MOLECULAR IDENTIFICATION OF MICROSPORIDIA SPECIES AMONG REFUGEE SCHOOL CHILDREN, SELANGOR, MALAYSIA

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Abstract. Microsporidia are obligate intracellular spore-forming protozoan parasites in humans. Two species commonly reported as etiology agents in humans are *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*. In Malaysia, microsporidiosis has been reported among hospitalized patients and the rural aboriginal community. However, recent concern has emerged regarding the health of refugee communities in Malaysia, due to their neglect by relevant governmental agencies. This study determined the prevalence of microsporidia among refugee school children in five districts of Selangor. Stool samples (n = 91) were collected and the presence of microsporidia was identified by diagnostic multiplex PCR of the small subunit ribosomal (SSU r) DNA. *Ent. bieneusi*, *Enc. intestinalis* and both species was present in 20, 2 and 2%, respectively. Phylogenetic analysis of the SSU rDNA sequences revealed each microsporidia species clustered into a single clade, with that of *Ent. bieneusi* having 87% similarity to a reference sequence from immunocompromised patients in Kuala Lumpur, while that of *Enc. intestinalis* a 38% sequence similarity to a reference sequence from diarrheal patient(s) in Pakistan. Thus, we suggest that sensitive and frequent surveillance together with intervention programs of microsporidia infection should be implemented in the marginalized communities in the country to minimize the potential emergence of public health issues.

Keywords: microsporidia, molecular detection, multiplex PCR, phylogenetic analysis, refugee children

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INTRODUCTION

Microsporidia is a class of obligate intracellular parasites that has a resemblance to the fungi kingdom, although the exact nature of this relationship is unclear. According to the Centers for Disease Control and Prevention, USA (CDC, 2019), microsporidia is recognized as one of the many opportunistic pathogens worldwide transmitting microsporidiosis, a health condition presenting chronic diarrhea, malabsorption and a series of disseminated diseases in patients with immunocompromised status (Han *et al*, 2021). However, individuals with normal immune system usually present self-limiting diarrhea (Franzen and Muller, 2001).

More than 1,400 microsporidia species belonging to 200 genera with invertebrates as hosts and capable of infecting various vertebrates have been reported (Ruan *et al*, 2021; CDC, 2019). However, approximately 17 microsporidia species have been identified to target humans as their primary host (Yusoff *et al*, 2021). The classical classification of microsporidia is based on ultrastructural features, such as the number of spirals in the polar tube, size, shape, morphology of the spores, life cycle features, and the host-parasite relationship (Yusoff *et al*, 2021). More recently, molecular phylogenetic analysis using small subunit (SSU) rDNA sequences or internal transcribed sequences (ITS) has enabled microsporidia to be assigned to specific genera as well as distinguished at the species level (Matos *et al*, 2012). The most common species associated with microsporidiosis in humans is *Enterocytozoon bieneusi* and to a lesser extent *Encephalitozoon*

intestinalis (Yusoff *et al*, 2021; CDC, 2019).

Currently, more than 500 genotypes of *Ent. bieneusi* have been reported based on ITS nucleotide sequences and are further classified into 11 total groups, Groups 1-11 (Qin *et al*, 2022). These *Ent. bieneusi* Groups are found in human microsporidiosis and in a broad spectrum of domestic and wild animals worldwide, such as poultry, ruminants, non-human primates, and birds (Anuar *et al*, 2013; Qin *et al*, 2022). The abundance of animals that act as reservoir host of *E. bieneusi* can help explain it being the primary etiology of human microsporidiosis especially in people residing in poor hygienic environments.

In Malaysia, microsporidiosis is limited to sporadic cases reported in hospitalized patients, HIV-infected patients and aboriginal communities in Pahang (Shehab *et al*, 2021). There have been limited studies of microsporidiosis in these marginalized Malaysian communities despite their susceptibility to this infection. To date, in Malaysia, there are only two studies on microsporidiosis prevalence in Orang Asli (aboriginal) people and HIV-infected patients (Ashikin *et al*, 2017; Hassan *et al*, 2018).

Marginalized communities are defined as vulnerable groups that require access to health facilities and constant monitoring of their health conditions since they are prone to systemic inequalities in the health services (Hanapi *et al*, 2024). In Malaysia, marginalized communities consist of Orang Asli communities, HIV-affected individuals, asylum seekers, refugees, and other similar groups who need special support and protection. As of June 2024, there are 190,370 registered refugees and asylum claimants in the country (UNHCR Malaysia, 2024), the majority being Myanmar refugees (n = 109,650), with the remaining from Afghanistan, Iraq, Pakistan, Palestine Somalia, Sri Lanka, Syria, and Yemen.

This study determined

microsporidia prevalence in refugee school children and identified the infecting species using a molecular approach to establish baseline data for further epidemiological research among marginalized populations in Malaysia.

MATERIALS AND METHODS

Study design, location and test population

This cross-sectional study was conducted in five different districts in the State of Selangor, Malaysia from February 2023 to June 2024. The test population comprised randomly recruited refugee children, 3-17 years of age, attending refugee schools located in the five abovementioned districts in Selangor.

