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Comparison of Calcofluor White M2R Fluorescence and Modified Gram Chromotrope Kinyoun Staining Methods for the Detection of Microsporidial Spores from Stool Samples

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ABSTRACT

Routine diagnosis of intestinal microsporidiosis in clinical diagnostic laboratories relies mostly on detection of microsporidial spores via special staining and microscopic techniques. This paper describes the comparative evaluation of Calcofluor White M2R method, with modified Gram-chromotrope Kinyoun method as the reference standard. One hundred and six stool samples were examined for the presence of microsporidial spores. Sensitivity, specificity, positive and negative predictive values of the Calcofluor White M2R method compared to the reference technique were 95.2%, 4.3%, 78.2% and 20.0%, respectively. The positive predictive value (PPV) was 78.2% and the negative predictive value (NPV) was 20.0%. Despite low specificity of the CFW method due to its ability to stain chitinous wall of microorganisms, the presence of distinct deep-blue horizontal or equitorial stripes in microsporidial spores in modified Gram-chromotrope Kinyoun would likely reduce the false positive results obtained in the Calcofluor White M2R. Hence, the simultaneous use of these two methods would give better performance and accuracy for the detection of microsporidial spores in patients with intestinal microsporidiosis.

Keywords: Microsporidia, Calcofluor White M2R, Gram-chromotrope Kinyoun

INTRODUCTION

Microsporidia are single-celled, obligate intracellular parasites that affect a broad range of invertebrates and vertebrates[1]. Currently, 14 out of 1300 microsporidia species are known to infect humans, while chronic diarrhoea is recognised as the most common presenting feature in immunocompromised individuals such as HIV, organ transplant and cancer patients[2,3]. Despite increased awareness of this emerging disease, clinical diagnosis remains a challenge to physicians as most of the clinical manifestations are non-specific.

Detection of microsporidial spores by electron microscopy used to be confirmatory, but it is not suitable for a routine use in diagnostic laboratories[3,4]. Furthermore, the presence of PCR inhibitors has also limited the use of molecular techniques for detecting for spores in stools[5]. Thus, we believe that the application of histochemical staining methods to visualize spores is still practical in our clinical settings with limited resources and technical manpower. Previous studies have shown that the use of fluorescent brightening agents in Calcofluor White M2R, Uvitex 2B or Fungifluor stain is only good for screening purposes. Thus, all microsporidia-positive slides would usually be confirmed by modified trichrome stain[6,7].

In Malaysia, screening of samples for microsporidial spores is not routinely done in most hospitals and only a few clinical diagnostic laboratories offer a modified trichrome or Gram-chromotrope staining method for the detection of microsporidial spores on request basis[8,9]. The detection of microsporidial spores as requested by clinicians is done based on the clinical signs and symptoms. For the past few years, we have been using the modified Gram Chromotrope Kinyoun stain to identify microsporidial spores in stools of our patients with excellent performances[9,10,11]. This special stain was developed at the Department of Parasitology and Medical Entomology, Universiti Kebangsaan Malaysia, Kuala Lumpur[12]. The present study was carried out with the aims to compare the sensitivity and specificity of Calcofluor White M2R (CFW) and modified Gram Chromotrope Kinyoun (MGCK) staining methods in detecting microsporidial spores using MGCK stain as the reference standard, and to assess the feasibility of CFW stain for a routine use in our laboratory.

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MATERIALS AND METHODS

One hundred and six fresh stool samples of hospitalized children were collected from the Institute of Paediatric, Kuala Lumpur, following written consent of their parents. Approval for the study was also obtained from Universiti Putra Malaysia's Ethical Committee and the Ministry of Health Malaysia (NMRR-08-1802-3037). Each faecal smear was divided into two parts. One part was stained with Calcofluor White M2R (CFW; Sigma Chemical Co., St. Louis, Mo., USA), and the other part with modified Gram Chromotrope Kinyoun (MGCK).

The CFW staining procedure was performed according to the manufacturer's instructions. The ready-mixed CFW was centrifuged for two minutes, while 1-2 drops were applied to methanol-fixed faecal smears for 2 to 3 minutes, along with 1 drop of 10% potassium hydroxide. The slides were rinsed under slow running tap water and counterstained with 0.1% Evans Blue in Tris-Buffer Saline (pH 7.2) for 1 minute. The slides were re-washed again under slow running tap water and air dried. Lastly, all the slides were viewed under a UV microscope (MOTIC BA400 fluorescence compound trinocular microscope with a D350/50x exciter filter at a wavelength of between 395-415 nm). The microsporidial spores were identified as bright green to bluish turquoise oval halos, as described by Garcia[13].

