Comparative Evaluation of Modified Trichrome and Uvitex 2B Stains for Detection of Low Numbers of Microsporidial Spores in Stool Specimens

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At present, the laboratory diagnosis of intestinal infections caused by microsporidia depends on the detection of the typical spores either with a modified trichrome stain (MTS) or by staining with fluorochromes. The purpose of the present study was (i) to compare staining with MTS (MTS method) and the staining with the fluorochrome Uvitex 2B (U2B method) with respect to their sensitivities and specificities, particularly in the presence of low numbers of spores, and (ii) to evaluate their reliabilities under routine laboratory conditions. First, 30 negative human stool specimens as well as 30 specimens enriched with a low concentration of microsporidial spores were examined. The U2B and MTS methods detected 27 and 30 of the positive samples, respectively (95% confidence intervals for sensitivity, 0.73 to 0.98 for the U2B method and 0.88 to 1.00 for the MTS method) without yielding false-positive results (95% confidence intervals for specificity, 0.88 to 1.00 for the MTS and U2B methods). In addition, analysis of serial dilutions of 17 stool specimens from AIDS patients containing microsporidia revealed comparable detection thresholds ($P = 0.52$) for both methods. Finally, 40 slides prepared from one stool specimen containing very few microsporidia and 40 negative slides were included in the routine diagnostic program during 1 month in order to monitor laboratory handling and run-to-run variations. Again, both methods exhibited comparable sensitivities (95% confidence intervals, 0.83 to 0.99 for the MTS method and 0.91 to 1.00 for the U2B method) and specificities (95% confidence intervals, 0.91 to 1.00 for the MTS and U2B methods). In conclusion, MTS and U2B methods are equally useful in the diagnosis of microsporidiosis. However, since detection thresholds for both methods differed considerably in all diluted stool specimens, performance of a combination of both methods may be more sensitive than the performance of only one procedure in the event of very low numbers of microsporidial spores.

Microsporidia are small, obligate intracellular protozoan parasites known for a long time as pathogens in various vertebrates as well as invertebrates (28). Recently, members of the phylum Microspora have also been described as parasites in humans. Infections with species of the genera Pleistophora and Nosema are rarely diagnosed (28), but Encephalitozoon spp. (8), including E. intestinalis (formerly Septata intestinalis) (4, 11), and particularly E. bieneusi (7), are frequent opportunistic agents in patients with AIDS (28). In these patients, E. bieneusi and E. intestinalis infect enterocytes of the small intestine as well as the biliary system, causing severe, chronic diarrhea and cholangitis or cholecystitis (2, 16, 17). In addition, E. intestinalis may infect other cells such as macrophages and endothelial cells, and infections may disseminate and affect the kidneys, liver, or lungs (4, 18, 19).

Because the microsporidial spores are very small (1 to 2 μm), the laboratory diagnosis of microsporidiosis was first based on the detection of spores in intestinal biopsy specimens by either light or electron microscopy (10, 14, 21). Recently, two different staining methods have been described for the microscopic detection of spores in stool specimens: a method with a modified trichrome stain (MTS; the MTS method) developed by Weber et al. (27), which has subsequently been modified with respect to the temperature and counterstain that are used (9, 13, 20), and fluorescence staining with fluorochromes such as Uvitex 2B (U2B; the U2B method) or Calcofluor White, which bind to the chitin in the endospore layer of the microsporidal spore (22, 23). Until now, very limited data have been available on the sensitivities and specificities of both methods. Therefore, we compared the MTS and U2B methods with respect to their sensitivities and specificities, as well as to the feasibility of their use in the laboratory routine.

MATERIALS AND METHODS

**Stool specimens.** Fecal specimens from AIDS patients routinely submitted to our laboratories containing spores from E. bieneusi and negative stool samples were divided, and one part of each specimen was preserved in formalin (27) the other part was preserved in ethanol, which is required for the performance of the U2B staining procedure (22). Microsporidiosis had previously been diagnosed in these patients by detecting microsporidial spores by either the trichrome or Uvitex method. All cases of microsporidial infection had additionally been confirmed by examination of intestinal biopsy specimens.