Sample collection

The minimum sample size (N) was calculated using the following equation (Lwanga and Lemeshow, 1991):

$$N = Z^2p(1-p)/e^2]$$

Where Z = the value for a 95% confidence interval which is 1.96

p = prevalence of microsporidia

e = sampling error

According to a previous study (Anuar *et al*, 2016), the prevalence of microsporidia among children in Malaysia was 27.1%. When allowing 10% sampling error (e = 0.1), the minimum sample size of N = 76. However, to reduce any potential of non-response and incomplete samples, the final target sample size was increased by 20% above the minimum calculated sample size, thus a total of 91 stool samples was needed and collected for subsequent analysis at the Parasitology Laboratory, Faculty of Medicine and Health Science, Universiti Putra Malaysia.

Identification of microsporidia species by multiplex PCR

DNA was extracted from the fecal material (200 g) using the NucleoSpin® DNA Stool kit (Macherey-Nagel GmbH, Duren, Germany) according to the manufacturer's instructions. DNA was collected in 50 µl of the elution buffer and stored at -30 °C until used.

Multiplex PCR was conducted using primers specific to the ribosome small subunit (SSU) rDNA rDNA) of *Ent. bieneusi* [EBIEF1 (5′ GAAACTTGTCCACTCCTTACG 3′) and EBIER1 (5′ CCATGCACCACTCCTGCCATT 3′)] and *Enc. intestinalis* [SINTF (5′ TTT CGAGTGTAAAGGAGTCGA 3′) and SINTR (5′ CCGTCCTCGTTCTCCTGCCCG 3′)] (Franzen and Muller, 2001). PCR assay was performed in a 15-µl reaction mixture containing 7.5 µl of 2×Rapid Taq Master Mix (Vazyme Biotech, Nanjing, PR China), 4.3 µl of nuclease-free water, 0.3 µl each of the two primers for *Ent. bieneusi* and *Enc. intestinalis*, and 1.5 µl of DNA template. Amplification was performed using a MyCycler thermal cycler (Bio-Rad, Hercules, CA) as follows: 94 °C for 5 minutes; 35 cycles of 94 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 90 seconds; with a final step of 72 °C for 10 minutes. Amplicons (607 and 560 bp for *Ent. bieneusi* and *Enc. intestinalis* respectively) were separated by 1.7% agarose gel-electrophoresis at 100 V for 45 minutes, stained with FloroSafe DNA dye (Stain Axil Scientific, Woodlands, Singapore) and visualized under UV illumination. PCR amplifications were performed in parallel with positive controls, *Ent. bieneusi* (Matrioux Science Lab, Selangor, Malaysia) and *Enc. intestinalis* ATCC 50651. Gel extracted amplicons were directly sequenced (1st Base Sequencing; Apical Scientific, Selangor, Malaysia) and submitted to GenBank, *Ent. bieneusi* accession nos. E.B PQ285199- E.B PQ285203 and *Enc. intestinalis* accession nos. E.I PQ285204 and E.I PQ285206.

Phylogenetic analysis

Nucleotide sequences obtained in the present study and reference sequences downloaded from GenBank, National Center for Biotechnology Information (NCBI), were aligned with each other using the Clustal X 2.0 program as implemented in MEGA 11 (Tamura *et al*, 2021) to determine the genotypes. Phylogenetic analysis was performed using MEGA software version 11 (Tamura *et al*, 2021). The maximum likelihood method was based on the Hasegawa (Kishino and Hasegawa, 1989) with bootstrap sampling at 1,000 replicates (Soltis and Soltis, 2003). The available reference sequences (RefSeq) for *Ent. bieneusi* and *Enc. intestinalis* were retrieved from GenBank and used as controls.

Statistical analysis

Descriptive analysis (frequency and percentage) was used to determine the occurrence of *Ent. bieneusi* and *Enc. intestinalis* and the socio-demographic factors, while Fisher's Exact test was used to identify the association between the two *Microsporidia* spp and socio-demographic factors. A *p*-value <0.05 is considered significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software version 29.0 for Microsoft Windows (IBM Corporation, Armonk, NY).

Ethical considerations

Ethical approval for the study protocol was obtained from the Medical Ethics Committee of Universiti Putra Malaysia (JKEUPM.2021.442). Prior written consent was obtained from the parent/legal guardian of each child participant.

RESULTS

Prevalence of *Ent. bieneusi* and *Enc. intestinalis* using multiplex PCR detection method

Of the 91 stool samples examined in this study, microsporidia were detected using multiplex PCR in 22 (24%) samples, with 18 (20%) and 2 (2%) infections of *Ent. bieneusi* and *Enc. intestinalis* respectively, and

2 (2%) infections of both species (Table 1). Al-Islamiyah Alternative Learning Centre, Kajang had the highest microsporidia prevalence (27%), followed by REC Puchong, REC Gombak, Refugees Jenjarom, and RISE Kajang (14-18% each school) (Table 1). The two cases with mixed infection were confined to REC Klang and REC Gombak.

