PREVALENCE AND RISK FACTORS FOR ASYMPTOMATIC INTESTINAL MICROSPORIDIOSIS AMONG ABORIGINAL SCHOOL CHILDREN IN PAHANG, MALAYSIA

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Abstract. The epidemiology and environmental factors affecting transmission of human microsporidiosis are poorly understood. We conducted the present study to determine the prevalence and risk factors associated with asymptomatic intestinal microsporidiosis among aboriginal school children in the Kuala Krau District, Pahang State, Malaysia. We collected stool samples from 255 school children and examined the samples using Gram-chromotrope Kinyoun stain. We also collected demographic, socioeconomic, environmental and personal hygiene information using a pre-tested questionnaire. Sixty-nine of the children was positive for microsporidia: 72.5% and 27.5% were low (1+) and moderate (2+) excretions of microsporidia spores, respectively. Univariate and multivariate analyses showed being aged ≥10 years ($p = 0.026$), using an unsafe water supply as a source for drinking water ($p = 0.044$) and having close contact with domestic animals ($p = 0.031$) were all significantly associated with microsporidial infection among study subjects. Our findings suggest asymptomatic intestinal microsporidiosis is common in the study population, more than previously reported. In the study population, control measures need to be implemented, such as good personal hygiene, proper sanitation and safe drinking water supply.

Keywords: microsporidia, epidemiology, asymptomatic, children, Malaysia

INTRODUCTION

Microsporidia is a single-celled, obligate intracellular organism belonging to the phylum Microspora, currently considered to be very closely related to fungi (Gill and Fast, 2007). It can be an opportunistic parasite in immunocompromised individuals. The first case of microsporidiosis in a person infected with human immunodeficiency virus (HIV) was identified in 1985 and led to the description of a new species: *Enterocytozoon bieneusi* (Desportes *et al*, 1985). This species is associated with chronic diarrhea,

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unexplained weight loss and cholangitis (Eeftinck Schattenkerk *et al,* 1991). Another species, *Encephalitozoon intestinalis*, is associated with intestinal manifestations with or without disseminated symptoms (Cali *et al,* 1991).

The prevalence of microsporidiosis varies based on geographic region, diagnostic method and characteristics of the population being studied (Didier *et al,* 2004). Increased awareness and improved diagnostic methods have resulted in microsporidiosis being detected in a wide range of human populations (Bryan and Schwartz, 1999). Microsporidia species capable of infecting humans have been identified in animals and water, which raises public health concerns about zoonotic and waterborne transmission of microsporidia (Cotte *et al*, 1999; Mathis *et al*, 2005). Up to this date, one outbreak of microsporidiosis, associated with waterborne transmission has been reported in the literature (Cotte *et al,* 1999).

A few studies have been published investigating the occurrence of microsporidiosis among immunocompromised individuals other than HIV-infected patients, such as bone marrow and organ transplant recipients and those undergoing immunosuppressive therapy (Hernandez-Rodriguez *et al,* 2012; Hocevar *et al,* 2014). Some immunocompromised patients have an increased probability of acquiring parasitic infections. In contrast to the hundreds of publications about microsporidia in immunocompromised patients, only a few have studied microsporidiosis in immunocompetent individuals (Abreu-Acosta *et al,* 2005; Sak *et al,* 2011). The most frequent clinical manifestations of microsporidia in AIDS patients are diarrhea, nausea, vomiting, malabsorption and weight loss, while in immunocompetent individuals it may cause self-limited diarrhea (Wanachiwanawin *et al,* 2002).

In Malaysia, despite sustained socioeconomic and infrastructural development, intestinal microsporidiosis is still highly prevalent among impoverished rural communities (Shahrul Anuar *et al,* 2013). Several studies have investigated the prevalence of microsporidia among HIV infected patients, hospitalized patients and in aboriginal communities (Norhayati *et al,* 2007, 2008; Lono *et al,* 2011; Salleh *et al,* 2011). These studies revealed high prevalences of microsporidiosis with considerable morbidity among immunocompromised patients in Malaysia. There have been few studies of microsporidial infection among primary school children in Malaysia. The present study was conducted to determine the prevalence of intestinal microsporidiosis and its associated risk factors among asymptomatic aboriginal school children in Kuala Krau District, Pahang, Malaysia.

MATERIALS AND METHODS

Study area

This cross sectional study was carried out between March and June 2014, in Kuala Krau District, Pahang State, 200 km northeast of Kuala Lumpur, Malaysia. The study area consisted of 15 villages located in a remote valley. There is a clinic in the study area with an ambulance to send critical cases to the nearest hospital at Temerloh, a town in Kuala Krau District (40 km from the study area). The aboriginal people in this area live in houses made of wood or bamboo. Most of the houses have electricity only at night and have supply water used for drinking. Water for domestic needs (bathing, washing clothes and utensils and feeding animals) is collected from rivers adjacent to the village. Most

of the residents in the study area work as farmers, laborers, rubber tappers or other agricultural jobs.

