Malays J Pathol 2024; 46(2) : 299 – 306

ORIGINAL ARTICLE

A comparative analysis of blood and faecal-based laboratory methods in the diagnosis of extraintestinal microsporidia infection

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Abstract

Diagnosis of extraintestinal microsporidiosis is always hampered due to non-specific symptoms and difficulty in diagnosis. This study aimed to compare the diagnostic utility of blood and faecal-based polymerase chain reaction (PCR) to detect microsporidiosis in immunocompromised patients. A total of 42 immunocompromised patients consisting of HIV-infected and chemotherapy-treated patients were enrolled. Paired faecal and blood samples were collected and subjected to PCR to detect *Enterocytozoon bieneusi* and *Encephalitozoon* spp. Faecal samples were microscopically screened for microsporidia spores. Overall, 42.9% (18/42) of patients were positive for microsporidiosis. Of this, 19.0% (8/42) and 4.8% (2/42) were positive by blood and stool PCR respectively. Meanwhile, 33.3% (14/42) of the faecal specimens were microscopically positive. Among the positive patients, 22.2% (4/18) had microsporidia confirmed by blood PCR and stool microscopy, suggestive of dissemination. Interestingly, the stool specimen in which microsporidia spores were detected via microscopy is not positive via PCR method. This highlights the limitation of the faecal-based detection method and the important use of blood samples for diagnosing extraintestinal microsporidiosis. Only *E. bieneusi* species were detected in all PCR-positive samples. This study highlights the diagnostic value of blood PCR in diagnosing extraintestinal microsporidiosis infections.

Keywords: Microsporidia; extraintestinal microsporidia; blood; faecal; PCR

INTRODUCTION

Microsporidia is an emerging and opportunistic fungi infection in immunocompromised persons. The two main microsporidia genus, *Encephalitozoon* spp., and *Enterocytozoon* sp. are associated with a wide range of clinical manifestations including prolonged diarrhoea, abdominal pain, and weight loss.[1] To date, microsporidiosis not only localizes in the enteric but has been reported to cause systemic infections.[2,3] Given that the clinical manifestations of extraintestinal microsporidiosis have a broad spectrum, the diagnosis of this disease is becoming increasingly challenging.

The incidence of extraintestinal microsporidiosis may be under-reported due to non-specific symptoms of the infection. With regard to laboratory diagnosis, the size of microsporidia spores is relatively small, requiring expert microscopists for verification. Yet, the fact is that microsporidia are often not included in the routine differential diagnosis as a potential cause for diarrhoea or systemic infections.[4] Thus, most cases of extraintestinal microsporidiosis are only diagnosed at autopsy.[5-8] Meanwhile, the differentiation of microsporidia species is crucial in deciding on treatment, as different species of microsporidia respond differently to

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the treatment provided. Although anthelmintic medication such as albendazole is effective against *Encephalitozoon* spp., fumagillin has been shown to have better efficacy in clearing infections for *Enterocytozoon bieneusi*.[4,9]

The selection of clinical specimens partially influences the effectiveness and accuracy of the diagnosis method, which include faeces, urine, bronchoalveolar lavage fluid, sputum, and biopsy of the suspected affected area such as muscle.[6] Faecal samples are generally used in detecting microsporidia spores due to their non-invasive and readily available for testing. Currently, light microscopy and polymerase chain reaction (PCR) assay are used for the detection of microsporidia in faecal samples.[4] Unlike microscopy, PCR assay can identify the infecting microsporidia species, which is essential in determining the treatment for the infection.[10] Nevertheless, reliable diagnostic methods are still needed to supplement routine microscopy or PCR, especially when spore excretion in faeces may be intermittent or present at a low level of detection rate.

Our previous study demonstrated the usefulness of blood samples for the diagnosis of disseminated microsporidiosis in HIV-AIDS patients.[3] In the current study, we further evaluate the diagnostic and clinical value of the blood-based PCR assay in comparison to stool PCR and microscopy for the detection of extraintestinal microsporidia infection in paired blood and faeces samples of immunocompromised patients.

MATERIALS AND METHODS

Blood and faecal specimen collection

A total of 42 paired venous blood (3 ml/person) and faeces samples from immunocompromised individuals were collected between January 2020 to March 2021 from Hospital Canselor Tunku Muhriz, Kuala Lumpur. The samples were from patients with HIV infection (CD4 count below 200 cells/mm^3) (n = 23), haematological malignancy (n = 10), and solid organ cancers (n = 9); the latter two patient groups were receiving chemotherapy and had undergone at least three cycles of chemotherapy at the time of sampling. Patients below 18 years old, with primary immunodeficiency disease, or with an autoimmune disease that is on immunosuppressants were excluded from this study. Data on patients' socio-demographic and clinical records were also retrieved. Written informed consent for participation in the study was obtained from participants before sample collection. Ethical clearance was obtained from the Research Ethics Committee of Universiti Kebangsaan Malaysia (UKM PPI/111/8/JEP-2019-732(2)).

