Original Article

Evaluation of different laboratory techniques for diagnosis of intestinal microsporidiosis in diarrheic children

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ABSTRACT

Background: Microsporidia are a group of obligate intracellular organisms that can infect all animals including man causing disease in both immunocompetent individuals and immunosuppressed patients. The most common microsporidia causing gastrointestinal infection worldwide are *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*.

Objectives: The present study was designed with the aim of estimating the rate of microsporidia in diarrheic children and the evaluation of different staining techniques and nested polymerase chain reaction (PCR) for the clinical diagnosis of intestinal microsporidiosis.

Subjects and Methods: One hundred and fifty diarrheic stool samples were collected from Abou El-Rich children hospital laboratory. Samples were examined by "Ryan-Blue" modified trichrome stain (MTS), modified Ziehl Neelsen (MZN), acid fast trichrome (AFT) stain, and nested polymerase chain reaction (nPCR).

Results: *Microsporidium* spp. was detected in 12 (8%) of samples using MTS. Compared with the other detection techniques the respective sensitivities and specificities of MZN stain was 100% and 98.57%; nPCR was 80% and 100%; and AFT was 85.71% and 97.87%.

Conclusion: Using specific staining techniques as MZN and AFT for diagnosis of intestinal microsporidiosis in diarrheal stool samples are nearly as efficacious as PCR, but even better in some cases with the added advantage of being an inexpensive diagnostic method compared to PCR. Another benefit is the detection of other oocyst forming parasites such as *Cryptosporidium*, which makes staining techniques very suitable for developing countries.

Keywords: acid fast trichrome; microsporidia; modified trichrome; nPCR; Ziehl Neelsen.

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INTRODUCTION

Oocysts forming apicomplexan protozoa including *Cryptosporidium* spp., *Cyclospora* spp., *Isospora* spp., *Sarcocystis* spp. and intestinal spore forming parasites including microsporidia spp. have emerged as a cause of opportunistic infections in human causing various degrees of diarrhea and extraintestinal manifestations[1].

Microsporidia are a group of obligate intracellular organisms that can infect all animals including man[2]. They are eukaryotic, unicellular organisms belonging to the phylum Microspora[3]. Over 1300 species of microsporidia are detected, of which 15 species were identified in human[4]. The most identifiable stage of microsporidia is a highly resistant small spore measuring 1-10 µm[5]. Microsporidiosis is considered an opportunistic infection causing severe chronic diarrhea in immunosuppressed patients especially the HIV infected, and in transplant recipients, children, and the elderly[6,7]. Microsporidia can also cause extraintestinal manifestations as bronchitis, keratitis and myositis[5]. *Enterocytozoon bieneusi* and the three *Encephalitozoon* spp. (*Encephalitozoon intestinalis*, *Encephalitozoon hellem* and *Encephalitozoon cuniculi*) were identified as opportunistic pathogens[8].

There are several methods for diagnosis of microsporidiosis using different stains including gram stain (microsporidia spores are gram-positive and stain dark violet), Hematoxylin and Eosin (H&E), Giemsa, Warthin-Starry silver and MTS[9]; as well as transmission electron microscopy (TEM), immunofluorescence assays (IFA) and molecular methods[5].

Under-reporting of *Microsporidium* spp. and intestinal coccidian parasites is common because the general diagnostic microscopic methods are inadequate for detection of these infections. Their detection requires the use of specific stains or the use of PCR techniques which are very expensive for developing countries[1]. To the best of our knowledge little is known about the frequency of intestinal microsporidiosis among diarrheal children in Egypt.

Thus, the present study was designed to estimate the rate of microsporidia in diarrheic children by using a battery of stains (MTS, MZN, AFT), and nPCR; and to evaluate the efficacy of these diagnostic techniques in clinical diagnosis of microsporidia. The presence of *Cryptosporidium* in these patients was also determined.

