, microsporidia have not been highlighted in the local HIV-positive population in Malaysia. This study aimed to provide preliminary information on the prevalence of microsporidia in the local HIV-population. Microsporidia were detected in 21/247 (8.5%) stool samples from the HIV-infected individuals, a significantly higher (P-value <0.05) prevalence than in the control group, in which 5/173 (2.9%) were positive. HIV patients were 3x more at risk for acquiring microsporidium (OR: 3.12; 95% CI 1.15–8.44). Spores were ellipsoid in shape with outlines that stained dark pink with the interior a lighter shade. Approximately 21% of the positive specimens were from individuals in the 40–49 years age group. Ten individuals who were positive for microsporidia were also positive for other enteric parasites such as Blastocystis hominis and Giardia lamblia. We detected Encephalitozoon intestinalis DNA following nested PCR from three of 10 samples analysed, as demonstrated by an amplicon of 370 bp. From the findings reported, it appears that microsporidial infection in humans may actually be more common than reported. We strongly advocate greater emphasis on personal hygiene through public education on personal hygiene and the consumption of boiled or filtered water.

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1. Introduction

In HIV-positive individuals, the progressive decline in the immune response increases susceptibility to a variety of common and opportunistic infections. Intestinal parasitic infections are a major cause of disease in HIV-positive individuals, particularly in the tropics; significant disease occurs in 50–96% of patients.1 Studies on the adult HIV-positive population in Malaysia, which makes up 0.4% of the population, have described opportunistic parasitic infections,2 but studies involving the detection and identification of microsporidia in the population have not been reported, even though it has been estimated that 7–50% of chronic diarrhea in AIDS patients may be associated with microsporidia.3,4 This may be a reflection of local diagnostic expertise or that the parasite is not included in routine examination,5 as microsporidia infections have been reported from similar HIV-positive populations in Thailand and Vietnam.6,7

2. Materials and methods

The study was carried out in May 2008. Two hundred and forty-seven stool specimens were collected from 233 male and 14 female HIV-positive patients in a hospital in Kajang, Selangor. The group consisted of 164 Malays, 33 Indians, 40 Chinese and 10 individuals of unspecified race, all in the 20–59 year age range. Eighty-eight contributors did not specify their age, but all volunteers were above the age of 18 years. All patients who were approached had

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E-mail address: suresh@um.edu.my (S. Kumar).
2.2. DNA extraction and nested PCR

Due to financial constraints, we opted to carry out nested PCR on only 10 specimens that were positive for microsporidia based on light microscopy examination. 100 µl purified stool sample were vortexed and suspended in 96 µl lysis buffer containing 100 mM Tris-HCl (pH 8), 100 mM EDTA, 2% SDS and 150 mM NaCl and pulse-centrifuged to ensure complete sedimentation. Freeze-thaw extraction was carried out based on methods described previously12,13 using 0.4 mg/ml of proteinase K (20 mg/ml).

Primary PCR employed a pan-microsporidian C1 (5′-CACCAGTGTTAATCTGCC-3′)/C2 primer pair (5′-GTGACGGGCCGGTGATGTC-3′) to amplify a 1200 bp region of microsporidian SSUrRNA. Nested PCR then employed primer pairs EB1EF1/EB1ER1 (GAAACTTGTCCACTTCTAG-3′/5′-CATGCACACTCCTGCACT-3′) to amplify a 607 bp fragment of Enterocytozoon bieneusi SSUrRNA14,15, V1/SI500 (5′-CCACAGTGTTAATCTGCC-3′/5′-CTCGCTCCTTTACACTCG-3′) to amplify a 370 bp fragment of Encephalitozoon intestinalis;17,18 EHELF/EHEL5R (5′-AGAAGTAGTATTTAGCA-3′/5′-GTAAAACACTCTCA-CACTCA-3′) to amplify a 547 bp fragment of Enc. hellem19 and ECUNF/ECUNR (5′-ATGAGAACTGATCTTGTGGCG-3′/5′-TGCACTGCAGCCAGCATC-3′) to amplify a 549 bp DNA fragment of Enc. cuniculi.19

Amplification was carried out in 50 µl volume containing 1x PCR buffer, 2.5 mM MgCl2, 200 µM dNTP mix, 12.5 pmol of each primer, 1 (primary PCR) or 0.5U (secondary PCR) Taq DNA polymerase (Fermentas), 2% Tween20 and 0.2 mg/ml bovine serum albumin. Each reaction set contained a negative control of ultra pure water and a positive control containing template DNA obtained from cultures. Amplicons were separated on a 1.5% agarose electrophoresis gel at 100 kV for 80 minutes and the gel was stained with 2 µg/ml of ethidium bromide and viewed under ultraviolet light.