Parasitology examination

All faecal specimens were subjected to microscopy examination for the presence of microsporidian spores using Gramchromotrope Kinyoun (GCK) stain as described by Salleh *et al.* (2011).[11] Briefly, each sample was smeared and stained in duplicate followed by examination under a light microscope at high magnification (×100) with oil. The stained faecal smears were examined by two experts in a blinded manner. Microsporidia spores were diagnosed based on the presence of specific morphological features that include pink-blue ovoid spores with a blue spore wall, and a belt-like stripe across the spores against a relatively clean or pale pink background. The result was recorded as positive and negative upon the presence or absence of spores and graded based on the number of spores per high power field as follows: 1+ (number of spores 1–10), 2+ (number of spores 11–20), and 3+ (number of spores more than 21).[12,13]

Genomic DNA extraction

DNA extraction for blood and faeces were carried out using the QIAamp DNA Mini kit and QIAamp DNA Stool Mini Kit (Qiagen, USA) respectively, according to the manufacturer's instruction. The quality and the concentration of DNA extracted were quantified using NanoDrop™ 2000/2000c Spectrophotometers (Thermo Scientific, USA).

Primers

The identification of *E. bieneusi* in both faecal and blood samples was verified using defined species-specific primer pair; EBIEF1 (5'- GAAACTTGTCCACTCCTTACG-3') and EBIER1 (5' - CCATGCACCACTCCTGCCATT-3'). These primers were based on nucleotides 295 to 315 and 881 to 901 of the *E. bieneusi* SSU-rRNA sequence and amplified product of 607bp.[14] For identification of *Encephalitozoon* spp. in both faecal and blood samples, primers int530f (5'-TGCAGTTAAAATGTCCGTAGT-3') and int580r (5'-TTTCACTCGCCGCTACTCAG-3') were used. These primers were based on sequence conserved between *E. cuniculi*, and *E. hellem* which amplify product with a size of 1000 bp.[15]

Faecal-based PCR

The PCR was conducted with HelixAmp Taq Polymerase (NanoHelix Co., Ltd, South Korea) and it was performed in a 25 µl volume with 50 ng of faecal DNA template, 10X Taq buffer, 10 mM of dNTP mix, 10 µM of each primer and 1.25 U Taq DNA Polymerase. Both negative control (reagent mixture without template DNA) and positive control were included in each set of the PCR. Positive control for *E. bieneusi* was prepared by extracting DNA from the negative faecal sample that was spiked with the synthetic plasmid of *E. bieneusi*. The *E. bieneusi* SSU-rRNA gene accession number (KF271507.1) was cloned by Bio Basic Inc. Positive control for *Encephalitozoon spp.* was prepared by extracting DNA from faecal that was spiked with *E. cuniculi* spore (ATCC 50602). For the faecal specimen, amplification for *E. bieneusi* DNA was performed using Mastercycler Pro S (Eppendorf, Germany) with initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec, 56.7°C for 30 sec, and 72° for 30 sec, and a 7 min-final extension at 72°C following the modified method from Didier *et al.* (1996). Amplification of *Encephalitozoon* spp. DNA in the faeces sample follows similar conditions except for annealing at 43.2°C for 30 sec and extension at 72°C for 60 sec. Prior to amplification of *E. bieneusi* and *Encephalitozoon spp.* DNA in faecal sample, gradient PCR were conducted with temperature ranging from 55°C to 65°C and 40°C to 60°C, respectively, to determine the optimum annealing temperature. Amplified DNA was analysed by electrophoresis in a 1.5% (w/v) agarose gel and visualized using Gel Doc EZ Gel Documentation system (Bio-Rad, USA).

