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A B S T R A C T
Microsporidia are ubiquitous parasites thought to be closely related to fungi. Their presence in the environment means that humans are frequently exposed to infection. Stool samples were collected from 151 indigenous villagers from the eastern state of Pahang in 2005. The samples were concentrated with water-ether sedimentation, stained with modified trichrome stain and examined under oil-immersion microscopy. Thirty-two specimens (21.2%) were positive for microsporidia. Microsporidia were observed as ovoid or rounded ovoid shapes measuring ∼1 μm, with a bright pink outline containing a central or posterior vacule. PCR amplification with specific primers on microscopy-positive specimens amplified Encephalitozoon intestinalis DNA from five of the ten specimens used.

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1. Introduction

Microsporidia are single-celled, obligate intracellular organisms belonging to the phylum Microspora, currently considered to be very closely related to fungi.¹ More than 1200 species from this phylum currently have been classified into approximately 150 genera. They are recognised as opportunistic parasites in immunocompromised individuals. Currently, attention is focused on elucidating their phylogeny and more efficacious treatment, as well as the development of tissue culture and animal models to aid in treatment development for Enterocytozoon bieneusi.

Prevalence of microsporidia varies based on geographic region, diagnostic method and characteristics of the population being studied.² Increased awareness and improved diagnostic methods have resulted in microsporidia infections being detected from a wide range of human populations. Additionally, microsporidia species that infect humans have been identified in animals and water sources, which raises public health concerns about zoonotic and waterborne transmission of microsporidia.²

The Orang Asli is a collective term for a group of indigenous people that usually reside in the interior regions of peninsula Malaysia. The term ‘Orang Asli’ literally means ‘(the) original people’, and they identify themselves by tribes, e.g. Negrito, Temuan, Mak Meri, Semaq Beri, etc.

The past two to three decades have seen the Orang Asli relocated from their homes in the jungle to more urban settlements in a bid to improve their living conditions. Such communities often lie in the hardcore poor category, having failed to integrate into such settlements. The quality of health care available to the Orang Asli is poor compared to that of a city dweller. This results in poor overall health for the Orang Asli, as shown by reports of high prevalence of infectious diseases such as malaria, tuberculosis and even leprosy.³ Nutritional deficits and attendant intestinal parasitism rates in Orang Asli are also higher compared to those of urbanites.³

There have been only two reports from Malaysia to date on detection of microsporidia in Orang Asli children and hospital patients, respectively.⁴ However, similar studies on Orang Asli adults could not be found.
In order to provide some baseline data, a prevalence study was carried out on a tribe of Temuan Orang Asli in the eastern state of Pahang in order to determine the prevalence of microsporidia in the community, as well as to attempt to specifically identify any microsporidia detected.

2. Materials and methods

2.1. Specimen collection

Specimen collection was carried out at one routine medical camp held in Bertang village in Raub town, located in the state of Pahang in 2005. The village housed the Temuan tribe of Orang Asli, who had been relocated from the interior of the Pahang jungle to a modern settlement more easily accessed by vehicles. One hundred and fifty-one fresh stool specimens were collected from 30 families. The group was made up of 70 males and 81 females, with ages ranging from 9 to 60 years. The age of 13 volunteers was not specified or unknown. The control group was a group of normal healthy individuals from Kuala Lumpur. A specified or unknown. The control group was a group of normal healthy individuals from Kuala Lumpur. A χ² test, with a P-value of <0.05 considered to be significant and Yates’ correction used where applicable, was carried out to determine the statistical significance of the findings. The screening results were relayed to the medical team with suggestions for treatment, and management and control.

2.2. Light microscopy screening

Specimens were concentrated by water-ether sedimentation and stained with the modified trichrome stain. One hundred observation fields were examined under oil immersion before a specimen was considered negative. Screening was carried out in duplicate. Ten samples were randomly selected from specimens positive by light microscopy for PCR amplification to be carried out to determine whether specific identification of the microsporidia was possible.

2.3. DNA extraction

DNA was extracted from 100 μl of purified stool sample, as described by Velasquez and colleagues. Further freeze–thaw extraction was carried out as described by Smith and Rose and van Eys and colleagues with 0.4 mg/ml of proteinase K enzyme (20 mg/ml).

2.4. PCR

A pan-microsporidian primer pair was used to amplify a 1200 bp fragment from the 16S or small subunit rRNA (SSUrRNA). The forward primer (C1) is based on nucleotide positions 1 to 18 in all microsporidian SSUrRNA, while the reverse primer (C2) is based on nucleotide positions 1169 to 1186 in Encephalitozoon intestinalis, positions 1173 to 1190 in Enc. cuniculi, positions 1188 to 1205 in Enc. hellem and positions 1152 to 1170 in Ent. bieneusi SSUrRNA, respectively.