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MATERIAL AND METHODS

In this descriptive analytical study, fresh fecal samples were collected from 150 diarrheic children at Abou El-Rich Children Hospital Laboratory, Cairo, Egypt, during the period from November 2018 to April 2019.

Stool sample collection, storage and staining techniques: Fecal samples were collected in 60 ml clean labeled containers, each sample was subsequently divided into two portions. A part was stored at -20°C for further molecular studies, and the remaining part was preserved using formalin saline fixative for subsequent microscopic examination and concentration by formalin-ether technique. Smears were made from the deposit, air dried, and fixed in methanol.

- **Modified Ziehl Neelsen (MZN) stain:** Methanol fixed smears were stained in working fuchsin for 45 min and rinsed in slow-running water. Malachite green (2%) was used as counter stain for 10 min. The slides were rinsed under slow-running water and allowed to dry at room temperature[10].
- **"Ryan Blue" modified trichrome (MTS) stain:** Methanol fixed smears were incubated for 90 min in trichrome stain, rinsed for 1 to 3 sec in acid-alcohol, dipped several times in 95% alcohol, immersed in 95% alcohol (two changes), 100% alcohol, and three changes of xylene for 5, 10, and 10 min, respectively[11].
- **Acid fast trichrome (AFT) stain:** Methanol fixed smears were placed in carbol fuchsin solution for 10min, briefly rinsed with slow-running water then decolorized with 0.5% acid alcohol, placed in trichrome stain for 30 min at 37°C, then rinsed in acid alcohol for 1 to 3 sec, dipped for several times in 95% alcohol, then placed for 30 sec in 95% alcohol[11].

Copro-PCR Assay: Genomic DNA was extracted from frozen fecal samples using the MO BIO's PowerSoil® DNA Isolation Kit according to the manufacturer's specifications. Eventually, the purified DNA was stored at −20°C for further analysis. Amplification of the 410-420 bp fragment of *Enterocytozoon bieneusi* and *Encephalitozoon* species was done using previously published forward primer Mic C (5′-GGTGCCAGCAGCCGCGG-3′) and reverse primer Mic D (5′-GCACAATCCACTCCT-3′) as per published protocol was done[12]. All the amplified products were electrophoresed on 2% (w/v) agarose gel (Promega Corporation: 2800 Woods Hollow Road-Madison, WI 53711-USA, cat no. V 3121) and stained with ethidium bromide to be visualized on a UV trans-illuminator. The sizes of fragments of the amplified products were compared to the standard 100 bp DNA ladder which contained fragments of known size.

Detection criteria: Identification of microsporidia spores under oil immersion field lens was based on their characteristic morphology as ovoid pink bodies measuring ~1-2 µm in length with a clear uncolored vacuole and a pink polar body[11]. Detection of spores of microsporidia using microscopy in one or more stool samples or its DNA during molecular analysis indicated that the sample was positive for microsporidia spp. The true positive samples were confirmed according to selection of MTS as gold standard test[13].

Statistical Analysis: The collected data was revised, coded, tabulated and introduced to a Personal Computer using Statistical package for Social Science. Data were analyzed using SPSS package version 15 (SPSS Inc., Chicago, IL). Descriptive statistics were frequency and percentage for qualitative data and kappa agreement test to test congruency of the two tests (PCR and microscopy examination).

Ethical consideration: An informed consent was taken from all patients for examining their stool samples. Positive results were relayed to their supervising physicians for prescribing the appropriate treatment. The study was approved by the Research Ethics Committee, Faculty of Medicine, Ain Shams University.