Blood-based PCR

DNA amplification from blood samples was executed following microsporidia DNA amplification in blood specimens by Zainudin *et al*. (2016). DNA amplification was conducted with HelixAmp Taq Polymerase (NanoHelix Co., Ltd, South Korea) and it was performed in a 25 µl volume with 150 ng of blood DNA template, 10X Taq buffer, 10 mM of dNTP mix, 10 µM of each primer and 1.25 U Taq DNA Polymerase. Modification from the previous method by Zainudin *et al* (2016) was performed to optimize the presence of unspecific bands. Both negative control (reagent mixture without template DNA) and positive control were included in each set of the PCR. Positive control for *E. bieneusi* was prepared by extracting DNA from negative blood sample that was spiked with synthetic plasmid of *E. bieneusi*. The *E. bieneusi* SSU-rRNA gene accession number (KF271507.1) was cloned by Bio Basic Inc. To prepare the positive control for *Encephalitozoon* spp., DNA was extracted from blood spiked with *E. cuniculi* spores (ATCC 50602). Initially, a 15 ml volume of *E. cuniculi* culture underwent centrifugation at 1500 XG for 15 minutes to gather the spores, which were then washed with PBS. Subsequently, the spore pellet was mixed with blood from a healthy individual to create the spiked blood sample. For the blood sample, amplification for *E. bieneusi* DNA was performed with an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec, 56.7°C for 30 sec, and 72° for 30 sec, and a 7 min-final extension at 72°C. Amplification of *Encephalitozoon* spp. DNA in the blood sample follows similar conditions except for 50 ng of blood DNA template, annealing at 46.5°C for 30 sec and extension at 72°C for 60 sec. To determine the optimum annealing temperature of *E. bieneusi* and *Encephalitozoon spp*. in blood sample, gradient PCR were conducted with temperature ranging from 55°C to 65°C, and 40°C to 60°C, respectively. Annealing and extension time were optimized from protocol describe by Zainudin *et al*. (2016)[3] to reduce the appearance of nonspecific band. Amplified DNA was analysed by electrophoresis in a 1.5% (w/v) agarose gel and visualized using Gel Doc EZ Gel Documentation system (Bio-Rad, USA).

Sequence Analysis

PCR-positive samples amplicons were sent for sequencing for further confirmation. All positive amplicons were purified and then subjected to DNA sequencing in both directions using the same primers used in PCR. Sequence results were analysed by BioEdit Sequence Alignment Editor version 7.2.5. Homology searches were carried out using the *E. bieneusi* and *Encephalitozoon* spp. sequences on the Basic Local Alignment Search Tool (BLAST), hosted by the National Center for Biotechnology Information (NCBI).

Statistical analysis

A chi-square test (X^2) or Fisher's exact test was used to test the association between the patient's characteristics and microsporidia diagnosis in the patient. A p-value of <0.05 was considered significant.

Ethical Approval Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Research Ethics Committee of UNIVERSITI KEBANGSAAN MALAYSIA (UKM PPI/111/8/JEP-2019-732(2) on 10th January 2020.

RESULTS

Microsporidia spores were able to be detected in stool samples of the immunocompromised patients using Gramchromotrope Kinyoun (GCK) stain (Figure 1). Further analysis by PCR for detection of *E. bieneusi* DNA amplified bands at approximately 607 bp. A summary of the microscopy and PCR screening results for matched blood and faeces samples of 42 immunocompromised patients is shown in Table 1. Overall, 42.9 % (18/42) were positive for microsporidia, of which microscopy detected spores in 33.3% (14/42) of the faeces samples, 19.0% (8/42) of microsporidia DNA were amplified in blood PCR, and 4.8% (2/42) by stool PCR. The combination of detection of blood PCR and microscopy resulted in 42.9% (18/42) detection, a 2.25-fold higher detection rate in comparison to a single blood PCR test (19.0%; 8/42). Among the positive patients, 22.2% (4/18) had microsporidia confirmed by both blood PCR and stool microscopy, suggestive of dissemination of the parasite (Table 2).

Interestingly, the stool specimen in which microsporidia cysts were detected via microscopy was not positive via PCR method. This highlights the limitation of faecal samples and the usefulness of blood samples to detect extraintestinal microsporidia. Of note, molecular screening of all blood and faeces samples identified microsporidia species of *E. bieneusi* only, indicating its notable prevalence in immunocompromised patients suspected of extraintestinal microsporidiosis.

Based on the sequencing results, the DNA from blood samples that were PCR-positive were matched to the published *Enterocytozoon bieneusi* sequences in the database. The gene amplified in blood and stool samples, belongs to the *E. bieneusi* small subunit ribosomal RNA gene. The sequence samples in blood exhibited 98.8% – 100% homology to the sequences in GenBank with accession numbers (MH027470.1) and (MG976584.1). Furthermore, the DNA from faeces samples also showed 99.34% similarity to *E. bieneusi* small subunit ribosomal RNA gene (GenBank accession no MH027462.1).