The MGCK staining procedure was performed according to the previously published protocol[12]. In brief, after all the faecal smears had been air-dried and fixed in methanol for 5 minutes, the slides were stained with crystal violet for 1 minute. The excess stain was rinsed off with Gram's iodine. The slide smears were then counterstained with Gram's iodine for 2 minutes and gently decolourized with acid-alcohol. Following this, the slides were washed under running tap water for 10-15 seconds and stained with chromotrope stain for 8 minutes, as described by Moura *et al*.[8]. Then, the slide smears were rinsed in 90% acid alcohol, counterstained with Kinyoun's carbol-fuchsin for three minutes and rinsed again in 90% acid alcohol. Finally, the slides were dipped in 95% and 100% alcohol twice for two minutes, drained and dried completely before mounting with DPX.

Microsporidial spores were identified according to the established criteria; the presence of one or more pinkish-blue ovoid structures with a belt-like stripe in the middle of the spore, in at least 100 fields examined under 100× magnification and confirmed by two parasitologists[14]. The spores were graded as follows: 1+ if the average number of spores seen was 1-10; 2+ if the average number of spores seen was 11-20; and 3+ if the average number of spores seen was more than 21[9].

The formula used to assess the diagnostic performance of CFW stain was based on Thomas and Michelle[15], as follows:

	Reference Method		
Test Method	Positive	Negative	Total
Positive	TP (True positive)	FP (False positive)	(TP+FP)
Negative	FN (False negative)	TN (True negative)	(TN+FN)
Total	(TP+FN)	(TN+FP)	

RESULTS AND DISCUSSION

Higher detection rate was detected in stool smears stained with CFW than MGCK (95.3% versus 78.3%; data not shown) in this study. However, the CFW method yielded more false positive results (95.7%), leading to reduced specificity (Table 1). Our findings corroborate with Didier *et al*.[3]. In their study, 50 formalinized stool samples

Table 1. Performance of Calcofluor White M2R staining technique in detecting microsporidial spores with Gram Chromotrope Kinyoun as the reference standard

	Gram Chromotrope Kinyoun		
Calcofluor White M2R	Positive	Negative	Total
Positive	79 (TP)	22 (FP)	101 (TP+FP)
Negative	4 (FN)	1 (TN)	5 (FN+TN)
Total	83 (TP+FN)	23 (FP+TN)	106

Sensitivity: TP/(TP+FN): 79/83 = 95.2%; Specificity: TN/(FP+TN): 1/23 = 4.3%; Positive predictive value: TP/ (TP+FP): 79/101 = 78.2%; Negative predictive value: TN/ (FN+TN): 1/5 = 20%. False positive rate: FP/(FP+TN): 22/23 = 95.7%. False negative rate: FN/(TP+FN): 4/83 = 4.8%.

containing serial 10-fold dilutions of microsporidial spores were examined by using three methods, namely, Calcofluor White M2R, Modified Trichrome Blue and indirect immunofluorescent antibody (IFA) staining. In particular, the CFW method was found to exhibit 100% of sensitivity that is similar to modified trichrome blue, but had a comparatively low specificity (77.4%) using transmission electron microscopy (TEM) as the reference standard. This finding is not surprising as most chemofluorescent brighteners bind to chitinous layer of microsporidial spores but they can also bind to the chitins found in yeast cells leading to non-selective staining. In contrast, Luna *et al.*[16] found that the CFW method was more sensitive than Modified Trichrome Blue but performed similarly in terms of its specificity. However, no standard reference method was included in their study.

Fluorescent stains, such as the CFW and others, are also useful for detecting spores in smears, and it is well known that the staining features of these fluorescent methods are influenced by the intensity and selectivity of fluorescence being used[13,16,17]. For instance, Conteas *et al.*[18] successfully reduced the false positive findings due to the background staining by using different UV wavelengths. The specificity of CFW can be improved if one considers the size (microsporidial spores are smaller) and the budding nature of yeasts seen in the stain (see Figure 1). Several modifications have been made to improve the specificity of CFW by using an alkaline solution (1N NaOH), which can recover the old faded spore and reduce the background problem[19]. The use of counterstain, such as Evan's blue, can diminish the tissue and cellular backgrounds, while the use of potassium hydroxide can enhance the visualization of the spore[20]. The CFW stain provides a good screening method for microsporidiosis as it is less time consuming (15 minutes) as compared to Modified Trichrome stain and Gram Chromotrope stain[20]. Furthermore, the CFW stain is also very practical and much cheaper as it does not require any series of solutions compared to the MTS and GCK stains, which require a series of solutions to perform[20]. In this study, MGCK was used as the reference standard because it has higher sensitivity (98.0%) and specificity (98.3%) levels compared to Weber Modified Trichrome method[12]. In addition, horizontal or equitorial deep-blue stripes that encircle the spores are more prominent and discriminatory in MGCK (Figure 2). Nonetheless, this particular characteristic feature is not seen in yeast cells or other intestinal protozoan.

