DOI: 10.2478/s11686-0110072-4

Acta Parasitologica, 2011, 56(4), 348–352; ISSN 1230-2821

VERSITA

Diagnosis of intestinal microsporidiosis in pediatric oncology patients in Egypt using modified acid fast trichrome staining versus PCR

Hadir El-Mahallawy[1], Mayssa Mohamed Zaki[2]*, Maha El-Arousy[3], Lobna Shalabi[4] and Tarek Mansour[5]

[1]Department of Clinical Pathology, National Cancer Institute, Cairo University, Egypt;
[2,3]Department of Medical Parasitology, Faculty of Medicine, Cairo University, Egypt;
[4]Department of Pediatric Oncology, National Cancer Institute, Cairo University, Egypt;
[5]Unit of Virology and Immunology, National Cancer Institute, Cairo University, Egypt

Abstract

Microsporidia are intracellular opportunistic parasites that cause chronic diarrhea in AIDS patients. Little is known about the prevalence of these pathogens in pediatric cases with cancer and diarrhea. Unidentified causes of chronic diarrhea were previously encountered in pediatric cancer patients at the National Cancer Institute in Egypt. Therefore, this study tried to search for the contribution of microsporidia as a causative agent of diarrhea in this population using acid-fast trichrome stain as a specific staining and the PCR in order to evaluate the staining technique in clinical diagnosis of microsporidia. Between January 2008 and June 2009, 271 diarrheic samples from pediatric patients with cancer were studied. Microsporidia were confirmed in 13 (4.8%) cases by both PCR and staining, and additional 2 samples were positive only by staining. As a negative control, stool samples from 60 diarrheic children without malignant cancer and no microsporidia detection were examined by these two methods. So, it can be concluded that adding a diagnostic test for microsporidia to the clinical laboratory work in hospitals concerned with cancer is essential. Acid fast trichrome staining technique as being nearly efficacious as the PCR, but simpler and less expensive, can replace the molecular techniques for the diagnosis of microsporidia.

Keywords

Microsporidia, pediatric cancer patients, acid fast trichrome stain, polymerase chain reaction (PCR)

Introduction

Microsporidia are obligate intracellular, spore-forming parasites that emerged as opportunistic pathogens since the onset of the AIDS pandemic. Infections caused by these fungal parasites have been predominantly identified in immunocompromised patients with chronic resistant diarrhea, a wasting syndrome, and malabsorption (Gamboa-Dominguez *et al*. 2003). *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are the most common causes of human infections and are associated with diarrhea and systemic disease, with *Enterocytozoon bieneusi* more commonly identified in patients with human immunodeficiency virus (HIV) infection (Stark *et al*. 2009). Systemic infections include pulmonary, nasal, ocular, muscular, and cerebral infections (Notermans *et al*. 2005).

These organisms appear to cause infection via waterborne transmission. Several microsporidia have been found in surface water samples and in solid waste landfill leachate, in sewage sludge in France and United States, and there was a report of a water-borne outbreak in Lyon (France) during summer (Cotte *et al*. 1997, Graczyk *et al*. 2007). Identification of microsporidia in water sources has resulted in their inclusion into the NIH Category B list of biodefense agents and the EPA microbial contaminant candidate list of concern for waterborne transmission (Didier *et al*. 2009).

Diagnosis of microsporidiosis is difficult owing the small size of the spores, *Enterocytozoon bieneusi* measuring from 0.8 to 1.4 µm and those of *Encephalitozoon* spp. from 1.5 to 4 µm (Didier *et al*. 1995). Traditionally, the diagnosis of microsporidiosis depends on direct visualization of the parasite by light microscopy after staining with special stains; however, the sensitivity and specificity of these techniques depend on the experience of the microscopist (Garcia 2002). On the other hand, application of antigenic techniques is limited.

*Corresponding autor: mayssaabcd@hotmail.com; Laila.Ossama@yahoo.com

Studies using specific PCR primers based on the region coding for small subunit ribosomal RNA (SSU rRNA) has shown the usefulness of such tool for identification of microsporidia to the species level (Da Silva *et al*. 1997).