2.3. Transmission electron microscopy

Transmission electron microscopy was carried out to describe the morphology of microsporidia in the specimens that were PCR-negative. Specimens were filtered three times through a 20 µm filter (25 mm diameter) to remove debris before being fixed overnight in 4% glutaraldehyde at 4°C prior to further processing.

The specimens were washed in 0.1 M cacodylate buffer (pH 7.4) and post-fixed with 1% OsO4 in cacodylate buffer (pH 7.4) at 4°C, then dehydrated in increasing concentrations of ethanol and embedded overnight in resin at 60°C. Thin sections were stained with toluidine blue for initial viewing under immersion oil light microscopy. Based on selected areas from the thin sections, ultra-thin sections were obtained and stained with lead citrate and mounted on a carbon-coated grid for viewing.

2.4. Statistical analysis

The statistical significance for microsporidia detection in the HIV-positive and normal controls was determined with SPSS Version 10 (SPSS Inc., Chicago, IL, USA), using a χ² test with Yates’ correction where applicable.

3. Results

3.1. Light microscopy detection

All stool specimens obtained were extremely soft or watery, indicative of diarrhea.

Microsporidia were detected in 21/247 (8.5%) of stool samples from the HIV-infected individuals, significantly higher (P-value < 0.05) than the prevalence in the control group, in which 5/173 (2.9%) were positive for microsporidia. HIV patients were 3x more at risk for acquiring microsporidia (OR: 3.12: 95% CI 1.15–8.44). While the number of specimens collected from males outnumbered that of females, the proportion of microsporidia in females was actually higher than that of males (Table 1). Approximately 21% (9) of the positive specimens were from individuals in the 40–49 years age group (Table 1). Microsporidia were detected in 10.4% of Malays (17/164), who also comprised the largest ethnic group in the sample (Table 1). Ten individuals who were positive for microsporidia were also positive for other enteric parasites such as Blastocystis hominis and Giardia lamblia (Table 1). Spores were ellipsoidal in shape with outlines that stained dark pink (Figure 1). The interior of the spores was usually stained a lighter shade than the spore coat.

3.2. Molecular detection

We detected Enc. intestinalis DNA following nested PCR from three of 10 samples analysed, as demonstrated by an amplicon of 370 bp (Figure 2).
### Table 1
Prevalence of microsporidia in local adult HIV-positive population

<table>
<thead>
<tr>
<th>Gender</th>
<th>Specimens collected</th>
<th>Microsporidia-positive n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>14</td>
<td>2 (14.3)</td>
</tr>
<tr>
<td>Male</td>
<td>233</td>
<td>19 (8.1)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20–29 years</td>
<td>37</td>
<td>2 (5.4)</td>
</tr>
<tr>
<td>30–39 years</td>
<td>75</td>
<td>8 (10.7)</td>
</tr>
<tr>
<td>40–49 years</td>
<td>43</td>
<td>9 (20.9)</td>
</tr>
<tr>
<td>50–59 years</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Unknown (≥21 years)</td>
<td>88</td>
<td>2 (2.3)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malay</td>
<td>164</td>
<td>17 (10.4)</td>
</tr>
<tr>
<td>Indian</td>
<td>33</td>
<td>1 (3.0)</td>
</tr>
<tr>
<td>Chinese</td>
<td>40</td>
<td>2 (5.0)</td>
</tr>
<tr>
<td>Unknown/not stated</td>
<td>10</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td>Single/multiple infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single infection</td>
<td>11</td>
<td>11 (52.4)</td>
</tr>
<tr>
<td>Multiple infectionsa</td>
<td>10</td>
<td>10 (47.6)</td>
</tr>
</tbody>
</table>

a e.g. other enteric pathogens such as Blastocystis hominis and Giardia lamblia.

### 4. Discussion

Opportunistic pathogens such as Cryptosporidium have been widely reported but this is one of the few studies to highlight the prevalence of microsporidia in the HIV-infected population in Malaysia. The number of volunteers in this study was much larger (247 vs 22) compared to a retrospective study carried out in Thailand, which reported microsporidia in 27% of stools from HIV-positive individuals with chronic diarrhea, which was higher than that reported in this study. However, there were some parallels with a German study reporting microsporidia in 5.9% of HIV-positive individuals with acute diarrhea, as the samples provided by the volunteers in this study were indicative of acute diarrhea, i.e., extremely watery. The number of samples collected (311) was also closer to the number collected in this study.

Not all instances of acute diarrhea could be directly attributed to microsporidia as 48% of the microsporidiosis-positive specimens were also infected with B. hominis and G. lamblia. We did not find any literature that attributed gender or racial bias toward a higher prevalence of microsporidia, although our analysis of these parameters determined the difference was not statistically significant. We were not able to determine how many of the volunteers had chronic diarrhea, although the watery consistency of the HIV-positive specimens indicated its presence.

