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Original Article

Comparison of Three Staining Methods for the Detection of Intestinal *Microspora* Spp.

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Received 05 Apr 2014
Accepted 21 Sep 2014

Keywords:
Microspora,
Staining,
Calcofluor,
Chromotrope,
Gram stain

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Abstract

Background: This study aimed to compare three staining methods including: Calcofluor white, Chromotrope and Quick Hot Gram chromotrope used in diagnosis of intestinal microsporidial spores.

Methods: One hundred and seventy five stool specimens were collected from patients referred to Laboratory of Intestinal Protozoology at the School of Public Health, Tehran University of Medical Sciences during 2012-2013. All of specimens were evaluated by nested PCR. The formalin–fixed stool samples were prepared from each specimen and dried at room temperature for 10 min, followed by 10 min methanol fixation. All the collected stool samples were evaluated blindly by calcofluor white, Chromotrope and Quick Hot Gram chromotrope staining methods separately.

Results: Microsporidial spores were recognized using Chromotrope, Quick Hot Gram chromotrope and Calcofluor white, in16 of 18 (88.8%), 17 of 18 (94.4%) and 18 of 18(100%) samples that were positive by nested PCR respectively. Regarding 14 stool samples that were negative by nested PCR, 14 cases were negative by chromotrope and Quick hot Gram chromotrope and 13 samples were negative by Calcofluor white. One discordant sample interpreted as false positive.

Conclusion: Calcofluor white staining had the best performance for the detection of intestinal *Microsprora* spores and can be used as initial screen test for the detection of intestinal *Microspora* spp.

Introduction

Microspridia are obligate intracellular spore-forming protozoa. They have been recognized as human pathogen particularly in immunodeficient patients (1). There are a number of methods available for diagnosing of microsporidial spores and con-

firmation of microsporidiosis. Most of these methods were developed for diagnosing infections in the immunocompromised population (2). The diagnosis of intestinal microsporidiosis has traditionally depended on direct visualization of the parasites by light and or electron microscopy (1, 3). Although in some studies sensitivity of PCR particularly nested PCR have been greater than light microscopy for the diagnosis of intestinal microsporidia (3-5), but molecular methods have been some limitations. Several diagnostic methods may be needed for diagnosing of microsporidial infection, especially when fecal specimens are tested (2). The chromotrope staining method using modified trichrome was first described by Weber et al. for diagnosis of microsporidia (1). In this staining method, fast green was the counterstain and microscopical slides were stained for 2 hours.

Later Ryan et al. (6) modified chromotrope staining method by using aniline blue as the counterstain. Further Kokoskin et al. introduced an improved version of Weber standard staining technique by modifying the staining temperature to 50°C. The procedure is known as hot chromotrope (7). The staining method with calcofluor white that stain chitin in endospore layer of microsproridia spores was described by Vavra, Chalupsky and Vicki (8-10). Another staining method for diagnosis of micrsporidia spores is Gram Chromotrope. This method is a combination of Gram staining and Weber's staining methods. This method follows the steps of Gram staining method except safranin step, continuing with chromotrope staining steps (11). The Gram chromotrope was adapted by changing chromotrope to hot chromotrope and short incubation time by Moura et al. (12).

In this study three different staining methods, calcofluor white, chromotrope and Quick hot Gram chromotrope were evaluated and compared for detection of microsporidial spores in the feces.

Materials and Methods

One hundred and seventy five stool specimens were collected from patients referred to laboratory of intestinal protozoology at school of Public Health, Tehran University of Medical Science. All patients had gastro-intestinal signs and symptoms such as chronic or intermittent diarrhea. All of specimens were evaluated by nested PCR (13). The specimens were prepared to evaluate blindly by staining methods. Conventional formalin-ether method was carried out for all samples. Thin smears were prepared from each specimen, dried at room temperature for 10 min, and followed by 10 minutes methanol fixation. All positive and negative stool samples were blindly evaluated by calcofluor white method, chromotrope and Quick Hot Gram chromotrope staining methods.

Calcofluor white staining method

All microcopical slides were stained with 0.05% wt/vol calcofluor white M2R (Sigma chemical co, St Louis, Mo) as described by Vicki et al. (10).

Formalin- fixed positive and negative control stool samples that confirmed by nested PCR were examined in test series. The slides were cover slipped and screened under 100X oil–immersion using epiflourescence microscope fitted 455 nm.

Quick Hot Gram chromotrope staining method

The Quick Hot Gram chromotrope staining method was used in this study as described by Moura et al. with some modifications (12).

Methanol–fixed smears were dipped into wells containing1% Methylene violet for 1min, slides then were washed and dipped in 0.5% Iodine solution for 1 min after decolonization and rinse off excess stain with water, slides were placed in warm chromotrope stain (chromotrope 2R, Mallinckroat) for at least 2 minutes and followed by acid alcohol decolor-

ize solution and ethyl alcohol and mounted with cytosol. All slides were observed under light microscope with high magnification (1000X).