Reports of microsporidia in pediatric patients with cancer are scarce and mostly in the form of case studies, especially in transplantation recipients (Orenstein *et al*. 2005, Lanternier *et al*. 2009). Previous studies concerned with identifying causes of diarrhea in cancer patients at the National Cancer Institute (NCI) revealed the presence of cases with no identifiable causative organism (El-Mahallawy *et al*. 2004). Therefore, the present study aims to search for the contribution of microsporidia as a cause of diarrhea in diarrheic pediatric oncology patients receiving therapy at the cancer institute of the Cairo University, and in addition, to evaluate the acid-fast trichrome stain specific for microsporidia using PCR as the reference standard technique.

Patients and methods

Patients

Stool samples were obtained from 271 children with different malignancies hospitalized in the NCI for chemo- and/or radiotherapy during the period of first of January 2008 and the end of June 2009. NCI is the main tertiary cancer center in Egypt receiving referrals from all Egypt. The patients' age ranged from one year to 18 years. The patients were complaining of gastro-intestinal symptoms mainly diarrhea (i.e., more than 4 bowel motions per day) with or without other symptoms such as vomiting or dehydration. As a negative control group, stool samples were also collected from 60 children of matching age and socioeconomic status but without malignancy. They were complaining of diarrhea and attending the Children Hospital in Cairo, Abou-El Riche. The study was approved by the Ethical Committee Board of the University and the parental consents were obtained.

Stool samples

After direct microscopic examination of the samples, each sample was divided into three parts. The first portion was destined for routine microbiological cultures including fungal culture on Sabaroud dextrose agar in order to exclude candida as stool colonizer which might cause false positive results in staining. Another part was preserved in formalin for concentration technique both for microscopic examination and for acid-fast trichrome (AFT) staining, whereas the third part was placed into sterile containers and kept at –20ºC for PCR analysis.

Concentration technique

All stool specimens were processed for wet mount examination after concentration using the formalin-ethyl acetate sedimentation procedure with centrifugation for 10 min (Melvin and Brooke 1982). A drop of the sediments was examined by light microscopy with and without iodine staining.

Modified acid-fast trichrome staining (MTS): This stain allows detection of microsporidia in addition to enteric coccidian parasites. From each of the previously prepared concentrates, thin films were spread on two slides using 0.01 ml of the sediments and air dried. Slides were immediately fixed in methanol for 5 min, stained according to Didier *et al*. (1995), and examined directly with an oil immersion microscope objective, the latter requiring approximately 10 min examination time for each slide.

Polymerase chain reaction (PCR): DNA was prepared by using a QIAmp tissue kit [Qiagen, Hilden, Germany]. Specimens were incubated in digestion buffer with 400 µg of proteinase K [Qiagen] and 0.4 U of chitinase [Sigma] at 55°C for 2 h; then vortexed for 1 min. DNA was prepared from the solution by using QIAmp spin columns in an Eppendorf microcentrifuge following the manufacturer's instructions.

Generally, the most frequently used target for PCRs is the microsporidial SSU rRNA gene. In this study, the primers used for diagnosis of *E. bieneusi* and *Encephalitozoon* were used (Notermans *et al*. 2005) to amplify specifically conserved regions of SSU rRNA, in terms of positions 4 to 23 for *E. bieneusi* [GenBank accession no. AF023245] 5'-CAGGTTGATTCTGCCTGACG, as the forward primer (FP), and positions 263 to 244 for *E. bieneusi*, 5'-ATCTCTCAGGCTCCCTCTCC, as the reverse primer (RP).

The PCR was performed in 50-µl of reaction mixture containing the following: 50 pM of each primer, 10X NH_4 PCR buffer, and 200 µM of each of dNTP, 2 mM $MgCl_2$, and 2 units of Taq DNA polymerase [Promega]. DNA amplification was performed using thermocycler [Bio RAD DNA Engine Dyad Peltier-Mexico] and each set of reactions included a negative control (i.e., reagent mixture without template DNA) and a positive control kindly supplied by Dr. Maha El-Arousy (El-Arousy *et al*. 2004). The amplification procedure included 35 cycles, each comprising 5 min of initial denaturation at 95°C, followed by 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C, and 90 sec of extension at 72°C. A 10 min extension at 72°C was added after the 35 cycles. Amplified products were electrophoretically resolved on a 2% agarose gel and stained with ethidium bromide to visualize the amplified PCR products under UV illumination.