Microsporidia are detected more commonly in cases of chronic rather than acute diarrhea in HIV-positive individuals, although it is also possible that chronic diarrhea can be attributed to intestinal changes due to advanced immunodeficiency. In contrast, the numbers of microsporidia-related acute diarrhea may be low because the symptoms are self-limiting and are unreported.

All samples were watery, and by assumption diarrheic, which agrees with studies that reported a strong correlation between intestinal microsporidiosis and diarrhea, as some investigators have suggested that asymptomatic carriage of microsporidia may precede the appearance of symptoms, more frequent specimen collection from the same individuals would clarify this matter. However, we were unable to reestablish contact with the volunteers following the end of the study which could have provided more information of the numbers of microspores and its correlation to diarrheic stools.

It has been established that patients with other protozoal infections, such as cryptosporidiosis, and less advanced immunodeficiency tend to have self-limited,
milder or even asymptomatic diseases, while patients with more advanced immunodeficiency tend to present with protracted and refractory diarrheal illnesses.21 As *Enc. intestinalis* was identified in the specimens, we have recommended that the corresponding individuals receive albendazole treatment.

The screening results of this study were passed on to the facilitators of the respective medical camps with recommendations for treatment for infected individuals. Guidelines for antiretroviral treatment exist, although HIV-positive individuals may not receive appropriate treatment due to ignorance of such options, or the long-term cost (even with pharmaceutical price cuts and government subsidies). The social stigma associated with HIV infection also discourages individuals from seeking medical follow-up, which minimizes the chance for detection and treatment of microsporidial infections.

It is possible that the PCR-negative spores did not belong to the *Enteroctyozoon* or *Encephalitozoon* genera, therefore, the primers chosen for the study were not specific to their SSUrRNA DNA sequences. Due to financial constraints faced by the study, we opted to narrow the focus down to the detection and identification of microsporidia commonly reported from HIV-positive individuals, i.e., *Enc. intestinalis* and *Ent. bieneusi*. The primers used were specific for the four most commonly identified microsporidia in humans (*Enc. intestinalis, Enc. hellem, Enc. cuniculi* and *Ent. bieneusi*), and do not amplify DNA from other organisms. As such, the presence of microsporidia not covered by the four specific primers would not have been amplified. The use of primers that facilitate the amplification of other microsporidia would certainly paint a more complete picture of their prevalence in this population group.

From the findings reported, it appears that microsporidal infection in humans may actually be more common than reported. The parasites may exist as a latent infection, and their numbers may be low in a normal healthy individual and may increase greatly when the individual is immunocompromised, but without causing serious disease. We would certainly have liked to correlate these findings with the individuals' CD4+ counts, this was not possible due to privacy rulings. However, we believe these findings are useful as they indicate the presence of microsporidia in the country's population of HIV-positive individuals, more so that of a human-pathogenic microsporidian as *Enc. intestinalis*, which should warrant attention from policy-makers.

Transmission electron microscopy examination was attempted on the specimens containing PCR-negative microsporidia, but efforts to locate spores were unsuccessful, perhaps due to their sparse numbers. Furthermore, the spores detected in the light microscopy specimens may have been empty spore coats, leading to the negative PCR result. The staining method used in this study facilitated quick processing of a large number of specimens, a task whose efficiency was unsatisfactory when the more precise fluorochrome staining was initially attempted. This could have had the unwanted drawback of producing false positive results in some samples under light microscopy detection.

The microsporidia can cause disseminated microsporidiosis, and has been isolated from urine, nasal mucosa, sputum, bronchoalveolar lavage fluid, and feces.29 Additionally, different *Enc. intestinalis* isolates have been shown to have similar molecular and antigenic properties, which indicate that it may be possible to transmit infection from man to animal and vice versa, giving rise to concern that this microsporidian may have zoonotic potential. The present study confirms that the prevalence of microsporidiosis was 8.5% in diarrhea stool specimens collected from HIV-positive prisoners in Malaysia and strongly advocates greater emphasis on personal hygiene and the consumption of boiled or filtered water, through public education. Not only are they general precautions that are easy to implement, they would greatly reduce the chances of people, especially those who are HIV-positive, in acquiring microsporidial infections.

**Authors’ contributions:** GSK and AL designed the study protocol; AL carried out the prevalence study, performed staining techniques and deployed various detection methods for other parasites; TTC and AL carried out PCR work and analysis; GSK and AL carried out the analysis and interpretation of all data pertaining to the study; AL, GSK and TTC drafted the manuscript. All authors read, revised and approved the final manuscript. AL, GSK and TTC are guarantors of the paper.

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**Funding:** None declared.

**Conflicts of interest:** None declared.

**Ethical approval:** Not required for collection of controls; the samples were obtained from a routine service medical camp, which was carried out as a voluntary community service rendered by a non-governmental organisation. All participants gave informed consent. We obtained ethical clearance from the relevant committees for specimen collection from HIV-positive individuals, who had all also granted informed consent for participation in this study.

**References**