Association among socio-demographic characteristics with microsporidia species

Of the *Ent. bieneusi*-positive children, there was nearly an equal distribution between the sexes, and those of 7-12 and 13-17 years of age had a higher prevalence (15% and 38% respectively) compared to the younger age group (10%) (Table 2). The majority of infected children had working parents (19%), with families of 2-5 other members (20%) and lived in flats/apartments (21%) than in low-cost terrace houses. No associations of *Ent. bieneusi* infection in children were discerned among the sociodemographic characteristics.

Of *Ent. intestinalis*-positive children (n = 2), one was a boy and the other a girl, one in 0-6 years and the other in the 13-17 years of age group; one each had a working and non-working parent, both with a family of two other members; and one each lived in a flat/apartment and a low-cost terrace house (Table 2). Given the small number of samples, it was not possible to calculate the statistical association of infection with the socio-demographic characteristics.

Apart from that, there were mixed infection of both species in 2 samples of refugee children from REC Klang and REC Gombak. Both samples were taken from the girls aged between 13 to 17 years old; had a working parent with a family of two and three to five members and lived in low-cost terrace (Table 2). No associations of mixed infection in children with their sociodemographic characteristics.

Phylogenetic relationships

Phylogenetic analysis based on SSU rDNA sequence of *Ent. bieneusi* samples from refugee school children revealed that the

Table 1

Enterocytozoon bieneusi and *Encephalitozoon intestinalis* among refugee school children, Selangor, Malaysia

Refugee school	Total number of samples (N)	Positive microsporidia by PCR *n* (%)	*Enterocytozoon bieneusi* *n* (%)	*Encephalitozoon intestinalis* *n* (%)	*Ent. bieneusi* and *Enc. intestinalis* *n* (%)
Refugees Education Centre (REC), Klang	19	2 (11)	1 (5)	0 (0)	1 (5)
Refugees Education Centre (REC), Gombak	18	4 (22)	2 (11)	1 (5)	1 (5)
Refugees Education Garden, Puchong	20	5 (25)	4 (20)	1 (5)	0 (0)
Refugees school, Jenjarom	6	2 (33)	2 (33)	0 (0)	0 (0)
Al-Islamiyah Alternative Learning Center, Kajang	7	6 (86)	6 (86)	0 (0)	0 (0)
Refugees Intellectual Skills Education, Kajang	21	3 (14)	3 (14)	0 (0)	0 (0)
Total	91	22 (24)	18 (20)	2 (2)	2 (2)

PCR: polymerase chain reaction

Table 2

Socio-demographic characteristics of refugee school children (N = 91) with *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* infections, Selangor, Malaysia

Socio-demographic characteristic	Frequency *n* (%)	*Enterocytozoon bieneusi* positive			*Encephalitozoon intestinalis* positive			Mixed infection (*Ent. bieneusi* and *Enc. intestinalis*)		
		Frequency *n* (%)	Fisher's exact test	*p*-value	Frequency *n* (%)	Fisher's exact test	*p*-value	Frequency *n* (%)	Fisher's exact test	*p*-value
Sex			2.031	0.185		0.073	1.000		2.035	0.189
Male	37 (41)	10 (27)			1 (3)			0 (0)		
Female	54 (59)	8 (15)			1 (2)			2 (4)		
Age			5.842	0.056		2.911	0.221		5.840	0.085
0-6 years	19 (21)	2 (10)			1 (5)			0 (0)		
7-12 years	48 (53)	7 (15)			0 (0)			0 (0)		
13-17 years	24 (26)	9 (38)			1 (4)			2 (8)		
Father or mother work			0.101	1.000		0.266	1.000		0.160	1.038
Yes	63 (69)	12 (19)			1 (2)			2 (3)		
No	28 (31)	6 (21)			1 (4)			0 (0)		

Table 2 (cont)

Socio-demographic characteristic	Frequency *n* (%)	*Enterocytozoon bieneusi* positive			*Encephalitozoon intestinalis* positive			Mixed infection (*Ent. bieneusi* and *Enc. intestinalis*)		
		Frequency *n* (%)	Fisher's exact test	*p*-value	Frequency *n* (%)	Fisher's exact test	*p*-value	Frequency *n* (%)	Fisher's exact test	*p*-value
Family members						1.182	0.618		0.010	0.910
2	53 (58)	9 (17)	0.001	1.000	2 (4)			1 (2)		
3-5	35 (38)	7 (20)			0 (0)			1 (3)		
>5	3 (4)	2 (67)			0 (0)			0 (0)		
Accommodation			0.645	0.816		3.047	0.476		0.860	1.024
Flat/apartment	68 (75)	14 (21)			1 (1)			0 (0)		
Low-cost terrace	23 (25)	4 (17)			1 (4)			2 (9)		

isolates were grouped in one cluster (similarity of 92-93%), and shared similarity with environmental, animal and human reference samples deposited in Genbank of 63% (Fig 1A), 60% (Fig 1B) and 61% (Fig 1C), respectively. It is worth noting that the sequences from the refugee school children revealed 87% similarity to that of a reference sequence (GenBank accession no. E.B MH027470.1) from immunocompromised patients in Kuala Lumpur (Fig 1C).

