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Microsporidia infection among various groups of the immunocompromised patients

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Abstract. While information with regards to the bacterial and viral infections are commonly available among clinicians, data on parasitic infection, particularly *Microsporidia* among immunocompromised patient is currently lacking in Malaysia. This study was conducted to determine the prevalence of *Microsporidia* among a various group of immunocompromised patient. Two hundred and eighty-eight archived stool samples were examined for the presence of *Microsporidia* with Gram-Chromotrope Kinyoun staining method. The overall prevalence of *Microsporidia* was 29.2 % (84/288; 95% CI=24.2-34.5). The end-stage renal failure (ESRF) patients (32.1%) recorded the highest infection rate, followed by cancer (26.2%), human immunodeficiency virus (HIV/AIDS) (22.6%) and acute gastroenteritis (AGE) (7.1%). Meanwhile, organ transplant recipients and autoimmune disease patients recorded the lowest prevalence rate (6.0%). Other intestinal parasites were *Strongyloides stercoralis*, *Trichuris trichiura*, *Ascaris lumbricoides* and *Cryptosporidium* species. Diarrhoea was the most common symptoms among patients with microsporidiosis. The present study showed that the prevalence of *Microsporidia* infection was relatively high among immunocompromised patients. This finding highlighted the importance to include detection of microsporidia infection as a routine differential diagnosis in immunocompromised patients, which serves the benefit of treatment to the patients.

INTRODUCTION

Opportunistic infections are diseases with a facultative pathogenic organism that includes a wide range of viruses, bacteria, and parasites. Such pathogens are capable of causing disease when the host's resistance is lowered, by other diseases or drugs such as in immunocompromised patients. The immunocompromised group comprises a wide range of patients with disordered immune systems including acquired immune deficiency syndrome (AIDS), human immunodeficiency virus (HIV), patients on medical treatment such as chemotherapy, organ or bone marrow transplantation.

The opportunistic intestinal parasites are one of the major causes of uncontrollable debilitating illness including diarrhoea in human. The infection is self-limiting in immunocompetent hosts, which readily clear the parasites. However, persistent diarrhoea and severe malabsorption are common clinical presentations among immunodeficient host. Reports indicated that diarrhoea occurs up in 60.0% of immunocompromised patients in developed countries and in approximately 90.0% in developing countries with opportunistic intestinal protozoa playing major roles (Kulkarni *et al.*, 2009). The variation in the prevalence may be because of differences in the diagnostic techniques, geographical location, socioeconomic status, immunity status as well change in personal and environmental hygiene (Teklemariam *et al.*, 2013).

Microsporidia are recognized as one of the most common emerging opportunistic parasitic pathogen among immunodeficient

hosts (Matos *et al.*, 2010; Lono *et al.*, 2011). *Microsporidia* are single-celled, intracellular spore-forming and eukaryotic protozoa parasite that belongs to phylum *Microspora*. Mature spores of the *Microsporidia* are tiny (1 to 4 µm). The spores possess a unique organelle, the polar tubule or polar filament, which is coiled inside the spores as demonstrated by its ultra-structure (Didier, 2005).

To date, more than 1,200 species belonging to 143 genera have been identified as parasites infecting a wide range of vertebrate and invertebrate hosts. There are at least 15 *Microsporidia* species that have been identified as human pathogens. This includes *Anncaliia algerae*, *A. connori*, *A. vesicularum*, *Encephalitozoon cuniculi*, *E. hellem*, *E. intestinalis*, *Enterocytozoon bieneusi*, *Microsporidium ceylonensis*, *M. africanum*, *Nosema ocularum*, *Pleistophora* sp., *Trachipleistophora hominis*, *T. anthropophthera*, *Vittaforma corneae*, and *Tubulinosema acridophagus* (Didier *et al.*, 2004; Didier & Weiss, 2006; Matos *et al.*, 2010). The most common species infecting human are *Encephalitozoon* and *Enterocytozoon* species (Didier & Weiss, 2006; Anane & Attouchi, 2010; Matos *et al.*, 2010). Although not known with certainty, transmission of *Microsporidia* is thought to be human to human (Bergquist *et al.*, 1984; Birthistle *et al.*, 1996) and animal to human contact (Bern *et al.*, 2005; Leelayoova *et al.*, 2005; Zhang *et al.*, 2011; Velásquez *et al.*, 2012). Infection may also result from ingestion or inhalation of spores from the environment including water (Li *et al.*, 2012; Galván *et al.*, 2013) and food (Hutin *et al.*, 1998; Didier *et al.*, 2004; Decraene *et al.*, 2012).