Study population

Penderas National School is the only primary school for aboriginal children at this area. The school was selected from the available official school list in collaboration with the Department of Orang Asli Development and meets all selection criteria such as located in the rural area, easy access from the main roads and school enrolment of more than 100 pupils. Although the total enrolment of the Penderas National School was 405 pupils, only 302 were present during sampling visits. Of the children present during sampling, 255 aged 7-12 years (138 boys and 117 girls) agreed to participate in the study and met the inclusion criteria (obtained written consent from their parents, completed the questionnaire and provided a fecal sample for examination). The minimum sample size calculated for this study used the formula of Lwanga and Lemeshow (1991). The minimum number of participants calculated to be required for this study was 246, based on a 20% prevalence of microsporidial infection among aboriginal children as reported previously (Norhayati *et al*, 2007).

Questionnaire

Demographic, socioeconomic, environmental information, personal hygiene practices, history of receiving anthelmintic treatment and health status of the participants were collected using a pretested questionnaire (Shahrul Anuar *et al*, 2013). The questionnaire was designed in English and then translated into Malay. Two research assistants from the Department of Medical Laboratory Technology, Universiti Teknologi MARA were trained to administer the questionnaire. The subjects and their parents were interviewed at their homes. During the interview, observations were made regarding the personal hygiene of the children (*eg*, cut fingernails, wearing shoes when outside the house and hand cleanliness), household cleanliness and the availability of functioning toilets and piped water.

Collection of fecal samples

After completing the questionnaire, each child provided a stool sample collected in a wide mouth screw cap, a 100 ml clean container pre-labeled with the individual's name and code. Their ability to recognize their name was counterchecked. A thumb sized fecal sample was placed into the container and then the container was placed in a zip-locked plastic bag. Parents and teachers were instructed to monitor the children during sample collection in order to ensure that they placed their fecal samples in the correct container.

Detection of microsporidia by Gram-chromotrope Kinyoun stain

A thin fecal smear was made from each stool sample and air dried, fixed with methanol and stained with crystal violet for one minute; the excess stain was rinsed off with Gram's iodine. The slides were then stained with Gram's iodine for 2 minutes. The Gram's iodine solution was removed by gently rinsing with a decolorizer until the flow become colorless. The slides were washed with tap water and stained with chromotrope stain and prepared as described by Moura *et al* (1996). The slides were rinsed in 90% acid-alcohol and counterstained with Kinyoun's carbol fuchsin stain for 3 minutes. The slides were then rinsed in 90% acid-alcohol, 95% alcohol for 5 minutes and 100% ethyl alcohol for 2 minutes (Salleh *et al*, 2011). The samples was then covered with DPX

medium (mixture of distyrene, plasticizer and xylene) and covered with cover slips.

The criterion used to identify a microsporidium was finding a pink-blue ovoid structure with a blue spore wall and a belt-like strip encircling the spore. At least 100 fields were examined for each slide at 1,000x magnification and positive specimens were confirmed by two technologists. The spore density was graded as follows: 1+ (1-10 spores seen), 2+ (11-20 spores seen) and 3+ (>21 spores seen) (Norhayati *et al*, 2008).

Data analysis

The data were reviewed and double-checked before and after data entry by two different researchers. Only subjects with complete data (results for Gram-chromotrope Kinyoun stain and completed questionnaire) were included in the analysis. The prevalence of infections and illnesses were expressed in percentages. Means [± standard deviations (SD)] and medians [interquartile ranges (IQR)] were used to present quantitative data. All quantitative variables were examined for normality using the Kolmogorov-Smirnov test prior to analysis. The Pearson's χ^2 test was used to test correlations between variables. On univariate analysis, the dependent variable was stool samples positive for microsporidia among all the study subjects, while the independent variables were gender, age, selected socioeconomic factors, behavioral risks, environmental sanitation and living condition characteristics. The ages of participants were categorized into two groups; (i) <10 years old, (ii) ≥10 years old similar to previous studies conducted among aboriginals in Malaysia (Al-Mekhlafi *et al*, 2010). Odd ratios (ORs) and 95% confidence interval (95% CI) were computed for all variables. All variables significantly associated with the presence of microsporidia on the univariate model were included in logistic regression analysis to determine factors associated with intestinal microsporidiosis controlling for possible confounders. Statistical analysis was done using SPSS for WINDOWS, version 20.0 (IBM, Armonk, NY). The level of statistical significance was set as $p < 0.05$.