Results

Modified acid-fast trichrome stain revealed 15 microsporidia-positive samples. Microsporidian spores were found to be oval or round bodies of different sizes (1.5–4 µm), distributed either solitary (6 samples), or found in clusters (9 samples). Also, they varied in their staining characteristic, appearing either with a medium dark band or with bipolar staining (Fig. 1), or simply as pinkish oval bodies. Macroscopic appearance of

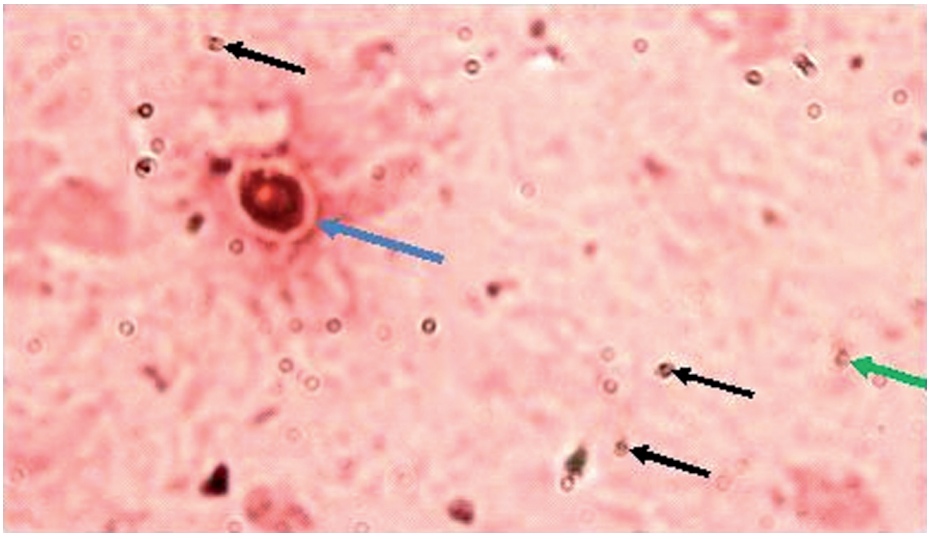

Fig. 1. Photomicrograph showing microsporidia (arrows) in stool stained with modified acid-fast trichrome

the positive stool samples showed that 5 samples were greenish and watery, 7 samples were watery and mucoid with or without blood, and 3 samples were semiformed. The staining cost per sample was calculated to be approximately 30 LE (i.e., 3 US$).

Of the 15 microscopy-positive stool samples, 13 samples (4.8% of all examined samples) were affirmed positive in both slides, while in two samples prepared of semiformed stools, microsporidia were only detected in one of the two slides.

After extraction, the DNA content was measured using Beckman coulter [DU^R 730] and UV/Vis spectrophotometer [Siemens med.]. The mean DNA concentration was 11.45 ± 6.35 µg/ml. Amplification of templates resulted in a diagnostic 260 bp specific DNA fragment according to the gene bank. Accordingly, PCR was positive in 13 cases (Fig. 2), that were affirmed positive in both stained slides. Macroscopic appearance, microscopic findings after staining with modified acid-fast trichrome stain, and PCR results of the positive group are summarized in Table I.

Other enteric pathogens detected by light microscopy were, *Blastocystis* in 14 (5.17%) cases, *Giardia lamblia* in 12 cases (4.43%), *Cryptosporidium parvum* in 7 cases (2.58%), *Cyclospora* in 2 cases (0.74%), and *Entamoeba histolytica* in 2 cases (0.74%). Out of the 15 cases found positive for microsporidia, 2 were harboring *Blastocystis*, and another 2 *Giardia lamblia* trophozoites, while *Cyclospora* oocysts were detected in one case.