Chromotrope staining method

The trichrome was applied for this study as described by Weber et al. (1) with some modification in the incubation time. The slides were prepared without coverslipping and viewed under a light microscope with high magnification (1000X).

Results

The chromotrope stain displayed transparent spores with pinkish wall of microsporidia and a background of faint green staining fecal bacteria. Some of ovoid 1.0-1.5µm spores had a belt-like strip in the middle or at the end of body whereas fungal elements were much larger and stained red intensively (Fig.1, A).

In the Quick Hot Gram chromotrope staining methods microsporidial spores appeared deep violet with ovoid structures. Yeasts were observed red in the background and easily recognized from microsporidial spores. The equatorial belt–like stripes as a diagnostic feature were seen in some spores (Fig.1, B).

In the calcofluor white staining, microsporidial spores had oval shape with enhanced peripheral fluorescence (Fig.1, C). Staining intensity was variable between fresh and old specimens. The fresh specimens fluoresced more brightly than old samples in our study. Internal structures were not visible in this method but peripheral staining pattern and small size and unique shape of spores were very characteristic. Yeast cells also displayed fluorescence but were more round shaped and larger than microsporidia spores.

Easy performance, cost benefit and stability of sample solution were advantages of using calcofluor in laboratory.

Microsporidial spores were recognized in16 of 18 (88.8%), 17of 18 (94.4%) samples that were positive by calcofluor white and nested PCR, using chromotrope and Quick Hot Gram chromotrope stain respectively. All 18 calcofluor positive stool specimens confirmed by nested PCR .Two slides that were negative by chromotrope were stained again and subsequent review on first slides and repeated ones, revealed few spores so these slides interpreted as false negative.

Fourteen stool samples that were negative by nested PCR were selected and analyzed to evaluate false positive in this study. All of the 14 specimens from these samples were negative by chromotrope and Quick Hot Gram chromotrope staining methods whereas the results of calcofluor White method revealed 13 negative samples and 1discordant sample interpreted as false positive. Performance of three staining methods compared with PCR in 175 individuals for the detection of intestinal microsporidia spores and agreements degree between results obtained from three staining methods in diagnosing of microsporidia spores are summarized in Tables 1 and 2.

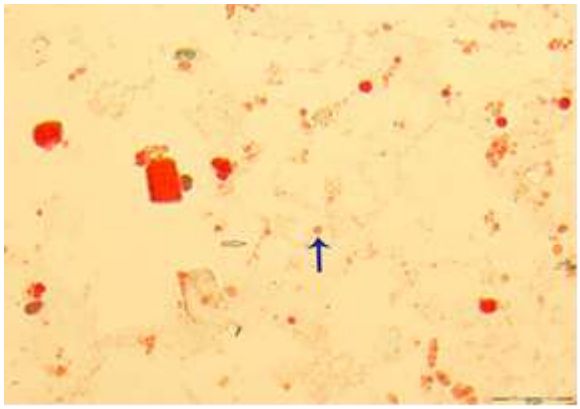
Table 1: Performance of three staining methods compared with PCR in 175 individuals for the detection of intestinal microsporidia spores

Methods	Number of true positive	Number of false negative	Number of true negative	Number of false positive
Chromotrope 2R	16	2	14	0
Quick hot gram Chromotrope	17	1	14	0
Calcofluor white	18	0	13	1
Nested PCR	18	0	14	0

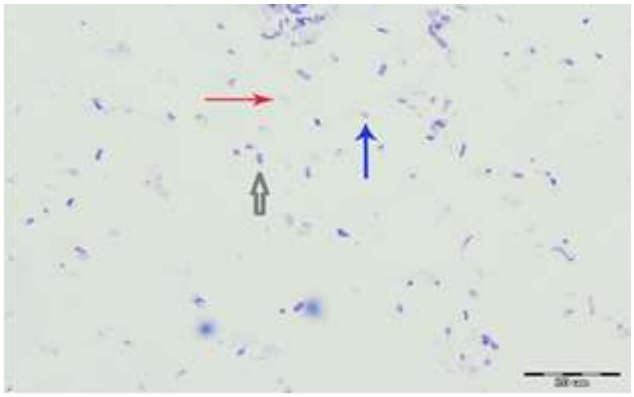
Table 2: Agreements among three staining methods in 175 individuals for the detection of intestinal microsporidia spores

Procedure	Calcofluor white	Chromotrope
Calcofluor white	1.0	**0.931**
Chromotrope 2R	0.87	**1.0**
Quick-hot gram Chromotrope	0.932	**1.0**

*Agreement calculated by Cohen's kappa test



A



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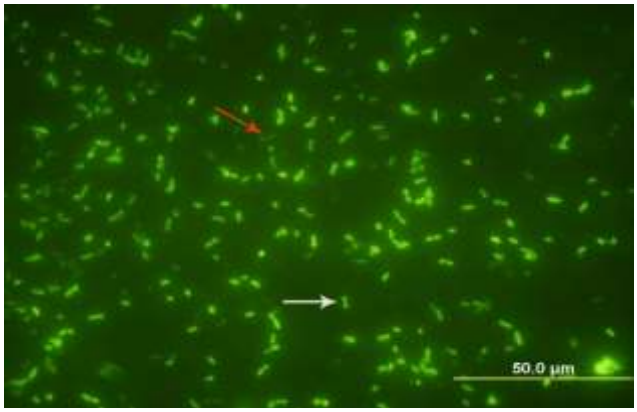