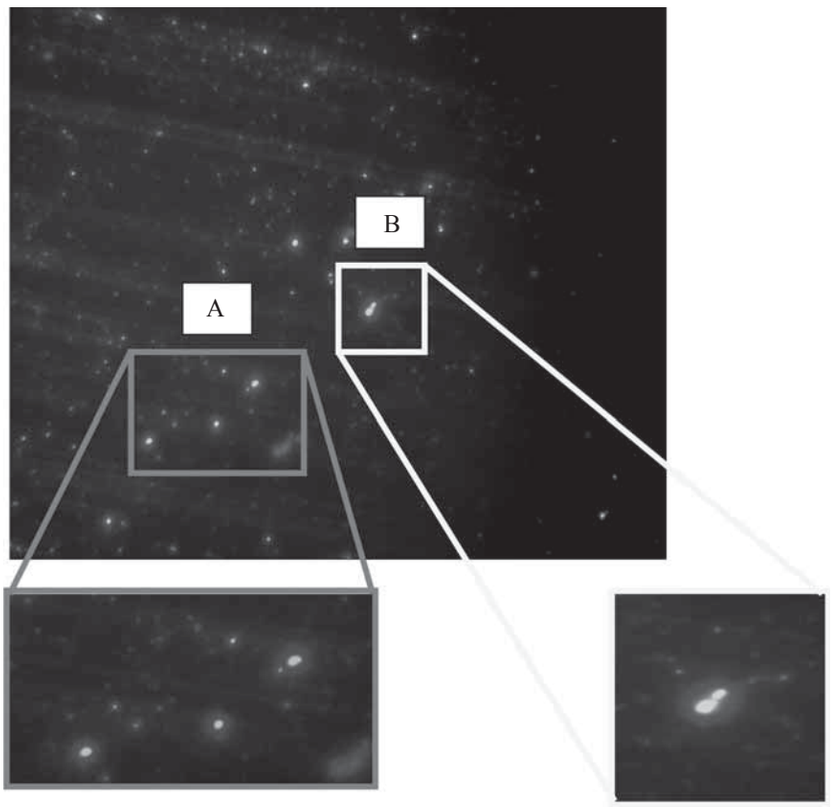

Figure 1. Microsporidia spores in Calcofluor White M2R stain (Viewed using Motic BA 400 fluorescence compound trinocular microscope, at x100 objective lens and a D350/50x exciter filter under 395-415 nm wave length); A: Microsporidial spores-oval shining ring with bluish white radiance, B: Yeast -bigger in size, rounded and budding in shape.

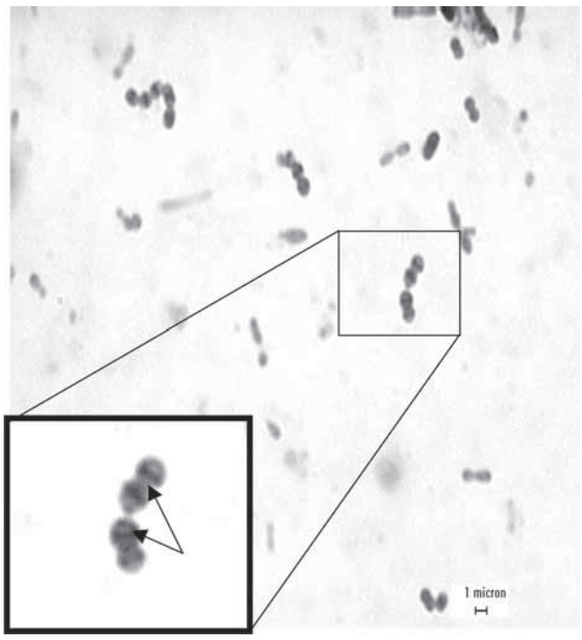

Figure 2. Microsporidial spores approximately measuring 0.5 x 1.0 µm present in a fecal specimen stained with Gram Chromotrope Kinyoun and viewed under light microscopy. Distinct deep-blue polar tubes were clearly seen encircling the spores (arrow sign).

It is known that the detection of microsporidial spores in stools is sometimes difficult when the intensity of infection is low[21]. In this study, however, the MGCK method could detect low spore counts of 1-10 per 100 fields/100 (99.0%; data not shown). Similar findings were also observed by Norhayati *et al.* (2008). Of 116 stool samples, 72.4% had low spore counts in their study. In addition, it has been reported that the screening method should be concurrently done with the confirmatory method to enhance the performance and accuracy of diagnosis, especially in patients with light infection[22]. Hence, we believe that the simultaneous use of CFW and MGCK methods could provide greater advantage and accuracy in patients with intestinal microsporidiosis.

CONCLUSION

In conclusion, the initial screening of microsporidial spores from the stool samples by using Calcofluor White M2R, followed by confirmatory method, modified Gram Chromotrope Kinyoun offers a feasible diagnostic in our study. However, due to its low specificity, a blinded, multicenter study should be employed on samples with positive microsporidia spores to ensure the validity of various staining methods in future. The reliability of these two staining methods can be improved by employing PCR or TEM as a reference standard.

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