In Malaysia, the information on the prevalence of *Microsporidia* infection among immunocompromised patients is not well studied. Information on other parasitic, bacterial and viral infections is more commonly available to the local physician. The lack of information regarding *Microsporidia* may be because it is currently not included in a routine diagnostic test which may indirectly result in it regarded as 'uncommon' infection. However, many previous studies have acknowledged that *Microsporidia* are widespread among the immunocompromised individuals, especially in AIDS/HIV patients (Didier & Weiss, 2006; Matos *et al.*, 2010; Sherchan *et al.*, 2013).

To date, there were few studies of *Microsporidia* infection in Malaysia with prevalence ranging between 8.5% and 21.2% (Norhayati *et al.*, 2007; 2008; Lono *et al.*, 2010; 2011; Anuar *et al.*, 2013). Of this, only one study was carried out among HIV individual (Lono *et al.*, 2011), while others in the general populations. This clearly showed that data on the microsporidiosis among immunocompromised hosts is currently lacking. With this in mind, this study was carried out to determine the prevalence of *Microsporidia* and its association with clinical manifestations among various groups of immunocompromised patient. The finding of this study will provide useful insights and information into the epidemiology of this infection, thus facilitating the understanding of clinical manifestation, diagnosis, and treatment management that will benefit the patients.

MATERIALS AND METHODS

Study design and subject

The stool samples were obtained from the archived pool of the Parasite Southeast Asian Diagnostic (Para: SEAD) Laboratory at Department of Parasitology, Faculty of Medicine, the University of Malaya. In brief, the stool sample was sent to the Para: SEAD laboratory for a routine diagnosis of parasitic infections from the University of Malaya Medical Centre (UMMC). Only samples with a confirmed diagnosis of immunodeficiency or treated with immunosuppressive therapy as stated in the standard request form and confirmed by the attended physician patients were examined. The sample in this study was not randomized. Information such as demographic, disease stage/type and clinical symptoms were obtained from the standard request form submitted along with the stool sample.

Ethical consideration and sample size calculation

The Medical Ethics Committee of the University Malaya Medical Centre (UMMC) (IRB Ref. No. 655.17) approved the methodology, considering there to be no risks against the physical well-being, integrity or right to anonymity of the participants. The sample size was calculated according to the anticipated and latest prevalence of intestinal *Microsporidia* infection among immunocompromised patients in Malaysia. According to the previous study, the overall prevalence was 8.5 % (Lono *et al.*, 2011). By using a significance level of 5% and confidence level of 95%, a minimum sample size of 120 was required in this study.

Detection of *Microsporidia* by Gram-Chromotrope Kinyoun staining

Gram-chromotrope Kinyoun (GCK) staining was used to detect the presence of *Microsporidia* spp. (Moura *et al.*, 1996). Stool smear was prepared on a glass slide and air dried. Then, the smear was fixed with methanol and stained with crystal violet for one minute. The excess stain was rinsed off with Gram's iodine. With the same reagent, the slide was then stained for two minutes. The Gram's iodine solution was removed by gently rinsing with a decolourized until the flow become colourless. The slide was washed with tap water and stained with chromotrope for 8 minutes. The slide was rinsed with 90% acid-alcohol and counter-stained with Kinyoun's carbol fuchsin for 3 minutes. The slide was rinsed with 90% acid-alcohol, followed by 95% alcohol for 5 minutes and finally 100% ethyl alcohol for 2 minutes. Finally, the slide was mounted with DPX medium and covered with a cover slip for microscopic examination. The slide was examined under 1000x magnifications.