Similar phylogenetic analysis of *Enc. intestinalis* samples from refugee school children showed a single cluster (similarity of 83-88%) that shared a weak similarity of 38% with a reference sample from patient(s) with chronic diarrhea in Pakistan (GenBank accession no. E.I JF932507.1) (Fig 2).

The sequence analysis part in this study used a strategic selection method aimed at schools with the highest and moderate rates of positive microsporidiosis. Financial constraints led us to continue with genetic characterization of seven representative positive samples which is five for *Ent. bieneusi* and two for *Enc. intestinalis* isolates. Working under budgetary limits, this subset was meticulously selected to reflect genetic diversity across the most impacted educational refugee schools.

DISCUSSION

Parasitic infections persist as a public health concern, particularly in impoverished communities. The prevalence of these infections varies depending on the target population, being higher in rural communities with poor hygienic practices (Sahimin *et al*, 2024). Microsporidiosis is commonly detected in immunosuppressed patients (Yusoff *et al*, 2021) but has been reported in immunocompetent individuals (Croppo *et al*, 1991; Abreu-Acosta *et al*, 2005; Sak *et al*, 2011). Microsporidia are ubiquitous in soil, environmental water sources and domestic and wild animals. The spores are microscopic and resistant to chlorinated water, and can evade water filtering

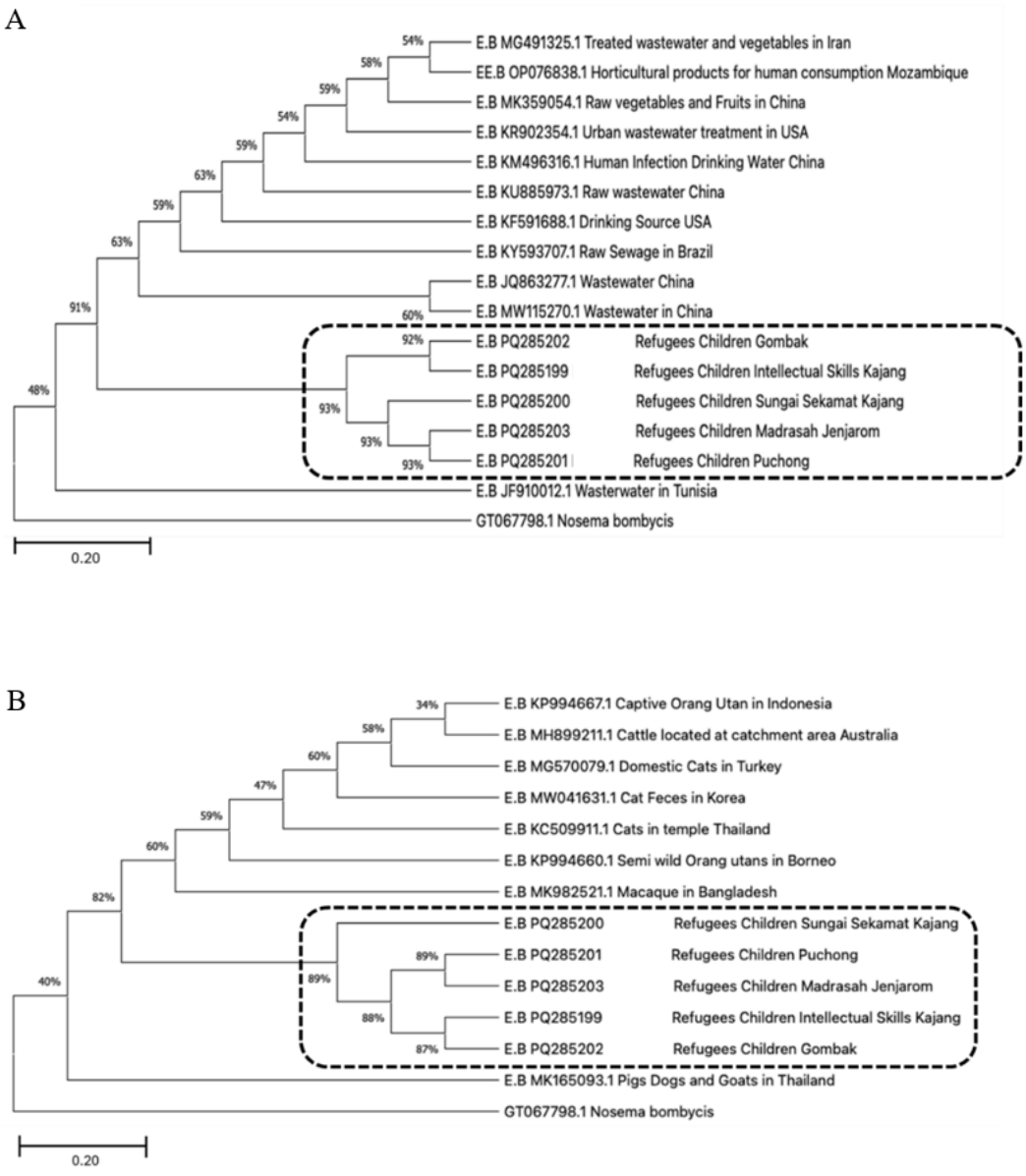

Fig 1 - Phylogenetic tree of *Enterocytozoon bieneusi* isolates from this study and from environmental samples (A), animals (B) and humans (C) deposited in GenBank