The patient's demographic data and symptoms associated with microsporidia were tested with the detection of microsporidia in blood and faecal using Fischer's exact test and listed in Table 3. There is a significant association between the microsporidia detected in faecal and fever

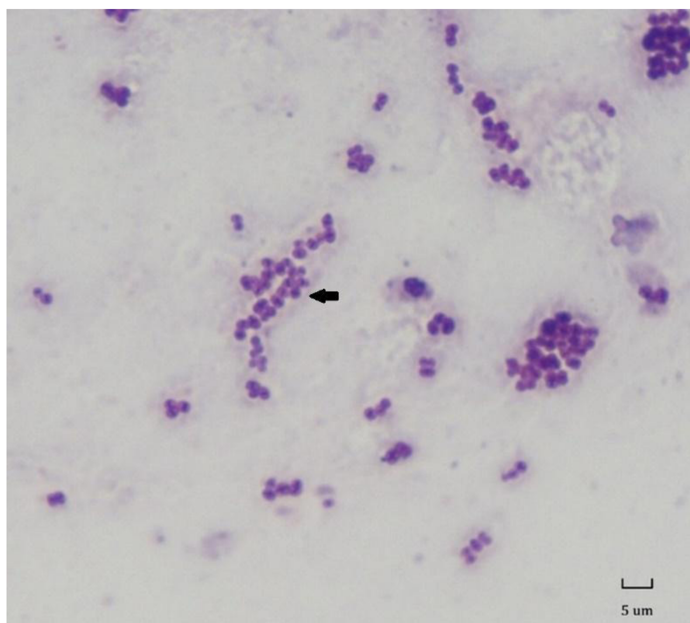

Figure 1. Microsporidian spores were detected in stool sample using Gramchromotrope Kinyoun (GCK) stain under a light microscope at high magnification (×100) with oil.

Table 1: Detection of microsporidia using microscopy and PCR technique for *E. bieneusi* and *Encephalitozoon spp* in matched faeces and blood samples

	Microscopy (%; n)	PCR (%; n)			
		Enterocytozoon bieneusi		*Encephalitozoon spp.*	
		Blood	Faecal	Blood	Faecal
Matched blood and faeces sample (n=42)	33.3%; 18	19.0%; 8	4%; 2	0	0

($\chi2 = 7.941$, p=0.008). No significant associations were found for other patients' demographic data and symptoms ($p > 0.05$) who were positive for microsporidia.

DISCUSSION

HIV and cancer patients are at higher risk of extraintestinal microsporidiosis due to their immunocompromised state. As the incidence of extraintestinal microsporidiosis is increasing worldwide,[2,6,10,16] greater efforts need to be made to contain the infection through the development of more sensitive diagnostic methods. The broad spectrum and non-specific symptoms of the disease, such as diarrhoea and fever, make it challenging to diagnose as it is not considered for differential diagnosis.[10] Besides, extraintestinal cases of microsporidia may not be detected during the screening of faecal samples.[17] Herein, we highlighted the value of blood-based PCR for

Table 2: The presence of microsporidia in matched faeces and blood samples was detected by microscopy and PCR

Positive sample no.	Microscopy	PCR *Enterocytozoon bieneusi*	
		Blood	**Faecal**
#1	√	n.d	n.d
#2	√	n.d	n.d
#3	√	n.d	n.d
#4	√	n.d	√
#5	√	√	n.d
#6	n.d	√	n.d
#7	√	√	n.d
#8	n.d	√	n.d
#9	√	√	n.d
#10	n.d	√	n.d
#11	√	√	n.d
#12	n.d	√	n.d
#13	√	n.d	n.d
#14	√	n.d	n.d
#15	√	n.d	n.d
#16	√	n.d	n.d
#17	√	n.d	√
#18	√	n.d	n.d

n.d = not detected

Table 3: Patient's demographic data and associated symptoms with microsporidia infection

Characteristics	Microsporidia positive in blood (n=8)	X^2 (p-value)	Microsporidia positive in stool (n=14)	X^2 (p-value)
Age (Years)				
20-40	2 (25.0)	0.309	6 (42.9)	0.857
>40	6 (75.0)	(0.697)	8 (57.1)	(0.490)
Gender				
Male (n=29)	7 (87.5)	1.574	11 (78.6)	0.891
Female (n=13)	1 (12.5)	(0.398)	3 (21.4)	(0.485)
Diagnosis				
HIV (n=23)	4 (50.0)	0.090	10 (71.4)	2.355
Cancers (n=19)	4 (50.0)	(1.000)	4 (28.6)	(0.191)
Diarrhoea	0	3.507 (0.086)	3 (21.4)	0.246 (0.723)
Fever	2 (25.0)	0.008 (1.000)	7 (50.0)	7.941 (0.008)
Nausea	0	1.040 (0.572)	2 (14.3)	0.553 (0.590)

the detection of extraintestinal microsporidiosis in immunocompromised patients.