Microsporidia were observed microscopically based on the unique morphological characteristics that have pinkish-blue ovoid with belt-like stripe microorganism against a relatively clean or pale pink background. The spore seen in the stained samples was graded as follows: 1+ (average number of spores seen was 1-10), 2+ (average number of spores seen was 11-20) and 3+ (average number of spores seen was >21) (Norhayati *et al.*, 2008). Additionnaly, the stool sample was analysed by formalin-ether concentration techniques for the presence of other intestinal parasites.

Data analysis

The collected data were analyzed using SPSS software (Statistical Package for the Social Sciences) program version 21. All the data entry was cross-checked consistently before the study. A simple descriptive analysis such as percentage and rate was applied to calculate the prevalence of *Microsporidia*. The degree of association was determined using chi-square test (x^2). The dependent variable was prevalence rate while the independent variables were demographic, sign and symptoms as well as the type of diseases. A significant level of $p<0.05$ was used for all tests.

RESULTS

Demographic and general characteristics of the study population

A total of 288 stool samples were examined in this study. Of this, stools of cancer patients were the most highest (69/288; 24.0%), followed by HIV/AIDS (65/288; 22.6%), acute gastroenteritis (AGE) (53/288; 18.4%), end-stage renal failure (ESRF) (51/288; 17.7%), organ transplant recipients (27/288; 9.4%), leukemia (15/288; 5.2%) and autoimmune disease (8/288; 2.8%). Males constituted 186 (64.6%) of the study population and females was 102 (35.4%) participants. Based on the age group, 43 (14.9%) were aged less than 18 years old and 245 (85.1%) aged 18 years old and above. The age range was to 93 years old (median age of 42 years) (Table 1). Of this, more than half (174; 60.4%) were presented clinical manifestations associated with gastrointestinal illness such as mild to chronic diarrhoea. Other symptoms such as nausea (7; 2.4%), fever (6; 2.1%) and weight loss (1; 0.3%) were also reported among these patients.

Table 1. Prevalence of *Microsporidia* infection based on the demographic characteristics among immunocompromised patients (N=288)

Variables	N	n	%	95% CI
Gender				
Male	186	60	32.3	26.0–39.3
Female	102	24	23.5	16.4–32.6
Age groups (years)				
<18	43	12	27.9	16.8–42.7
≥18	245	72	29.4	24.0–35.4
Race				
Indian	49	15	30.6	19.5–44.5
Chinese	112	34	30.4	22.6–39.4
Malay	117	34	29.1	21.6–37.9
Others	10	1	10.0	7.9–40.4
Sign and symptoms				
Diarrhea	174	58	33.3	48.7–67.8
Fever	6	2	33.3	0.6–0.7
More than one symptoms	100	24	24.0	16.9–33.5
Nausea	7	0	0	0
Weight loss	1	0	0	0
Total	288	84	29.2	24.2–34.5

N: number examined; n: number positive; %: percentage positive; 95% CI: 95% Confidence Interval.

Prevalence of *Microsporidia* infection

The overall prevalence of *Microsporidia* was 29.2% (84/288; 95% CI=24.2–34.5). The highest prevalence was recorded among ESRF patient (32.1%; 95% CI=23.1–42.7), followed by cancer (26.2%; 95% CI=18.0–36.8), HIV/AIDS (22.6%; 95% CI=15.0–32.7), and AGE (7.1%; 95% CI=3.3–14.7). Organ transplant recipients and autoimmune disease patients showed similar prevalence of 6.0% (95% CI=2.6–13.2) (Table 2). Of this positive samples, 32.3% (95% CI=26.0–39.3) were male, which was slightly higher compared to female (23.5%; 95% CI=16.4–32.6). Based on age group, approximately 29.4% (95% CI=24.0–35.4) were aged 18 years old and above compared to 27.9% (95% CI=16.8–42.7) for those aged less than 18 years old. Based on the ethnic group, 30.6% (95% CI=19.4–44.5) were Indian, followed by 30.4% (95% CI=22.6–39.4) Chinese, 29.1% (95% CI=21.6–37.9) Malay and 10.0% (95% CI=1.79–40.4) of other races (Table 1).