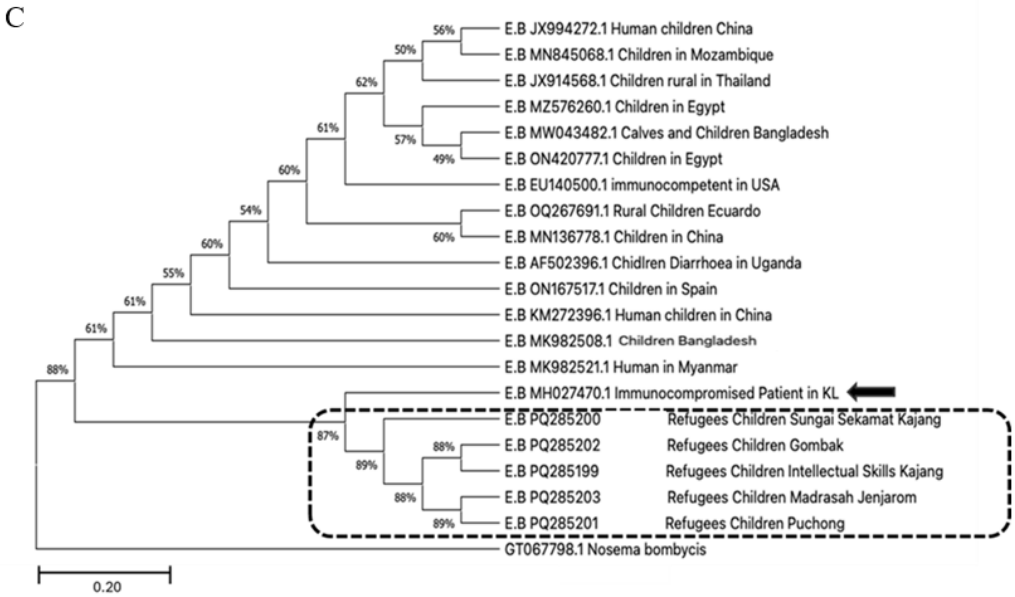

Fig 1 - (cont)

Note: The tree was constructed based on the Maximum Likelihood test and Kishino-Hasegawa model using MEGA10 software with 1,000 bootstrap replicates. Values >70% indicate high confidence level (Karimi *et al*, 2020).

The dash box in Figs 1(A), 1(B) and 1(C) showed *Enterocytozoon bieneusi* found in the refugee school children were grouped in its own cluster and shared similarity with environmental, animal and human reference samples deposited in Genbank while the black arrow in Fig 1(C) indicates the shared similarity of 87% between the cluster of *Ent. bieneusi* in the refugee school children and immunocompromised patient in Kuala Lumpur (KL).

The scale bar index indicates a genetic distance of 0.20 substitutions per nucleotide position.

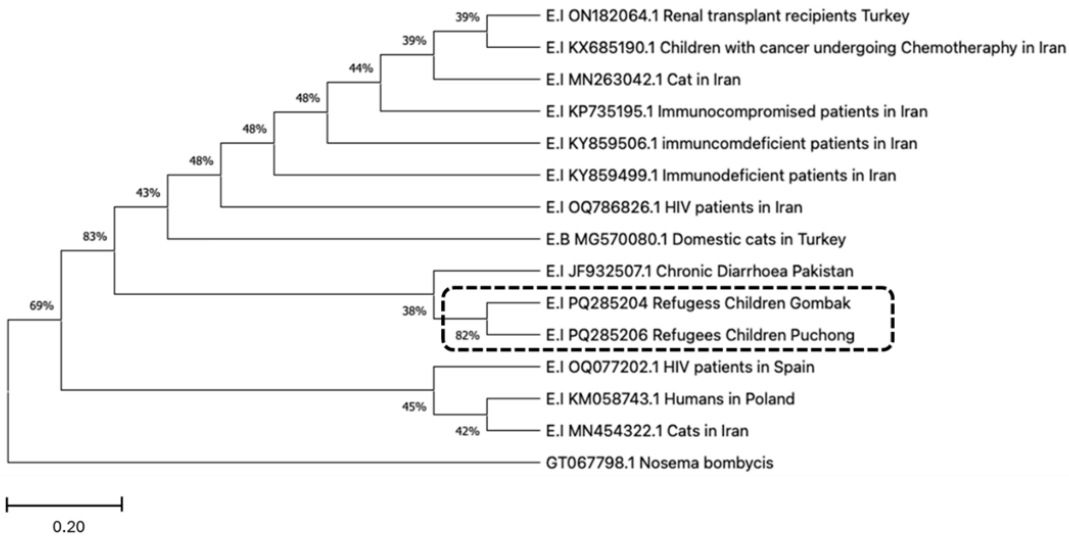

Fig 2 - Phylogenetic tree of *Encephalitozoon intestinalis* isolates from this study and from humans and animals deposited in Genbank

