SPECIES IDENTIFICATION OF INTESTINAL MICROSPORIDIA USING IMMUNOFLUORESCENCE ANTIBODY ASSAYS

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Abstract. The species identification of *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* is only possible using transmission electron microscopy (TEM), molecular techniques and immunofluorescence antibody assays (IFA). In this study, 50 positive and 50 negative fecal specimens for microsporidial spores using the Weber modified trichrome (WMT) staining technique were examined using IFA-MAbs. Of the 100 specimens examined, the microsporidial spores identified by IFA-MAbs were *Enterocytozoon bieneusi* 42 (75%) *Encephalitozoon intestinalis* 7 (12.5%) and mixed infections 7 (12.5%). The sensitivity and specificity of IFA-MAbs in detecting microsporidial spores were 98% and 86%, respectively. The agreement between the WMT staining technique and IFA-MAbs was statistically significant by Kappa statistics (K=0.840; p<0.001). *E. bieneusi* was the commonest *Microsporidia* species isolated from the studied population; the presence of microsporidial spores detected by IFA-MAbs should be confirmed by other methods.

Keywords: microsporidia, identification, IFA-MAbs

INTRODUCTION

Microsporidiosis is an opportunistic infection caused by obligate intracellular spore-forming organisms. *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are the primary microsporidium species associated with humans. A diagnosis of *Microsporidia* is traditionally based on direct visualization of the microsporidial spores using light and fluorescent microscopy. A number of stains have been used to demonstrate spores using light microscopy, such as Gram's stain, Giemsa stain, Gram-chromotrope and modified trichrome stain. Weber's modification of trichrome (WMT) stain (Weber *et al*, 1992) has allowed more definitive identification of *Microsporidia* by light microscopy in fecal specimens and is used as the standard technique in the diagnosis of microsporidiosis (Moura *et al*, 1997). Clinical specimens stained with chemofluorescent reagents, such as Uvitex 2B and Calcofluor White M2R, as described by van Gool *et al* (1993) and Didier *et al* (1995), respectively, have been used in the identification of microsporidian

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spores (Zulu *et al*, 2002; Endeshow *et al*, 2005; Nikinin *et al*, 2007). These techniques may generate false-positive results due to similarly staining small yeast cells, resulting in lower specificity (Didier *et al*, 1995). The simultaneous use of two or more of the above methods enhances the sensitivity and specificity of the tests and may provide greater accuracy, especially in patients with light infections with microsporidiosis.

These techniques cannot distinguish among the various species of *Microsporidia*. A more sensitive and specific assay is PCR, which is considered the method of choice for species identification; however, it is not routinely used since it is time consuming and labor intensive. An immunofluorescence antibody assay (IFA) using a specific monoclonal antibody is used for the identification of *Microsporidia* species spores in fecal specimens (Beckers *et al*, 1996). This study was carried out to determine the validity of IFA-MAbs for the identification of intestinal microsporidia compared with WMT staining as a reference technique. IFA-MAbs was used for species identification of intestinal microsporidial spores in this study.

MATERIALS AND METHODS

Fecal specimens

Fecal specimens received from hospitalized patients and patients seen in the clinics of Hospital Universiti Kebangsaan Malaysia were examined for the presence of microsporidial spores using the Weber modified trichrome (WMT) staining technique (Weber *et al*, 1992). Fifty positive and 50 negative fecal specimens for microsporidial spores using the WHT were examined for the presence of microsporidial spores using the immunofluorescence antibody assay (IFA-MAbs). This study was approved by the Medical Research and Ethics Committee of the Universiti Kebangsaan Malaysia.