RESULTS

Among 150 samples included in the study, the 12 (8%) samples found positive for microsporidia by MTS (Figure 1), were confirmed by MZN stain (Figure 2); while 10 (7%) samples were positive using AFT stain (Figure 3). Using nPCR 9 (6%) samples were positive for *Microsporidium* spp. (Figure 4). The 138 (92%) samples which were negative by all of the above-mentioned techniques were considered "true negative" (Table 1). The three false positive samples detected by AFT, and two false positive samples detected by MZN stain, were not statistically counted among the true positive results. Sensitivity of MZN stain was the highest at 100% followed by AFT stain at 85.71% (95% confidence interval [CI], 0.57 to 0.98), and PCR at 80% (95% CI, 0.51-0.95). The specificity of nPCR was the highest at 100% followed by MZN stain (98.57%) and AFT stain (97.87%).

Table 1. Comparison of MZN, MTS, AFT stains and nested PCR for diagnosis of intestinal microsporidiosis.

MTS	MZN	AFT	nPCR	Total
-	-	-	-	138
+	+	+	+	7
+	+	-	+	2
+	+	+	-	3

AFT: Acid fast trichrome, **MTS:** "Ryan-blue" modified trichrome, **MZN:** Modified Ziehl Neelsen, **nPCR:** Nested polymerase chain reaction.

A kappa agreement test was done to test congruency of the PCR and different staining techniques. An almost perfect agreement was found between nPCR and MTS by kappa test (0.81) (Table 2). Structures morphologically compatible with *Cryptosporidium* spp. were also detected in 5 (3.3%) samples using MZN stain with diagnostic

Table 2. Inter rater reliability of MTS, AFT and MZN staining and PCR for detection of microsporidia in stool samples.

	MTS +ve	MTS -ve	Interrater reliability*
nPCR+	7	0	0.81 (almost Perfect
nPCR-	3	138	agreement)
	nPCR+	**nPCR-**	
AFT+	7	3	0.71 (substantial
AFT-	2	138	agreement)
	AFT+	**AFT-**	
MTS+	10	2	0.90 (almost Perfect
MTS-	0	138	agreement)
	MZN+	**MZN-**	
nPCR+	7	0	0.81 (almost Perfect
nPCR-	3	138	agreement)
	AFT+	**AFT-**	
MZN+	10	3	0.90 (almost Perfect
MZN-	2	138	agreement)

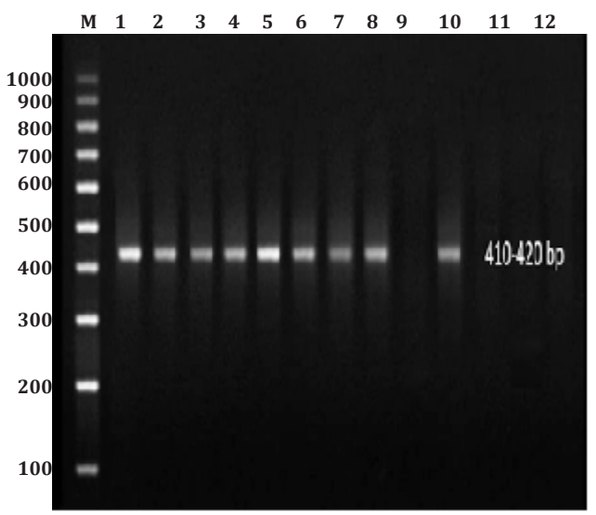
Kappa: 0 poor agreement, 0.01-0.2 slight agreement, 0.21-0.40 Fair agreement, 0.41-0.60 Moderate agreement, 0.61-0.80 Substantial agreement, 0.81-1.00 almost perfect agreement. **AFT:** Acid fast trichrome, **MTS:** "Ryan-blue" modified trichrome, **MZN:** Modified Ziehl Neelsen, **nPCR:** Nested polymerase chain reaction.

yield (100%), and in 4 (2.6%) samples using AFT stain with diagnostic yield (89%), while the distinct morphological character for the oocysts couldn't be confirmed using MTS. No Mixed infection was recorded between microsporidia and *Cryptosporidium* spp. (Table 3).

Table 3. Comparison of MTS, MZN and AFT staining techniques for diagnosis of cryptosporidiosis.