For the nested PCR, the following pairs of primers were used. (1) Enterocytozoon bieneusi: amplification of Ent. bieneusi templates with the primer pair EBIEF1/EBIER1 produces a 607 bp fragment; the forward primer (EBIEF1) corresponds to nucleotide positions 295 to 315, and the reverse primer (EBIER1) corresponds to positions 881 to 901 of a conserved region of Ent. bieneusi SSUrRNA. (2) Encephalitozoon intestinalis: amplification of Enc. intestinalis templates with the primer pair V1/SI500 produces a 370 bp fragment; the forward primer (V1) corresponds to positions 1 to 22, and the reverse primer (SI500) corresponds to position 500, based on the alignment of Enc. intestinalis with Escherichia coli, of a conserved region of Enc. intestinalis SSUrRNA. (3) Encephalitozoon hellem: amplification of Enc. hellem templates with the primer pair EHEL/EHEL'R produces a 547 bp fragment; the forward primer (EHEL') corresponds to positions 358 to 378, and the reverse primer (EHEL'R) corresponds to positions 884 to 904 of a conserved region of Enc. hellem SSUrRNA. (4) Encephalitozoon cuniculi: amplification of Enc. cuniculi templates with the primer pair ECUNF/ECURN produces a 549 bp fragment; the forward primer (ECUNF) corresponds to positions 344 to 364, and the reverse primer (ECURN) corresponds to positions 872 to 892 of a conserved region of Enc. cuniculi SSUrRNA. Amplification was carried out in 50 μl reactions containing 1 x PCR buffer, 2.5 mmol/l MgCl₂, 200 μmol/l dNTP mix, 12.5 pmol of each primer, 1U Taq DNA polymerase (Fermentas, Kuala Lumpur, Wilayah Persekutuan, Malaysia), 2% Tween20 and 0.2 mg/ml bovine serum albumin. Each reaction set contained a negative control of ultrapure water and a positive control containing template DNA obtained from cultures. Amplified products were run on a 1.5% gel and stained with 2 μg/ml ethidium bromide for viewing under ultraviolet light.

3. Results

Microsporidia observed in the specimens were stained a dark pinkish-purple. The interiors of the spores did not take up the stain and remained clear. Some of the spores were observed with darker polar staining than others, and an equatorial belt-like stripe could be discerned in other spores (Fig. 1). The microsporidia were ovoid or ellipsoid in shape and were approximately 1 μm in length. Overall, microsporidia were detected by light microscopy in 21.2% of the Orang Asli community examined (Table 1). Equal numbers of males and females were infected with microsporidia. More adults were found to be infected with microsporidia than children. Microsporidia were the sole parasites in six of the 32 individuals. The stool specimens obtained from those six individuals were all non-diarrhoeic. The occurrence of diarrhoea among some of the remaining 26 samples could not be directly attributed to microsporidia, owing to co-infections with other enteric pathogens detected by light microscopy: Ascites lumbricoides (6/26, 23.1%), Blastocystis hominis (5/26, 19.2%) and helminths (1/26, 3.8%), as well as multiple infections involving up to four other enteric pathogens, including Trichuris trichiura (14/26, 53.8%). The prevalence of microsporidia in the Orang Asli was significantly higher than that of the normal population (5/173, 2.9%).
Fig. 1. Microsporidia in an Orang Asli specimen. Some spores have darker polar staining (arrows and inset). An equatorial belt-like stripe is faintly visible in a larger spore (block arrow and inset). Scale bar = 5 μm.

Table 1

<table>
<thead>
<tr>
<th>Microsporidia among the Temuan Orang Asli, Pahang</th>
<th>Prevalence (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>16/81 (19.8)</td>
<td>0.79</td>
</tr>
<tr>
<td>Male</td>
<td>16/70 (22.9)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤19</td>
<td>17/85 (20.0)</td>
<td></td>
</tr>
<tr>
<td>≥20</td>
<td>15/66 (22.7)</td>
<td>0.838</td>
</tr>
<tr>
<td>Microsporidia infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single infection</td>
<td>6/32 (18.8)</td>
<td></td>
</tr>
<tr>
<td>Multiple infection</td>
<td>26/32 (81.3)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>32/151 (21.2)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Microsporidia DNA was not visualised after the primary amplification. Following the nested PCR, bands of approximately 370 bp were visualised, corresponding to the fragment size targeted from *Enc. intestinalis* DNA. The targeted microsporidian DNA was amplified from five of the ten samples used in the study (Fig. 2). No amplification was visualised for the targeted fragments from *Enc. hellem*, *Enc. cuniculi* and *Ent. bieneusi*.