With regards to the signs and symptoms, 69.0% microscopically positive patients showed diarrhoea (95% CI=58.5–77.9) (Table 1). In addition, almost all of the *Microsporidia* positive samples (96.4%) were reported with low spore counts of 1 to 10 per fields. Three (3.6%) samples were recorded with moderated spore counts (11 to 20 per fields) with no substantial spore counts. The prevalence of microsporidiosis was significantly higher among human immunodeficiency virus (HIV/AIDS) (p=0.048), autoimmune disease (p=0.035), acute gastroenteritis (AGE) (p=0.002) and ESRF (p<0.001) compared to the uninfected individuals.

Prevalence of other intestinal parasites

Other intestinal parasitic infections (IPIs) were also reported among these immunocompromised patients including *Strongyloides stercoralis* (14.2%; 95% CI=10.7–18.7), *Trichuris trichiura* (5.2%; 95% CI=3.2–8.4), *Cryptosporidium* spp. (3.8%; 95% CI=2.1–6.7) and *Ascaris lumbricoides* (1.7%; 95% CI=0.7–4.0) (Table 3). As for *S. stercoralis*, the highest prevalence was reported among HIV/AIDS patients (48.8%; 95% CI=34.3–63.5), followed by cancer (29.3%; 95% CI=17.6–

Table 2. Prevalence of *Microsporidia* based on the type of immunosuppression among immunocompromised patients

Type of immunosuppression	N	n	%	95% CI
Infectious				
HIV/AIDS	65	19	22.6	15.0–32.7
Chemotherapy/Drugs				
Cancer	69	22	26.2	18.0–36.8
Organ transplant receiver	27	5	8.4	2.6–13.2
Leukemia	15	0	0	0
Physiological disorder/Others				
ESRF	51	27	32.1	23.1–42.7
AGE	53	6	8.4	3.3–14.7
Autoimmune diseases	8	5	8.4	2.6–13.2

N: number examined; n: number positive; %: percentage positive; 95% CI: 95% Confidence Interval.

Table 3. Prevalence of other intestinal parasites among the immunocompromised patients

Variables	*S. stercoralis*			*Cryptosporidum* spp.			*T. trichiura*			*A. lumbricoides*		
	n	%	95% CI	n	%	95% CI	n	%	95% CI	n	%	95% CI
HIV/AIDS	20	48.8	34.2–63.5	3	27.3	9.8–56.6	5	33.3	15.2–58.3	1	20	3.6–62.5
Cancer	12	29.3	17.6–44.5	4	36.4	15.2–64.6	2	13.3	3.7–37.9	–	–	–
ESRF	5	12.1	5.3–25.5	2	18.2	5.1–47.7	5	33.3	15.2–58.3	1	20	3.6–62.5
AGE	2	4.9	1.4–16.1	–	–	–	3	20.0	7.1–45.2	3	60	23.1–88.2
Organ transplant receiver	2	4.9	1.4–16.1	1	0.9	1.6–37.7	–	–	–	–	–	–
Autoimmune disease	–	–	–	1	0.9	1.6–37.7	–	–	–	–	–	–
Leukemia	–	–	–	–	–	–	–	–	–	–	–	–

n: number positive; %: percentage positive; 95% CI: Confidence Interval.

44.5), ESRF (12.1%; 95% CI=5.3–25.5), organ transplant recipients and AGE (4.9%; 95% CI=1.4–16.1). *Cryptosporidium* spp. was mainly recorded among cancer patients (36.4%; 95% CI=15.2–64.6), followed by HIV/AIDS (27.3%; 95% CI=9.8–56.6) and ESRF (18.2%; 95% CI=5.1–47.7). On the other hand, *T. trichiura* was detected equally among HIV/AIDS and ESRF patients (33.3%; 95% CI=15.2-58.3). About 20.0% (95% CI=7.1–45.2) of this nematode was also reported among AGE patients followed with cancer patients (13.3%; 95% CI=3.7–37.9). Of the *A. lumbricoides* positive, 60.0% (95% CI=23.1–88.2) were reported among AGE patients followed with HIV/AIDS and ESRF, of each reported as 20.0% (95% CI=3.6–62.5).