Immunofluorescence antibody assay (IFA-MAbs)

Fresh fecal samples were diluted (1:2) with phosphate-buffer saline (PBS pH7.4) and filtered through many layers of gauze. A slide (14-wells) was prepared by depositing 2 µl of the diluted fecal samples into each well; then air dried at room temperature for an hour in an incubator. The slide was fixed with methanol for few seconds and left to air dry. Then, the slide was dipped for ten minutes in acetone at -20ºC and left to air dry. Each well of the slide was rehydrated with a drop of PBS and left for five minutes at room temperature. The PBS was aspirated and each well was covered with 20 µl of monoclonal antibody (Bordier Affinity Products SA, Crissier, Switzerland), incubated for 30 minutes in a moist chamber at room temperature at high humidity. The slide was then washed three times with PBS and each well was covered with 20 µl of conjugated fluoresceine isothiocyanate (FITC) and incubated for 30 minutes in a moist chamber in the dark. Conjugated FITC (Jackson ImmunoResearch Laboratories, West Grove, PA) labeled anti-mouse IgG was diluted 1/100 in PBS containing evans blue (Sigma-Aldrich Lyon, France) to a final concentration of 5 mg/l. The conjugate was then aspirated and the slide washed three times with PBS and dried. Cover slips were mounted with anti-fading fluorescence mounting medium in the wells and the slide was examined with a standard fluorescent microscope (Leica Digital DM 5000B, Leica Microsystems CMS GmbH, Germany), connected to a computer and a digital camera (ProgRes®). The smear was examined at 1,000x magnification with ultra-violet light. Double-blind examination

Table 1
Sensitivity and specificity of IFA-MAbs for detecting microsporidial spores in feces compared to the reference technique (WMT).

Immunofluorescence antibody assays (IFA-MAbs)	Weber modified trichrome (WMT)		
	Positive	Negative	Total
Positive	49	7	56
Negative	1	43	44
Total	50	50	100

Sensitivity: true positive: 49/50 = 98%; Specificity: true negative: 43/50 = 86%; Positive predictive value: 49/56 = 87.5%; negative predictive value: 43/44 = 97.7%

of IFA-stained fecal slides was conducted to determine the presence of microsporidial spores which were detected by visualizing bright green spores.

Data analysis

Data analysis was performed using the Statistical Package for Social Sciences for Windows SPSS (version 13, March 2004). Sensitivities, specificities, and positive and negative predictive values were calculated with their 95% confidence intervals. Kappa statistics were used to assess the agreement between tests. A p-value < 0.05 was considered significant.

RESULTS

Using IFA-MAbs staining, the microsporidial spores appear as bright apple-green oval halos, with an orange to brownish yellow background. The monoclonal antibodies react exclusively with the spore walls of microsporidia. *E. bieneusi* spores (1.3 x 0.7 μm) and *E. intestinalis* spores (1.7 x 1.0-1.1 μm) are surface labeled with a marked peripheral fluorescence. Although yeast cells and diplococcus-like bacterial spores exhibit dull fluorescence, they can easily be distinguished from microsporidial spores by the bright fluorescence as shown in Fig 1 (a). Of the 100 fecal specimens examined with IFA-MAbs, 56 samples were positive for microsporidia. The majority of microsporidial spores were *E. bieneusi* 42 (75%), followed by *E. intestinalis* 7 (12.5%), and mixed species 7 (12.5%). Fig 1 (b) shows the microsporidial spores stained with WMT stain.

The sensitivity and specificity of IFA-MAbs for detecting microsporidial spores were calculated by comparing its validity with WMT as a reference standard. Of the 50 fecal specimens testing positive by WMT, 49 were detected with IFA-MAbs and one was a false negative (Table 1). There were 7 false positive cases with IFA-MAbs. The sensitivity and specificity of the IFA-MAbs staining technique were 98% and 86%, respectively. The positive and negative predictive values were 87.5% and 97.7%, respectively. The agreement between the WMT and IFA-MAbs techniques was statistically significant by Kappa (K=0.840; p<0.001).