MTS	MZN	AFT	Total
-	-	-	145
-	+	+	4
-	+	-	1

Other enteric pathogens detected by light microscopy were, *Giardia lamblia* in 12 cases (8%), *Blastocystis* spp. in 9 (6%) cases. No helminthic or mixed infections were recorded.



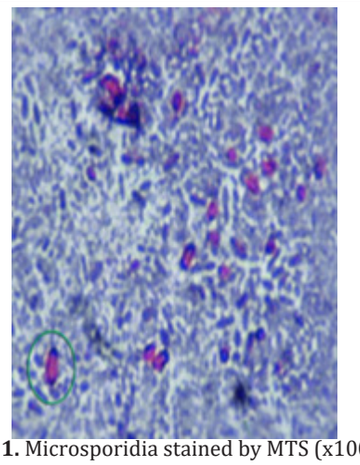


Fig. 1. Microsporidia stained by MTS (x100).

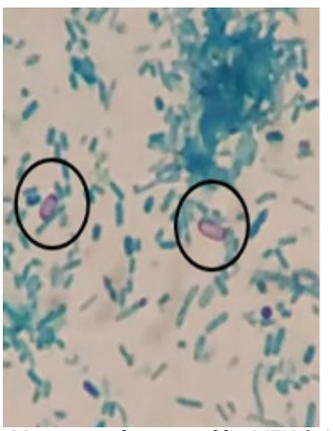


Fig. 2. Microsporidia stained by MZN (x100).

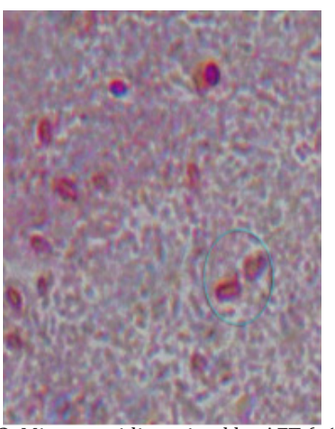


Fig. 3. Microsporidia stained by AFT (x100).

Fig. 4. Agarose gel 2% for products of the nested PCR of microsporidia isolates at 410-420 bp.
Lane (M) DNA molecular weight marker (100 bp);
lanes 1-8 and 10 positive samples;
lanes 9, 11 and 12 negative samples.

DISCUSSION

Microsporidium spp. infect human and an extensive range of animals worldwide[14], and may cause infection in both immunocompetent and immunosuppressed patients[15]. Presence of microsporidiosis in 17% of elderly patients and in 10% of Indian HIV negative individuals with diarrhea was recorded in Spain and India, respectively[6,16]. In developing countries enteric microsporidiosis is underestimated as its diagnosis is not included in routine examination of stool for ova and parasites. This is due to the need for special staining techniques which are rarely included, and because of technical inexperience and/or lack of physicians' awareness of its clinical significance[17]. Also, the application of antigenic techniques is limited[18]. According to Da Silva *et al*.[19] using specific PCR primers based on the region coding for small subunit ribosomal RNA (SSU rRNA) has shown the usefulness of such a tool for identification of microsporidia to the species level. Garcia[20] strongly assumed that with the use of more sensitive and specific methods, the number of patients who test positive for microsporidial infection might show dramatic increase.

In the present study, examination of 150 diarrheic stool samples under light microscopy using MTS detected microsporidia spp. in 12 (8%) of the samples, while MZN stain showed similar results as regard sensitivity (100%) with slight difference in specificity (98.57%). A previous study by El-Sayed *et al*.[17] in Egypt, reported the detection of microsporidia spores using MTS in (15.6%) of apparently immunocompetent individuals with diarrhea. However, MZN stain showed a better advantage in not only distinguishing microsporidia but also other oocysts-forming parasites as *Cryptosporidium*; proving that it is a better technique for use in developing countries minimizing the time and expenses needed. Additionally, Joseph *et al*.[10] were able to detect microsporidia (93.3%) in corneal scraping by MZN. Furthermore, AFT stain showed a promising role in diagnosis of both microsporidia and *Cryptosporidium* spp., but it needs fixation of the decolorization time, as sometimes the parasite may be washed off producing false negative results. On the other hand, under decolorization will result in false positive results. Garcia[11] mentioned that the thickness of the smear, the percentage of acid in the decolorizer and the time the smear is in contact with the decolorizer control the stain intensity.