C

Fig. 1: Appearance of microsporidial spores stained with chromotrope 2R (A), Quick Hot Gram chromotrope (B), Calcofluor white (C). Microspridia (white arrows), bacterial elements (red arrows), yeast cells (blue arrows). Final magnification 1000X

Discussion

In this study three staining methods, calcofluor white, chromotrope and Quick Hot Gram chromotrope stains were evaluated in the diagnosis of microsporidial spores. The results indicate the sensitivity of Chromotrope, Quick Hot Gram chromotrope and calcofluor white staining methods in comparison with nested PCR were (88.8%), (94.4%) and (100%) respectively.

Microsporidia are reported in patients with acquired immunodeficiency syndrome (14-16). The prevalence of intestinal microsporidiosis is reported to be 12-50% in European countries and 2-64% in United States depending on the study population and methods of diagnosis (17, 18). The chromotrope base stain is a routine diagnostic method (1, 19, 20), but because of small size of microporidia species sometimes they are overlooked in this procedure, particularly in the cases with low concentration of microsporidial spores (21). The Quick Hot Gram chromotrope technique is

another staining method that reported as a useful technique in diagnosis of microsporidial spores especially in cultured samples and tissue sections (11, 12). The calcofluor white described as a staining method with high sensitivity in some studies (10, 21).

Gram staining has been used occasionally to identify microsporidia spores in cell cultures and clinical samples such as urine that are either free of or have relatively low bacterial contamination (11).

There were some advantages found in Gram chromotrope staining method in this study. The microsporidia spores could be easily detected even they are present in small numbers; the staining time is short and could be photographed easier in comparison with Chromotrope.

The important advantages of calcofluor white were high sensitivity, speed, easiest performance between three staining method and stability of solution in the laboratory. Calcofluor solution could be stored for long time in laboratory with little loss of fluorescence if solution stored in darkness. Another advantage of this staining method is, individual microsporidia can be distinguished in thick area of smear that may be overlooked by chromotrope or Gram chromotrope staining methods. Chromotrope staining method is a method with high specificity but is time consuming.

Sensitivity of chromotrope in comparison with calcofluor white and nested PCR was 88% in our study. In a study that were conducted by Vicki et al. sensitivity of chromotrope stain was (75%) in comparison with calcofluor white staining method and the calcofluor white is more sensitive than chromotrope that is consistent with our study (10).

Dider et al. compared modified trichrom blue, calcofluor white and Transmission Electron Microscopy (TEM) in detecting of microsporidia. The sensitivity of calcofluor white, trichrom blue and TEM were 100%, 100% and 90.2% respectively. The calcofluor white and chromotrope stains are more sensitive than TEM for detecting of microsporidia spores and the specificity of calcofluor white and modified trichrom blue and TEM were reported 90.5%, 100% and 100% respectively (21). These findings are similar to our results with high level of sensitivity and less specificity for calcofluor white in comparison with chromotrope staining method.

The positive staining reaction of chitin containing objects in feces like yeasts and fungal elements and some parasites decrease specificity of calcofluor white staining that has been mentioned in previous studies (1, 12, 22) and false positive interpretations of calcofluor white reported in some studies (1, 9). Despite of these problems, the unique shape, small size, brightness of fluorescence and enhanced peripheral staining are very characteristic in diagnosing of microsporidial spores in this method that we observed in this study.

In fecal staining of Gram chromotrope microsporidial, spores appeared as deep violet to pink violet with ovoid structure and sometimes spores have equatorial belt like stripes whereas yeasts were stained pink-red and easily distinguished from microsporidial spores. Although in some studies the reported bacteria can be confused with microsporidia spores in fecal staining of Gram Chromotrope, they stained weakly with this technique in this study. The variability in some microsporidian spores with Gram stain is related to over-decolonization during staining or maturity of microporidian spores. Sporoblasts and immature spores are Gram intermediate or Gram negative but mature forms tend to be Gram positive. This matter described in some previous studies (11).

In this study, calcofluor white showed the best performance with high sensitivity in detecting microsporidial spores. The microsporidial spores had enhanced peripheral fluorescence but fungal elements were large and sometimes stained orange that mentioned in some previous studies (21). Our observations are similar to the earlier studies, which reported lower stain intensity in immature spores than mature

spores. The reason for this low intensity has been explained through late development of chitinous endospore layer during maturation (8, 9).

Conclusion

Our study shows that calcofluor white staining method has high sensitivity in comparison with other examined methods hence can be used as primary screen test in diagnosis of intestinal microspridia. Further, we recommend Chromotrope and Gram chromotrope staining methods for specificity improvement and result confirmation.

Acknowledgement

This study was self-funded. The authors declare that there is no conflict of interest.

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