For the negative control group, out of 60 individuals, 4 (6.67%) were positive for *Blastocystis*, whereas 4 (6.67%) for *Giardia lamblia.*

Table I. Macroscopic appearance, parasites detected by microscopic examination and the PCR results

Patient number	Organism detected by light microscopy	Result of PCR using primer pair (AF023245)
1	Microsporidia	Positive
2	Microsporidia, *Blastocystis*	Positive
3	Microsporidia	Positive
4	Microsporidia	Positive
5	Microsporidia	Positive
6	Microsporidia, *Cyclospora* oocyst	Positive
7	Microsporidia	Negative
8	Microsporidia	Positive
9	Microsporidia, *Giardia lamblia*	Positive
10	Microsporidia, *Blastocystis*	Negative
11	Microsporidia	Positive
12	Microsporidia, *Giardia lamblia*	Positive
13	Microsporidia	Positive
14	Microsporidia	Positive
15	Microsporidia	Positive

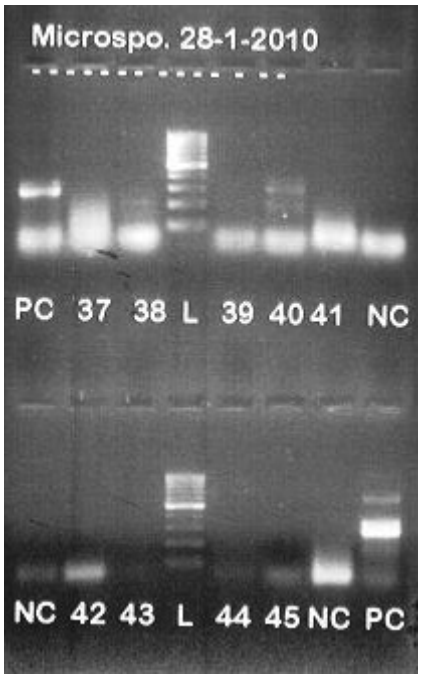

Fig. 2. PCR amplified products of microsporidia (*Enterocytozoon bieneusi* and *Encephalitozoon*) from stool samples

Discussion

In the present study, the prevalence of microsporidia in pediatric cancer patients with gastrointestinal manifestations approached 5%. It was previously reported that microsporidia were detected as causative agents of chronic diarrhea in immunocompromised patients with cancer (Angela *et al*. 2008). A prevalence of 10.5% was recorded in HIV-infected patients and adult patients with multiple myeloma in Tunisia by PCR with universal primer V1/PMP2 (Charchoubi *et al*. 2009). A prevalence of 10% was also detected in adult patients with cancer in Turkey (Karaman *et al*. 2008). Thus, it is evident that rates of infection vary among different geographical areas. In agreement, it was previously stated that the geographical distribution and the high prevalence among immunocompromised patients suggest that microsporidia may be natural parasites of humans (Franzen and Müller 1999). In addition, it was strongly suspected that with the use of more sensitive and specific methods, the number of patients who test positive for microsporidial infection may increase dramatically (Garcia 2002).

Other protozoa **(***Giardia*, *Cryptosporidium*, *Cyclospora* oocysts, and *Entamoeba* cysts have been detected) may have shared in the pathogenesis of diarrhea in few cases in this study. According to Moore *et al*. (2002), there have been very limited data describing the frequency and etiology of mixed enteric infections even in the developed world.

The search for the previous protozoa as causative agents for diarrhea is a routine practice in the labs of the cancer institute which is expected (Egypt is known to be epidemic for intestinal protozoa in rural areas which constitute the majority of our patient), however *Microsporidium* has not been given enough attention by physicians in diagnosis and treatment of immunocompromised diarrheic patients. In addition, the attribution of the diarrhea to *Microsporidium* needs to be further studied.

The primers used in the present study allow the detection and identification of two the most important species of microsporidia, *Enterocytozoon bieneusi* and *Encephalitozoon* spp., with a single PCR. These primers amplified the conserved region of SSU rRNA and produced positive results in 13 pediatric patients with malignant diseases at the National Cancer Institute in Egypt. Utilizing the modified acid-fast trichrome stain, 15 samples were positive. A false positive diagnosis by microscopy cannot be excluded when considering the more or less large variation in size and staining characteristic of these organisms, making a definitive identification difficult. Generally, small size of the microsporidial spores and close resemblance to other fungal spores and some streptococci might contribute to the ambiguity of microscopic results. PCR has shown the sensitivity of 95% and specificity of 100% when compared to microscopy (Notermans *et al*. 2005). In addition, PCR with universal primers, V1/PMP2, detected more intestinal microsporidia infections in immunocompromised patients (9 cases) than did light microscopy (5 cases). It was reported that the sensitivity of PCR allows the detection of parasitic load of 10^2 spores per gram of stool (Chabchoub *et al*. 2009). The authors of the latter study (Chabchoub *et al*. 2009) recommended the use of PCR for determining the true prevalence of microsporidia in immunocompromised patients.