Co-infection of *Microsporidia* with other intestinal parasites

With regards to the co-infection between *Microsporidia* and other intestinal parasites, 38.1% (95% CI=28.5–48.8) were infected with more than one species. Of this, *S. stercoralis* was the most common double infection detected (87.5%; 95% CI=71.9–95.0), followed by *T. trichiura* (18.8%; 95% CI=8.9–35.3), *Cryptosporidium* spp. (12.5%; 95% CI=5.0–28.1) and *A. lumbricoides* (6.3%; 95% CI= 1.7–20.2).

DISCUSSION

In recent years, *Microsporidia* infection has become a common parasitic pathogen in human (Samie *et al.*, 2007). *Encephalitozoon* spp. was the first case of human microsporidiosis reported in 1959 (Matsubayashi *et al.*, 1959). However, this first documented case of microsporidiosis in human are not gained much attention until the AIDS pandemic in the 1980s (Brasil *et al.*, 1996). Only serology techniques are available to identify the species of *Microsporidia* at that particular time (Hollister & Canning, 1987). Thus, the prevalence data at that time is based on the serology of anti-*Microsporidia* (Franzen & Muller, 1999; Didier 2005). To date, *Microsporidia* infection has a worldwide distribution with prevalence varying widely depending on the geographical region, studied population and diagnostic method (Franzen & Muller, 1999; Didier & Weiss 2006).

The present study showed that the prevalence of *Microsporidia* among immunocompromised patients was slightly higher compared to previous local studies (Norhayati *et al.*, 2007; 2008; Lono *et al.*, 2010; 2011; Anuar *et al.*, 2013). Previous studies of *Microsporidia* among indigenous groups reported that the prevalence ranged from 15.0% to 21.2% (Norhayati *et al.*, 2007; Lono *et al.*, 2010; Anuar *et al.*, 2013). Another study among patients with and without gastrointestinal symptoms showed a prevalence of 13.0% (Norhayati *et al.*, 2008). The only available local survey of the *Microsporidia* among the immunocompromised patient (i.e., HIV individual) reported a prevalence of 8.5% (Lono *et al.*, 2011). A similar finding was reported in Germany (0.7%) (Muller *et al.*, 2001) and Peru (8.0%) (Anane *et al.*, 2011). Other studies in Spain (17%) (Lores *et al.*, 2002) and Thailand (14.9%) (Wanachiwanawin *et al.*, 2002), however, showed a slightly higher prevalence rate among immunocompromised individuals.

In the present study, Gram-chromotrope Kinyoun (GCK) staining was used for the identification of *Microsporidia* spores. The GCK staining has higher sensitivity and specificity of 98.0% and 98.3% compared to the reference technique (i.e., Weber modified trichrome) as shown in a previous study (Salleh *et al.*, 2011). In addition, this could be associated with the relatively high level of immune status of the patients after taking some antiretroviral medication (Maggi *et al.*, 2000). The study has demonstrated that effective anti-retroviral therapy was associated with restoration of immune response with accompanying the resolution of opportunistic infection including *Microsporidia* (Maggi *et al.*, 2000). The resolution of diarrhoea seemed to be related to an increase of CD4+ cell count (Maggi *et al.*, 2000). Unfortunately, the information on CD4+ count was not available in the present study due to privacy ruling, which warrants future investigation.

The present study showed no significant difference in the prevalence of *Microsporidia* according to the gender, age group and race, a finding that is in accordance with previous local studies (Norhayati *et al.*, 2007; 2008; Lono *et al.*, 2010; 2011). Similar observations were also reported from other countries (Rukman *et al.*, 2008; Sak *et al.*, 2011). In contrast, a study among Malaysian indigenous group stated that there was a significant difference between participant aged 15 years old and above (Anuar *et al.*, 2013), most probably due to the differences in the sample size and age-cut-off value.