Note: The tree was constructed based on the Maximum Likelihood test and Kishino-Hasegawa model using MEGA10 software with 1,000 bootstrap replicates. Values >70% indicate high confidence level (Karimi *et al*, 2020).

The scale bar index indicates a genetic distance of 0.20 substitutions per nucleotide position.

systems. Transmission can be water-borne, food-borne, zoonotic, and anthroponotic (Mathis *et al*, 2005; Li *et al*, 2012). Determination of risk factors and underlying causes of infection in the general population is challenging because the prevalence data are restricted to specific risk-prone groups.

As a stateless population, refugee children are vulnerable to any infections due to a lack of legal recognition and rights, low socioeconomic status, language barriers, and inadequate health knowledge, thus hindering them from access to healthcare services (Chuah *et al*, 2018; Alaribi *et*

al, 2020). Moreover, Malaysia lacks a legal framework for the registration, documentation and acknowledgment of refugee rights. Furthermore, the country is not an affiliate of the 1951 Convention on the Status of Refugees and the 1967 Protocol, and as a result, does not prioritize the protection of refugees' essential needs and fundamental rights (Hanapi *et al*, 2024). Hence, this study was conducted to obtain baseline data for supporting government health policies of refugees' healthcare and for raising public awareness on their health situation, particularly of parasitic infections.

Our study, the first using molecular techniques, showed that microsporidiosis in refugee children was high (25%), with *Ent. bieneusi* as the predominant species (78%). Our results are in agreement with previous studies on microsporidiosis in children detected via microscopic observation, which ranges 1.3-27.1% (Tumwine *et al*, 2002; Leelayoova *et al*, 2005; Norhayati *et al*, 2007; Zhang *et al*, 2011; Anuar *et al*, 2016).

The prevalence of microsporidiosis in humans can be influenced by a variety of factors, such as sanitation, consumption of contaminated water, interaction with zoonotic hosts, and immune status (Wang *et al*, 2018). A meta-analysis conducted by Wang *et al* (2024) reported that global human microsporidiosis caused by *Ent. bieneusi* may be related to human proximity to the animal environment and communities in rural areas are highly susceptible to contracting *Ent. bieneusi* because of their poor living conditions and lack of good hygiene practices. These are comparable circumstances in refugee communities, despite being located in urban settings.

The findings from our study showed that children older than 13 years of age have a higher prevalence of *Ent. bieneusi* infection than younger children. It is in line with a previous study in Pahang, Malaysia, which reported that Orang Asli children above 15 years of age have a higher chance of contracting microsporidiosis compared to the younger group

(Anuar *et al*, 2013). This could be explained based on the age of development, which involves more independent and vigorous lifestyle activities of older compared to younger children. In addition, microsporidia spore shedding may also occur more readily in the lower age group (Anuar *et al*, 2013). Moreover, the density of the refugee population is high in the urban areas, particularly in Klang Valley, Selangor where they reside in flats/ apartments, the majority of whom share with multiple families, which results in a congested environment, a situation conducive to the rapid transmission of disease (Hanapi *et al*, 2024).

The SSU-rDNA sequence serves as a sensitive tool for the identification of microsporidia species, owing to its species-specific characteristics, although it does not offer as much insight into sequence variations compared to the ITS region (Coyle *et al*, 1996; Notermans *et al*, 2005; Hu *et al*, 2017). This technique allowed the detection of two refugee children with *Enc. intestinalis* infection that is rarely reported in previous studies in Malaysia (Hassan *et al*, 2018). *Enc. intestinalis* has been occasionally detected globally among both immunocompromised and immunocompetent patients in Iran (Karimi *et al*, 2020), HIV patients undergoing HAART therapy in Spain (Chozas *et al*, 2023) and diarrheal patients in Korea (Kim *et al*, 2015). However, there are insufficient data to explain the low *E. intestinalis* prevalence in humans (Joseph *et al*, 2005).

Phylogenetic analysis of the SSU rDNA sequence of *Ent. bieneusi* and *Enc. intestinalis* samples from refugee children in different school locations revealed the two microsporidia species each formed a single cluster, the former with ~ 60% similarity to global reference isolates from humans, animals and the environment deposited in GenBank. Of note, *E. bieneusi* samples of refugee children had 87% similarity with a reference sequence from immunocompromised patients in Kuala Lumpur. Certain genotypes

of *Ent. bieneusi* and *Enc. intestinalis* have been suspected to infect only humans, whereas other potentially zoonotic genotypes are found in both humans and animals (Mathis *et al*, 2005; Ruan *et al*, 2021).