On the other hand, a false negative PCR results are possible not due to the lack of its sensitivity, but because of the restricted number of specific primers utilized, which although expected to detect most of the important species, do not detect other microsporidian species implicated in causation of microsporidiosis. Similarly, Franzen and Müller (1999) reported negative PCR results for samples originally positive for *E. bieneusi* spores by microscopy. In the latter study, the authors considered the possibility of false positive microsporidial staining results, and also false negative PCR results due to the presence of inhibitors during DNA extraction. In addition, they raised the possibility that the storage of some samples for years at 4°C prior to performing the PCR analysis might have resulted in degradation of the spores and microsporidial DNA.

In relation to the efficiency of modified acid-fast trichrome staining as compared to the PCR in the present study, the stain by being a cheaper (costing about 30 LE/sample, i.e., about 3 US$) and simpler technique than the PCR, makes staining and microscopic examination more feasible as an initial test. Thus, staining could be recommended as a routine test for oncology children if they have resistant diarrhea with no other detected organisms, providing that the laboratory workers have appropriate training. Nevertheless, the PCR remains to be an alternative in some cases, if this can be financed. In addition,

choosing the modified acid fast trichrome staining instead of simple modified trichrome staining allows for instantaneous examination of stool samples for coccidian oocysts not uncommonly found in such immunocompromised patients. This could save time by omitting separate acid fast staining for coccidians and by being less time consuming, requiring only 30 min for the immersion step in Didier's trichrome solution instead of 90 min necessary for the modified trichrome staining technique.

In conclusion, microsporidiosis is an important cause of diarrhea in pediatric cancer patients in Egypt, and additional diagnostic test for microsporidia applied to the patients with diarrhea is essential. PCR results are very close to those of the specific staining techniques. Therefore, the use of specific stains is driven by the facts that these stains are being less expensive and less labor extensive when compared to the molecular techniques.

As false positive diagnosis by microscopy cannot be excluded, strict identification by staining is difficult and PCR sensitivity is affected by the restricted number of specific probes (even if the used probes are specific for the commonest two species of intestinal microsporidia), thus further study to evaluate both procedures so as to select a reference standard for microsporidial infections, in addition to identifying the exact contribution of microsporidia to diarrhea in cancer pts in relation to other pathogens in a community known to be endemic for protozoal infections is recommended.

References

Angela R., Kumar S.L., Chye T.T. 2008. Incidence of Microsporidia in Cancer Patients. *Journal of Gastrointestinal Cancer*, 39, 124–129.

Chabchoub N., Abdelmalek R., Mellouli F., Kanoun F., Thellier M., Bouratbine A., Aoun K. 2009. Genetic identification of intestinal Microsporidia species in immunocompromised patients in Tunisia. *American Journal of Tropical Medicine and Hygiene*, 80, 24–27.

Cotte L., Rabodononirina M., Raynal C., Chapuis F., Piens M.A., Trepo C. 1997. Outbreak of intestinal microsporidiosis in HIV-infected patients in relation to town water distribution system, abst. I-147. In: *Program and Abstract of the 37th Interscience Conference of Antimicrobial Agents and Chemotherapy (Toronto). Washington*, *DC*, *American Society for Microbiology*.

Da Silva A.J., Slemenda S.B., Visvesvara G.S., Schwartz D.A., Mel Wilcox C., Wallace S. 1997. Detection of *Septata intestinalis* (Microsporidia) using polymerase chain reaction primers targeting the small subunit ribosomal RNA coding region. *Journal of Molecular Diagnosis*, 2, 47–42.

Didier E.S., Louis M. Weiss, Cali A., Marciano-Cabral F. 2009. Overview of the Presentations on Microsporidia and Free-Living Amebae at the 10th International Workshops on Opportunistic Protists Eukaryotic Cell 2009 April, 8(4), 441–445.