Fig. 2. Secondary PCR on Orang Asli specimens for *Encephalitozoon intestinalis*. Lane 1: 100 bp marker; lane 2: negative control; lane 3: positive control; lanes 4–12: DNA amplified from the 10 fecal specimens. Lanes 4, 5, 8, 9 and 12 display a targeted 370 bp band.

4. Discussion

Indigenous communities usually reside in the interior or remote parts of the jungle and are usually marginalised in terms of education, career opportunities and welfare. In the village that was sampled in this study, only 17 individuals had received education up to primary level (e.g. grades 1 to 6), while three others were still schooling at secondary level. Additionally, only eight of the villagers were steadily employed, and no information was forthcoming on the employment status of the other villagers. These factors (the distance of the village from schools, rudimentary hygiene standards, level of poverty) are reduced compared with modern standards of living and hygiene and have a detrimental effect on the villagers’ living standards.

As stated in the results, the prevalence of microsporidia in the Orang Asli was significantly higher than that of the normal healthy individuals in the control group. However, microsporidia in the Orang Asli were not significantly prevalent compared to the other enteric pathogens that were detected in this study (results not shown). This finding is in line with the earlier assertion that intestinal parasitic infection, in general, among the Orang Asli is higher than in the normal healthy population.

Tapioca is the main food group for the people in this study, and the villagers forage in the jungle for additional sustenance, where there is a risk of acquiring microsporidial infection, owing to the parasite’s ubiquity in nature. It is possible that malnourished individuals, as observed among the villagers, may be more susceptible to microsporidial infection, as there appears to be a relationship between nutrition and (other) enteric parasite infections in children. Other risk factors associated with human microsporidiosis include exposure to recreational water, occupational water and drinking water.

To our knowledge, prevalence studies on microsporidia in local indigenous communities have not been carried out on the same scale as that for other enteric pathogens. Indeed, there is a lack of prevalence data based on parasite detection worldwide, as opposed to serological detection. A search for similar studies yielded an epidemiological survey carried out on two rural highland villages in Mexico, where anti-*Encephalitozoon* antibodies were detected in faecal samples from 7.8% of villagers (20/255), and 21.4% of households (15/70) had at least one infected member. It was suggested that consumption of unpurified water from an untreated water supply was the only factor that influenced the appearance of spores in the stools.

In this study, 60% of the 30 families sampled possessed at least one member who was positive for microsporidia: a figure that is higher than that detected by Enriquez and colleagues. Similarly to the Enriquez study, the river water that supplies Bertang village is also untreated. Water samples from the river were negative for microsporidia by light microscopy and PCR (results not shown), but periodic sampling should be carried out, as human-pathogenic microsporidia have been detected from water sources. Furthermore, the village outhouses located near the river...
posed a potential transmission risk, as their contents were emptied into the river, which is the only source of water for the village. It has been suggested to the relevant authorities that sanitation facilities be improved and appropriate treatment be rendered to the villagers involved in the study.

*Encephalitozoon intestinalis* is the second most prevalent microsporidian reported in immunocompromised individuals after *Ent. bieneusi,* although similar studies in other populations are scanty. *Encephalitozoon intestinalis* can cause disseminated microsporidiosis and has been isolated from urine, nasal mucosa, sputum, bronchoalveolar lavage fluid and faeces.

*Encephalitozoon intestinalis* DNA was amplified from five of ten samples in this study. However, we were not able to compare our findings with other studies because, as with the light microscopy, data from parallel studies are lacking. However, the findings indicate that amplification with the specific primers was successful and that *Enc. intestinalis* may be prevalent in the Bertang village environment.

It is possible that the samples that did not produce any amplification at all may have contained spores that do not belong to the *Enterocytozoon* or *Encephalitozoon* genera, and therefore are not compatible with the primers chosen for the study. The primers used were specific for the four most commonly identified microsporidia in humans, and no cross-reactivity has been reported. Although any diarrhoeal symptoms could not be directly correlated to the microsporidia, owing to the presence of other enteric pathogens, it has been suggested that microsporidiosis may exist as a latent infection, and low parasite burden in a normal healthy individual may increase greatly when the individual is immunocompromised, but without causing serious disease.

This is the first known report of molecular identification of microsporidia detected in an Orang Asli community. It is hoped that these findings will allow the formulation of better health-management and disease-prevention advisories, and improvement in the standards of health in similar communities.

**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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**Conflicts of interest:** None declared.

**Ethical approval:** Not required; the samples were obtained from routine service medical camps, which were carried out as a voluntary community service rendered by a non-governmental organisation. All participants gave informed consent.

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