In conclusion, this study revealed a significant prevalence of microsporidia among the marginalized community, with *Ent. bieneusi* being the highest reported species among them. It is also interesting to note that the infected children remained asymptomatic despite harboring microsporidia. The findings also confirmed the occurrence of mixed infection by both of the species. Information concerning this infection among refugee children is still limited. Baseline data and further molecular epidemiological studies are crucial to addressing the gaps of knowledge in identifying significant and relevant causes and risk factors of microsporidiosis in refugee children and adults. Future studies should utilize next-generation sequencing techniques to provide insight into the various genetic variations present in *Ent. bieneusi* and *Enc. intestinalis* in risk populations. The data will enable an understanding of the relationships among strains of both species, their adaptations, cellular metabolisms, virulence and transmission capabilities. In addition, cooperation among stakeholders, such as non-governmental organizations and government agencies, is needed to harmonize programs designed to improve the health and well-being of marginalized communities.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

Abreu-Acosta N, Lorenzo-Morales J, Leal-Guio Y, *et al*. *Enterocytozoon bieneusi* (microsporidia) in clinical samples from immunocompetent individuals in Tenerife, Canary Islands, Spain. *Trans R Soc Trop Med Hyg* 2005; 99(11) :848-55.

Alaribi FI, Unyah NZ, Misni N, Masri SN, Osman M. The prevalence of soil-transmitted helminths infection and its association with anaemia among refugee school children in the Klang Valley, Malaysia. *Mal J Med Health Sci*. 2020; 16(4): 46-53.

Anuar TS, Al-Mekhlafi HM, Salleh FM, Moktar N. New insights of microsporidial infection among asymptomatic aboriginal population in Malaysia. *PLoS One* 2013; 8(8): e71870.

Anuar TS, Bakar ANH, Al-Mekhlafi HM, Moktar N, Osman E. Prevalence and risk factors for asymptomatic intestinal microsporidiosis among aboriginal school children in Pahang, Malaysia. *Southeast Asian J Trop Med Public Health*. 2016; 47(3): 441-9.

Ashikin A, Al-Mekhlafi HM, Moktar N, Anuar TS. Molecular detection and species identification of *Enterocytozoon bieneusi* isolated from immunocompetent Orang Asli in Malaysia. *Parasitol Int* 2017; 66(2): 163-5.

Centers for Disease Control and Prevention (CDC). Microsporidiosis, 2019 [cited 2025 Jan 07]. Available from: URL: https://www.cdc.gov/dpdx/microsporidiosis/index.html

Chozas M, Dashti A, Prieto-Pérez L, *et al*. *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* (microsporidia) in HIV-positive patients in central Spain. *Med Mycol* 2023; 61(4): myad039.

Chuah FLH, Tan ST, Yeo J, Legido-Quigley H. The health needs and access barriers among refugees and asylum-seekers in Malaysia: a qualitative study. *Int J Equity Health* 2018; 17(1): 120.

Coyle C, Wittner M, Kotler DP, *et al*. Prevalence of microsporidiosis due to *Enterocytozoon bieneusi* and

Encephalitozoon (Septata) intestinalis among patients with AIDS-related diarrhea: determination by polymerase chain reaction to the microsporidian small-subunit rRNA gene. *Clin Infect Dis* 1996; 23(5): 1002-6.

Croppo GP, Gomez Morales MA, Pozio E. Microsporidia infections in immunocompetent and immunosuppressed subjects. *Parasitologia* 1991; 33(2-3): 209-18. [In Italian.]

Franzen C, Müller A. Microsporidiosis: human diseases and diagnosis. *Microbes Infect* 2001; 3(5): 389-400.

Han B, Pan G, Weiss LM. Microsporidiosis in humans. *Clin Microbiol Rev* 2021; 34(4): e0001020.

Hanapi IRM, Behnke JM, Sahimin N, *et al*. Intestinal parasitic infections and risk analysis among urban refugees in the Klang Valley, Malaysia. *Trans R Soc Trop Med Hyg* 2024; 118(1): 18-32.

Hassan NA, Lim YAL, Mahmud R, Mohd-Shaharuddin N, Sulaiman WYW, Ngui R. Molecular diagnosis of microsporidia among immunocompromised patients in Kuala Lumpur, Malaysia. *Am J Trop Med Hygiene* 2018; 99(6): 1562-6.

Hu S, Liu Z, Yan F, *et al*. Zoonotic and host-adapted genotypes of *Cryptosporidium* spp., *Giardia duodenalis* and *Enterocytozoon bieneusi* in dairy cattle in Hebei and Tianjin, China. *Vet Parasitol* 2017; 248: 68-73.

Joseph J, Vemuganti GK, Sharma S. Microsporidia: emerging ocular pathogens. *Indian J Med Microbiol* 2005; 23(2): 80-91.