Besides the study from Zainudin *et al.* (2016),[3] a study by Menotti *et al.* (2009) also demonstrated the use of blood-based PCR for the detection of microsporidiosis, specifically by *Encephalitozoon intestinalis.*[18] In this study, the real-time PCR technique assessed the dissemination of infection by the positivity of PCR in serum or whole blood. Meanwhile, in animal studies, disseminated infection of *E. cuniculi* in mice also can be detected using blood by PCR.[19] Aside from the PCR technique, microsporidia spores have also been detected in fish blood by IFAT and amphibian blood by smear.[20,21]

Molecular and microscopy screening on the 42 matched blood and faeces samples determined a positivity rate of 42.9% of microsporidia in immunocompromised patients, which is in accordance with the published data on microsporidiosis prevalence that ranges from 0.7 to 81.3%.[4,22] A higher positivity rate of microsporidia was detected in faeces samples by light microscopy (33.3%) in comparison to molecular detection of blood (19.0%) and faeces (4.8%) samples. This is not surprising as microscopy identified spores of any microsporidia species, whereas the PCR method is much more specific, targeting only species-specific microsporidia DNA. The high positivity rate of microscopy may also be due to false-positive results caused by the morphological similarity of microsporidia spores with other fungal spores or yeast.[23,24,25] Predictably, only *E. bieneusi* species were detected in both faeces and blood samples. This finding is in agreement with several studies stating that *E. bieneusi* is the predominant microsporidia species for gastrointestinal infection.[22,26] Infection with *Encephalitozoon spp.* is less common in the gastrointestinal and is often found in urine.[27] The 19.0% (8/42) detection rate by blood PCR in this study was 1.5-fold greater than those reported by Zainudin *et al* (2016), which could be due to the differences in sample size and a broader variety of immunocompromised populations studied whereby their study focused only on HIV subjects.[3] Four out of eight positive blood PCR samples were also positive by microscopic detection in stool samples, confirming the dissemination of microsporidia. Meanwhile, the other four samples that were positive by blood PCR, were found negative by faecal-based PCR and microscopy, suggesting the limitation of the faecal-based method to diagnose disseminated microsporidia infection. Besides microsporidia spores being absent in faecal samples yet detected in other types of specimens, the faecal-based detection method is also limited by the intermittent shedding of microsporidia spores.[27,28,29]

Although *E. bieneusi* is usually localized in the small intestine, extraintestinal dissemination

has been observed in the lung, biliary tract and gall bladder.[30] Out of 14 positive faeces samples with microsporidia spores, only two detected *E. bieneusi* DNA by faecal-based PCR. It is hypothesized that the remaining negative stool-PCR might be due to the misidentification of spores or yeast cells that appeared similar to those of *Encephalitozoon* and *Enterocytozoon* genera spores.[31,32] Additionally, it could also be due to the presence of other microsporidia genera affecting humans in the faeces samples such as *Nosema*, *Pleistophora,* or *Trachipleistophora*, hence it was not amplified by the PCR method.[33] The limitation of the study is the small number of sample but despite this, the study demonstrates the usefulness of blood-based PCR assay for microsporidia detection in immunocompromised patients.

HIV and cancer patients are at higher risk of extraintestinal microsporidiosis due to their immunocompromised state. Besides, extraintestinal cases of microsporidia may not be detected during the screening of faecal samples.[17] In conclusion, this study highlights the diagnostic value of a blood-based PCR assay for the detection of disseminated or extraintestinal microsporidiosis, which may be overlooked when using only stool-based PCR assay or routine microscopy.

Acknowledgement: The authors would like to thank the Ministry of Higher Education Malaysia (MoHE), under Fundamental Research Grant Scheme (FRGS), grant number [FRGS/1/2019/SKK12/UKM/02/1] for the funding and the Department of Parasitology and Medical Entomology, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia for the research facilities and technical support.

Funding Statement: This work was supported by Ministry of Higher Education Malaysia (MoHE), under Fundamental Research Grant Scheme (FRGS), grant number [FRGS/1/2019/SKK12/UKM/02/1].

Conflicts of Interest: The authors declare that they have no conflicts of interest.

Data Availability: All data supporting the findings of this study are included within the paper; however, details of the full data may be obtained from the corresponding author upon request.

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