DISCUSSION

The immunofluorescence antibody assay with monoclonal antibodies (IFA-MAbs) is highly sensitive and specific

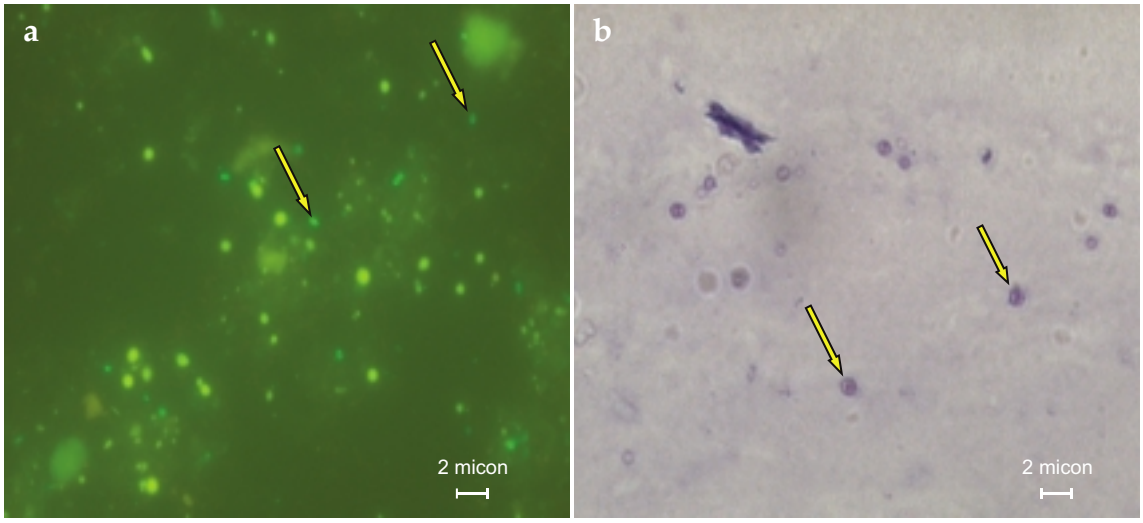

Fig 1–(a) Microsporidial spores from a fecal specimen stained with IFA-MAbs seen under fluorescent microscopy (magnification, x1,000). (b) Microsporidial spores from fecal specimen stained with Weber modified trichrome stain (magnification, x1,000). Scale bar = 2 μm.

for identifying microsporidia species (Enriquez 1997; Cisse *at al*, 2002). The IFA-MAbs has been successfully tested for detecting microsporidian spores of *E. bieneusi* on fecal specimens (Accoceberry *et al*, 1999); our findings show a lower specificity of IFA-MAbs in detecting microsporidial spores. Thus, the presence of microsporidial spores detected by IFA-MAbs must be confirmed by other methods. This is consistent with a previous study by Garcia *et al* (1994), who reported the IFA assay is not specific for microsporidia. However, Cisse *et al* (2002) reported IFA-MAbs had 100% sensitivity and 100% specificity for detecting of microsporidian spores when compared with primary polymerase chain reaction (PCR). Singh *et al* (2005) found a specificity of 100% with the IFA-MAbs compared to PCR and the IFA was more sensitive than PCR for detecting microsporidial spores. Our study found the IFA-MAbs has a high sensitivity for detecting microsporidial spores.

Identification of microsporidia to the species level is essential for prompt and proper pharmacological therapy to reduce the risk of progression to disseminated infection with a fatal outcome in immunocompromized patients. Species identification is possible using a transmission electron microscope (TEM) and molecular techniques (PCR). However, these techniques are tedious, time-consuming and not always available at diagnostic laboratories. The TEM may not be sensitive enough to detect a small number of microsporidial spores in feces (Lan and Michel, 2004). Therefore, it is advantageous to develop species-specific IFA-MAbs that can be used for species identification of microsporidia.

Our study showed the main cause of intestinal microsporidiosis was *E. bieneusi*, in 75% of positive cases. A previous study in Spain by Lorse *et al* (2002), detected 8 cases of microsporidiasis among 60 elderly HIV-negative patients: all the cases were

diagnosed as *E. bieneusi* infection by WMT and PCR. Similarly, *E. bieneusi* was identified using PCR in 12.9% and 4.5% of South African hospitalized patients and primary schoolchildren, respectively (Samie *et al*, 2007).

In conclusion, this study revealed that IFA-MAbs staining is a useful tool for species identification of microsporidia in fecal specimens. *E. bieneusi* was the commonest *Microsporidia* species isolated in the studied population. In view of the low specificity of IFA-MAbs obtained in this study, the presence of microsporidial spores detected by IFA-MAbs must be confirmed by other methods.

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