Didier E.S., Orenstein J.M., Aldras A., Bertucci D., Rogers L.B., Janney F.A. 1995. Comparison of the three staining methods for detection of microsporidia in fluids. *Journal of Clinical Microbiology*, 33, 1318–1345.

El-Arousy M.H., Abd El-Aal A., El-Badry A.A., Rashed L., El-Mahallawy H. 2004. Diagnosis of intestinal microsporidiosis by calcofluor white staining and PCR. *Egyptian Journal of Medical Science*, 25, 135–146.

El-Mahallawy H., Aly El-Din N., Salah F., El-Arousy M., Abou El-Naga S. 2004. Epidemiologic profile of symptomatic gastroenteritis in pediatric oncology patients receiving chemotherapy. *Pediatric Blood & Cancer*, 42, 338–342. DOI: 10.1002/pbc.10394.

Franzen C., Müller A. 1999. Microsporidiosis: human disease and diagnosis. *Microbes and Infection*, 3, 389–400. DOI: 10.1016/S1286-4579(01)01395-8.

Gamboa-Dominguez A., De Anda J., Donis J., Ruiz-Maza F., Visvesvara G.S., Diliz H. 2003. Disseminated *Encephalitozoon cuniculi* infection in a Mexican kidney transplant recipient. *Transplantation*, 75, 1898–1900. DOI: 10.1097/01.TP.0000064623.57821.22.

Garcia L.S. 2002. Laboratory Identification of the Microsporidia. *Journal of Clinical Microbiology*, 40, 1892–1901. DOI: 10.1128/JCM.40.6.1892-1901.2002.

Graczyk T.K., Kacprzak M., Neczaj E., Tamang L., Graczyk H., Lucy F.E., Girouard A.S. 2007. Human-virulent microsporidian spores in solid waste landfill leachate and sewage sludge, and effects of sanitization treatments on their inactivation. *Parasitolology Research*, 101, 569–575. DOI: 10.1007/s00436-007-0515-x.

Karaman U., Daldal N., Atambay M., Colak C. 2008. The epidemiology of microsporidias in humans (Malatya sample). *Turkish Journal of Medical Science*, 39, 281–288. DOI: 10.3906/sag-0803-32.

Lanternier F., Boutboul D., Menotti J., Chandesris M.O., Sarfati C., Mamzer Bruneel M.F., Calmus Y., Mechai F., Viard J.P., Lecuit M., Bouqnoux M.E., Lortholary O. 2009. Microsporidiosis in solid organ transplant recipients: two *Enterocytozoon bieneusi* cases and review. *Transplant Infectious Disease*, 11, 83– 88.

Melvin D.M., Brooke M.M. 1982. Laboratory procedures for the diagnosis of intestinal parasites. 3rd ed. U.S. Department of Health and Human Services publication no. (CDC) 82–8282, p. 36, 75–83, 105–106, 113 and 128. Centers for Disease Control, Atlanta.

Moore J.E., Crothers L., Millar B.C., Crothers E., Rooney P.J. 2002. Low Incidence of Concurrent Enteric Infection Associated with Sporadic and Outbreak-Related Human Cryptosporidiosis in Northern Ireland. *Journal of Clinical Microbiology*, 40, 3107–3108. DOI: 10.1128/JCM.40.8.3107-3108.2002.

Notermans D.W., Peek R., De Jong M.D., Wentink-Bonnema E.M., Boom R., Van Gool T. 2005. Detection and identification of *Enterocytozoon bieneusi* and *Encephalitozoon* species in stool and urine specimens by PCR and differential hybridization. *Journal of Clinical Microbiology*, 43, 610–614. DOI: 10.1128/JCM.43.2.610-614.2005.

Orenstein J.M., Russo P., Didier E.S., Bowers C., Bunin N., Teachey D.T. 2005. Fatal pulmonary microsporidosis due to *Encephalitozoon cuniculi* following allogeneic bone marrow transplantation for acute myelogenous leukemia. *Ultrastructural Pathology*, 29 (3–4), 269–276.

Stark D., Van Hal S., Barratt J., Ellis J., Marriott D., Harkness J. 2009. Limited genetic diversity among genotypes of *Enterocytozoon bieneusi* strains isolated from HIV-infected patients from Sydney, Australia. *Journal of Medical Microbiology*, 58, 355–357.

(Accepted October 18, 2011)