Symptoms such as diarrhoea, nausea, malabsorption and weight loss are commonly reported among immunocompromised patients with intestinal parasitic infection including *Microsporidia* infection (Wanachiwanawin *et al.*, 2002). Reports indicated that diarrhoea is the most common, with approximately 90.0% of diarrhoea cases associated with intestinal parasitism (Kulkarni *et al.*, 2009). In such cases, chronic diarrhoea more commonly reported in HIV-positive individuals compared to acute diarrhoea. The numbers of *Microsporidia*-related acute diarrhoea may be low because the symptoms are self- limiting and usually unreported (Lono *et al.*, 2011). It is critical to determine if asymptomatic and persistent *Microsporidia* infection occurs in humans, and if so, improved and reliable diagnostic

methods are needed to prevent the transmission to others at risk.

The aetiology of diarrhoea in the present study, however, could not be confirmed due to *Microsporidia* infection alone. It is well noted that other intestinal pathogen infections were also associated with diarrhoea. In the present study, more than half of *Microsporidia* positive samples were also co-infected with other intestinal parasites including *S. stercoralis*, *T. trichiura*, *Cryptosporidium* spp. and *A. lumbricoides*. These parasites were common pathogens for diarrhoea. Moreover, more than half of the co-infected patients with both *Microsporidia* and other parasite were presented with diarrhoea.

Accurate and specific diagnosis is essential for the formulation of effective control measures in any infectious diseases. Most research conducted on the epidemiology of *Microsporidia* and other intestinal parasites still relied on the use of microscopy technique for the identification of spores in a stool sample. The benefits of this method are mainly due to technical simplicity and low cost. The utilization of microscopic technique, however, is limited by the fact that the *Microsporidia* spores are morphologically indistinguishable from those of other species, laborious, time-consuming and requires relatively skilled personnel. In addition, this may lead to an incorrect diagnosis and unnecessary treatment to the patients. There is a need for a practical, highly sensitive and specific diagnostic and analytical tool, particularly those based on polymerase chain reaction (PCR) to be applied to strengthen the surveillance, treatment and control measure, a study that needed further investigation in the future investigation.

Previous studies based on PCR have demonstrated that the most common *Microsporidia* species infecting human belong to the genera of *Encephalitozoon* and *Enterocytozoon*. There are, however other species from other genera such as *Vittaforma*, *Anncaliia* and *Trachipleistophora* with similar morphology characteristic were reported in human (Didier *et al.*, 2004; Didier & Weiss, 2006; Matos *et al.*, 2010). In addition, PCR method has high sensitivity and specificity compared to microscopy technique (Muller *et al.*, 1999). The lowest detection limit of PCR for *Microsporidia* is 10^2 spores/g of the stool as reported in the previous study. In contrasts, the optical microscopy cut off is around 10^4 to 10^6 spores/g of stool (Franzen *et al.*, 1999; Didier *et al.*, 2004). Apart from the specific species differentiation, information at a molecular level possibly will provide new insights of other virulence species of *Microsporidia* infecting humans.

CONCLUSION

The present study showed that the prevalence of *Microsporidia* infection is relatively high among immunocompromised patients. The highest incidence was recorded among end-stage renal failure (ESFR) patient, followed by cancer, human immunodeficiency virus (HIV/AIDS), acute gastroenteritis (AGE), organ transplant recipients and autoimmune disease patients. Diarrhea is the most common clinical manifestation reported among patients with *Microsporidia*. These findings demonstrated the importance of identification of *Microsporidia* in stool samples of immunocompromised patients which in form will help in instituting appropriate treatment and better management of the patients. The diagnosis of *Microsporidia* should also be included as part of routine laboratory diagnosis to prevent these infections from becoming more severe health problems in immunocompromised patients due to it greater susceptibility.

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