Ethical considerations

This study was conducted following Declaration of Helsinki guidelines. All procedures involving human subjects were approved by the Universiti Teknologi MARA Research Ethics Committee [reference number: 600-RMI (5/1/6)]. Permission was also obtained from the Department of Orang Asli Development (reference number: JAKOA/PP.30.032 Jld. 12) and the Department of Education, Pahang (reference number: CBA 7151/100-2/2/2).

Prior to commencement of this study, meetings were held with the heads of villages and school headmaster and teachers to provide information about the objectives of and protocol for this study and their consent was obtained to perform the study. During the fieldwork, the purpose and procedures of the study were explained to the children and their parents. They were informed their participation was voluntary and they could withdraw from the study at any time without giving a reason. Written informed consent was obtained from the parents of all study subjects prior to participation. At the end of the study, all the results were submitted to the relevant authorities for appropriate treatment.

RESULTS

General characteristics of participants

Two hundred fifty-five school chil-

Table 1
Prevalence of stool microsporidia among study subjects by age and gender.

	No. examined	No. positive (%)
Age group in years		
<10	103	17 (16.5)
≥10	152	52 (34.2)
Gender		
Boys	138	36 (26.1)
Girls	117	33 (28.2)
Total	255	69 (27.1)

dren aged 7-12 (median 9, IQR 8-11) years were included in the study. Of these, 54.1% were boys. Fifty-three percent of the families had low monthly income (<RM500, USD127.37); the poverty income threshold in Malaysia (Department of Statistics Malaysia, 2015). Moreover, 68.6% and 63.2% of the mothers and fathers, respectively, had no formal education. Forty percent of the mothers and 28.6% of the fathers are not working. Those working were mainly engaged in agriculture (rubber and oil palm plantations), forestry, fishing and related occupations. Thirty-eight percent of the houses are without toilets and it was found that 58.8% of the aboriginal people preferred to defecate at the site of the streams. None of the study subject reported receiving anthelmintic treatment during the previous 6 months.

Prevalence of microsporidia among stool samples

Twenty-seven point one percent of the study subjects (69/255) had microsporidia in their stool sample (Table 1). Of these, 72.5% (50/69) had a microsporidia spore count of 1-10 per 100 fields at 100x magnification and 27.5% (19/69) had a microsporidia spore count of 11-20 per fields at 100x magnification. There was no significant difference in the prevalence of microsporidia in the stool by gender (OR = 0.89; 95%CI: 0.52-1.56; $p = 0.704$).

Factors associated with finding microsporidia in a stool sample

Univariate analysis showed the prevalence of micropsoridia in the stool was significantly more common among children aged ≥10 years (OR = 2.63; 95%CI: 1.42- 4.88; $p = 0.002$) than those aged <10 years. The prevalence of microsporidia in the stool was significantly greater among children from a family with a household monthly income <RM500 (OR = 1.79; 95%CI: 1.02-3.18; $p = 0.042$) and from a household with an unsafe drinking water supply (OR = 3.55; 95%CI:1.97-6.38; $p < 0.001$). Children with close contact with domestic animals had 3.8 times greater odds of having microsporidia in their stool (95%CI: 2.12-6.72; $p < 0.001$).

Multiple logistic regression analysis found being aged ≥10 years (OR = 2.22; 95%CI: 1.10-4.48; $p = 0.026$), having an unsafe drinking water supply (OR = 2.33; 95%CI: 1.02-5.28; $p = 0.044$) and having close contact with domestic animals (OR = 2.33; 95%CI: 1.08-5.02; $p = 0.031$) were significantly associated with finding microsporidia in the study among study subjects (Table 2).

Co-infection with other intestinal parasites

Of the 69 fecal samples with microsporidia, 76.8% also had an additional parasites found in their stool. *Trichuris trichiura* (57.9%) was the most common intestinal parasite found in association with microsporidia, followed by *Entamoeba coli* (36.2%), *Giardia intestinalis* (30.4%), *Entamoeba histolytica/dispar/moshkovskii* (24.6%), *Blastocystis* sp (20.2%), *Ascaris lumbricoides* (17.4%), hookworm (14.5%), *Iodamoeba butschlii* (11.6%) and *Entamoeba hartmanni* and *Chilomastix mesnili* (7.3%).

Table 2
Logistic regression analysis of factors associated with finding microsporidia in the stool of the study subjects.

Variable	OR	95% CI	*p*-value
Aged ≥10 years	2.22	1.10-4.48	0.026
Having an unsafe drinking water supply	2.33	1.02-5.28	0.044
Close contact with domestic animals	2.33	1.08-5.02	0.031

OR, odds ratio; CI, confidence interval.