Karimi K, Mirjalali H, Niyyati M, *et al*. Molecular epidemiology of *Enterocytozoon bieneusi* and *Encephalitozoon* sp., among immunocompromised and immunocompetent subjects in Iran. *Microb Pathog* 2020; 141: 103988.

Kim K, Yoon S, Cheun HI, Kim JH, Sim S, Yu JR. Detection of *Encephalitozoon* spp. from human diarrheal stool and farm soil samples in Korea. *J Korean Med Sci*. 2015; 30(3): 227-32.

Kishino H, Hasegawa M. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in hominoidea. *J Mol Evol* 1989; 29(2): 170-9.

Leelayoova S, Subrungruang I, Rangsin R, *et al*. Transmission of *Enterocytozoon bieneusi* genotype A

in a Thai orphanage. *Am J Trop Med Hyg* 2005; 73(1): 104-7.

Li N, Xiao L, Wong L, *et al*. Molecular surveillance of *Cryptosporidium* spp., *Giardia duodenalis* and *Enterocytozoon bieneusi* by genotyping and subtyping parasites in wastewater. *PLoS Negl Trop Dis* 2012; 6(9): e1809.

Lwanga SK, Lemeshow S. Sample size determination in health studies: a practical manual. Geneva, Switzerland: World Health Organization; 1991.

Mathis A, Weber R, Deplazes P. Zoonotic potential of the microsporidia. *Clin Microbiol Rev* 2005; 18(3): 423-45.

Matos O, Lobo ML, Xiao L. Epidemiology of *Enterocytozoon bieneusi* infection in humans. *J Parasitol Res* 2012; 2012: 981424.

Norhayati M, Al-Mekhlafi MS, Azlin M, *et al*. Intestinal microsporidial infections among Orang Asli (aborigine) children from Malaysia. *Ann Trop Med Parasitol* 2007; 101(6): 547-50.

Notermans DW, Peek R, de Jong MD, Wentink-Bonnema EM, Boom R, van Gool T. Detection and identification of *Enterocytozoon bieneusi* and *Encephalitozoon* species in stool and urine specimens by PCR and differential hybridization. *J Clin Microbiol* 2005; 43(2): 610-4.

Qin Y, Chen C, Qin YF, *et al*. Prevalence and related factors of *Enterocytozoon bieneusi* in cattle: a global systematic review and meta-analysis. *Prev Vet Med* 2022; 208: 105775.

Ruan Y, Xu X, He Q, *et al*. The largest meta-analysis on the global prevalence of microsporidia in mammals, avian and water provides insights into the epidemic features of these ubiquitous pathogens. *Parasit Vectors* 2021; 14(1): 186.

Sahimin N, Abu Bakar N, Lim YAL, *et al*. Entry of migrant workers to Malaysia: consideration to implement mass drug administration against intestinal parasitic infections. *Int J Health Policy Manag* 2024; 13: 7842.

Sak B, Kváč M, Kučerová Z, Květoňová D, Saková K. Latent microsporidial infection in immunocompetent individuals - a longitudinal study. *PLoS Negl Trop Dis* 2011; 5(5): e1162.

Shehab AM, Moneer EA, Allam AF, Khalil SS, Tolba MM. Intestinal microsporidia infection in

leukemic children: microscopic and molecular detection. *Acta Parasitol* 2021; 66(2): 346-53.

Soltis PS, Soltis DE. Applying the bootstrap in phylogeny reconstruction. *Stat Sci* 2003; 18(2): 256-67.

Tamura K, Stecher G, Kumar S. MEGA 11: Molecular Evolutionary Genetic Analysis version 11. *Mol Biol Evol* 2021; 38 (7): 3022-7.

Tumwine JK, Kekitiinwa A, Nabukeera N, Akiyoshi DE, Buckholt MA, Tzipori S. *Enterocytozoon bieneusi* among children with diarrhea attending Mulago Hospital in Uganda. *Am J Trop Med Hyg* 2002; 67(3): 299-303.

United Nations High Commissioner for Refugees Malaysia (UNHCR Malaysia). Figures at a glance in Malaysia, 2024 [cited 2025 Jan 10]. Available from: URL: https://www.unhcr.org/en-my/figures-at-a-glance-in-malaysia.html

Wang Y, Li XM, Yang X, *et al*. Global prevalence and risk factors of *Enterocytozoon bieneusi* infection in humans: a systematic review and meta-analysis. *Parasite* 2024; 31: 9.

Wang ZD, Liu Q, Liu HH. Prevalence of *Cryptosporidium*, microsporidia and *Isospora* infection in HIV-infected people: a global systematic review and meta-analysis. *Parasit Vectors* 2018; 11(1): 28.

Yusoff PSM, Osman E, Sabudin RZAR. Disemminated microsporidiosis: an underdiagnosed and emerging opportunistic disease. *Malays J Pathol* 2021; 43(1): 9-18.

Zhang X, Wang Z, Su Y, *et al*. Identification and genotyping of *Enterocytozoon bieneusi* in China. *J Clin Microbiol* 2011; 49(5): 2006-8.