A properly designed multiplex PCR would exclude highly trained expertise, reduce the diagnostic time, and detect multiple pathogens simultaneously without cross reaction. According to Lee *et al*.[12] the nested PCR primers that were primarily constructed for use in multiplex PCR with *Cryptosporidium* and *Cyclospora* were also capable of detecting both *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*, when followed by restriction fragment length polymorphism. In the present work, primers were tested using nPCR to evaluate their effectiveness in diagnosis of *Microsporidium* spp. owing to the high cost of multiplex PCR. As a result, the nPCR showed highest specificity (100%), but with lesser sensitivity (80%) than other staining techniques, which should be re-evaluated in comparison to the conventional PCR. False-negative results for microsporidiosis by PCR can be attributed to the presence of inhibitors during DNA extraction[15]. PCR sensitivity is also affected by the restricted number of specific probes, even if these probes are specific for the commonest two species of intestinal microsporidia[18]. Moreover, DNA extraction from microsporidia spores in stool are affected by the exceedingly small size of spores, rigid doubled-layer wall and the low counts in stool samples. In addition, the geographical and socioeconomic factors, source of infection, and diagnostic tests used, can have an impact on the molecular results[21].

The difference in diagnostic yield between microscopic examination and PCR was in accordance with Ghoyounchi *et al*.[15] who reported that out of 135 samples, 17 (12.8%) were microscopically positive for *Microsporidium* spp. infection, whereas only 14 (10.6%) cases were positive based on nPCR results. Similarly, El-Mahallawy *et al*.[18] reported that AFT staining technique is nearly as competent as the PCR, but simpler and less expensive, and can replace the molecular techniques for the diagnosis of microsporidia.

Moreover, Abdel-Hamid *et al*.[22] reported that permanent staining techniques and the use of ×100 objective allows accurate identification of important details needed for diagnosis of microsporidia. On the contrary, Kazemi *et al*.[21] confirmed that PCR technique was more sensitive than MTS. In a recent report microscopic examination of Weber's modified trichrome and modified Ziehl Neelsen stained stool samples recorded a total positivity of 13.9% microsporidiosis of which 14.5% were immunocompromised and 13.3% were immunocompetent; while nPCR and RFLP PCR scored 97.8% sensitivity and 100% specificity in detection of single and mixed infections with *Enterocytozoon bieneusi* and *Encephalitozoon* species[23].

In conclusion, this study demonstrated that using staining specific techniques as MZN and AFT in diagnosis of intestinal microsporidiosis in diarrheal stool samples are nearly as efficient as PCR. These stains may even be considered better in some cases with the added advantage of being an inexpensive diagnostic method compared to PCR. In addition, the mentioned stains are capable of detecting other oocyst forming intestinal parasites such as *Cryptosporidium*, which makes them very suitable for use in developing countries. Thus, it is relevant to endorse the selection of a standard reference test for detection of microsporidiosis. This

would allow identification of the exact contribution of microsporidia to diarrhea in pediatrics' in relation to other pathogens in a community known to be endemic for protozoal infections as Egypt. Additionally, testing for intestinal microsporidiosis should be part of patients' evaluation for diarrhea especially among immunocompromised population.

Author contribution: Zahran F shared in choosing the aim of study, designing the plan of work, performing staining techniques, analyzing the data and revising the manuscript. Ibrahim AN wrote and revised the manuscript, Abou-Seri HM conceived and designed the plan of work, shared in specimen collection, performed PCR, analyzed the data, wrote and revised the manuscript.

Conflict of interest: We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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