DISCUSSION

Microsporidia may be overlooked or misdiagnosed because they are not specifically searched in most diagnostic laboratories; they are small and do not stain well with modified trichrome stain. Most of what is known about human microsporidiosis is from HIV infected patients (Dowd *et al*, 1998). However, with increased awareness and improved diagnostics, microsporidia have become more frequently reported among immunocompetent individuals, in whom they are primarily asymptomatic (Nkinin *et al*, 2007). The incidence of microsporidial infections in the general population maybe much higher than previously reported and microsporidia maybe a cause of a more common disease (Sak *et al*, 2011).

Twenty-seven point one percent of the studied aboriginal school children from Kuala Krau District, Pahang, Malaysia in this evaluation had microsporidia in their stools, different from a previous study (20.7%) (Norhayati *et al*, 2007). Studies from Thailand (4.1%) (Leelayoova *et al*, 2005) and Turkey (7.8%) (Calik *et al*, 2011) found lower prevalences. The prevalence of microsporidiosis among aboriginal school children in Malaysia is significantly higher than among other groups in the Malaysian population such as from the local HIV-infected individuals (8.5%) (Lono *et al*, 2011). It is possible decreased immunity due to a higher prevalence of protein-energy malnutrition (37.3%) in the study population increase the risk of microsporidial infection since there appears to be a relationship between malnutrition and other enteric parasitic infections in this community (Al-Mekhlafi *et al*, 2008).

Our findings showed a well-supported correlation between low and moderate spore presence in excretions and positivity among school children, which distinguishes the actual latent microporidial infection from simple consumption and passage of spores through the intestinal tract. By contrast, considerably higher percentage with low (72.4%), moderate (23.3%) and high (4.3%) spore counts were reported by Norhayati *et al* (2008) among hospitalized patients. This suggests the density of infection may be greater among immunosuppressive patients.

We found no difference in the prevalence of finding microsporidia in the stool of children by gender, similar to previous studies conducted by Wanachiwanawin *et al*, (2002) and Norhayati *et al*, (2007). In our study, children aged ≥10 years were significantly more likely to have a microsporidial infection than children aged 7-9 years. Previous local study carried out by Norhayati *et al* (2008) demonstrated a high prevalence (26.1%) of microsporidial

infection among children aged 0-6 years. The high prevalence rate of microsporidia among those aged ≥10 years in the present study might be explained by the fact that they are more active and have more independent eating habits compared with their younger counterparts. However, it is also possible that microsporidia persisted but was shed less consistently or at levels below detection in younger age group.

In the present study, involvement of zoonotic transmission routes has been observed. Close contacts between children and their domestic animals *eg*, dogs, cats and chickens are very frequent in aboriginal communities. Study done by Mori *et al* (2013) suggested that opportunities for zoonotic transmission are assumed to be higher in developing countries, especially in rural areas due to frequent animal contact. In fact, human microsporidia species have been isolated from a large number of domestic and wild animals (Mathis *et al*, 1999; Haro *et al*, 2005). This zoonotic transmission is supported by phylogenetic studies which demonstrate that several genotypes can infect both humans and animals (Lobo *et al*, 2006; Henriques-Gil *et al*, 2010). Cama *et al* (2007) also reported possible zoonotic transmission from domestic guinea pigs to a child with no evidence of immunosuppression.

Waterborne transmission has been speculated as a mode of transmission for microsporidia especially those conducted in tropical countries and in travelers who just returned from these countries (Muller *et al*, 2001). In our study, using multivariate analysis we found microsporidiosis was two times more likely to occur in children who used unsafe drinking water. Aboriginal people in Malaysia often live close to rivers and use the river water for drinking and cooking. Rivers are also common defecation site for children. River water may become contaminated by parasites. Microsporidia are small and may not be removed by some water filters (Sparfel *et al*, 1997). The spores of microsporidia have been shown to be resistant to chlorine at the concentrations found in tap water (Dowd *et al*, 1998). Microsporidia should be considered as a potentially waterborne disease.

This study had some limitations. First, standard light microscope cannot detect species of microsporidia. Transmission electron microscopy or polymerase chain reaction testing are required to do this. Therefore, species of microsporidia were not determined in our study. Second, we only studied human samples. Therefore, we could not evaluate zoonotic transmission in this study. The current study is a preliminary study. We plan on conducting a larger longitudinal study to include animal and environmental (water) samples, in addition to human samples.

In conclusion, the findings of the present study show microsporidia are commonly found in the study population of asymptomatic aboriginal school children in rural Malaysia. There is an urgent need to implement satisfactory control program to reduce the prevalence of microsporidia in the stool of asymptomatic aboriginal children in Malaysia.

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