**MTS procedure.** The MTS procedure was performed as described originally (27), but without the final incubation in xylene and covering. Briefly, 20 μl of formalin-fixed specimens were thinly spread on a glass slide, and the slides were air dried and fixed with methanol. The slides were stained with the MTS solution for 90 min and then rinsed for 10 s with xylene and covering. Briefly, 20 μl of formalin-fixed specimens were thinly spread on a glass slide, and the slides were air dried and fixed with methanol. The slides were stained with the MTS solution for 90 min and then rinsed for 10 s with acid alcohol, rinsed briefly with 95% alcohol, and incubated for 5 min in 95% ethanol and for 10 min in 100% ethanol. The slides were air dried and examined microscopically with oil immersion for approximately 10 min (magnification, ×1000).
U2B procedure. The U2B procedure was performed as described previously (22). Briefly, 20 µl of ethanol-fixed stools was thinly spread on a glass slide and air dried. After fixation with methanol, the slides were covered for 10 min with 1% U2B (Ciba-Geigy, Basel, Switzerland) in phosphate-buffered saline (PBS; pH 7.2), rinsed with PBS, counterstained for 30 s with 0.05% Evans blue in PBS, and rinsed with PBS. The slides were air dried, and whole slides were examined completely under a Zeiss fluorescence microscope (50-W mercury bulb; excitation filter, 390 to 420 nm; barrier filter, 450 nm) with oil immersion (magnification, ×1,000).

The U2B reagent used for this study had been produced by Ciba-Geigy. Because it is no longer available from this source, it may be now obtained from Pfersee Chemie, Langwedl, Germany.

Preparation of slides. For testing of the sensitivities and specificities of both methods, 30 negative human stool specimens were collected and divided into two parts in order to produce 60 negative slides as well as 60 positive slides by enriching each of the negative stool with a positive specimen containing microsporidia at low concentration (one to five spores per 10 oil-immersion fields) in a 1:1 (vol/vol) ratio. Subsequently, all slides were coded, randomized before being tested, and stained with either MTS or U2B.

For the second part of the study, slides with serial dilutions of 17 patients’ stool specimens known to contain microsporidia and negative human stool were prepared and stained with either MTS or U2B.

Statistical analysis. Sensitivities, specificities, and positive and negative predictive values were calculated with their 95% confidence intervals (CIs). Two nonoverlapping CIs indicate a significant difference on a 5% level. Paired samples were compared by the Wilcoxon signed-rank test.

RESULTS

Determination of sensitivities and specificities of the MTS and U2B methods. Staining 60 positive slides containing low numbers of microsporidia (for details, see Materials and Methods) and 60 negative slides by both methods, we found that the MTS and U2B methods detected 30 and 27 of 30 positive slides, respectively (95% CI for sensitivity, 0.88 to 1.00 versus 0.73 to 0.98). Both methods identified all negative slides without yielding false-positive results (95% CI for specificity, 0.88 to 1.00 for the MTS method versus 0.88 to 1.00 for the U2B method). The positive and negative predictive values for the U2B method (95% CIs, 0.88 to 1.00 and 0.76 to 0.98, respectively) and for the MTS method (95% CIs, 0.88 to 1.00 for both values) were comparable.

Determination of the thresholds for detection of microsporidial spores by the MTS and U2B methods. In order to determine the threshold of both methods with regard to the detection of microsporidial spores, serial dilutions of 17 positive stool specimens were examined. The results presented in Table 1 demonstrate the comparable overall thresholds of the MTS and U2B methods. However, as indicated in Table 2, detection thresholds for both methods differed for all 14 individual specimens in which microsporidia were detected by both the U2B and MTS methods. The U2B method showed a lower threshold than the MTS method for 6 samples, whereas the MTS method detected microsporidia in a higher dilution than the U2B method in 11 samples. Additionally, the U2B and MTS methods did not yield positive results with 2 and 1 of 17 specimens, respectively (the difference was not statistically significant [P = 0.52]).

Evaluation of MTS and U2B methods under routine laboratory conditions. In order to evaluate laboratory handling and to monitor the run-to-run variations of both methods, 40 positive slides from a stool specimen containing very few microsporidia (one or fewer spores per 10 oil-immersion fields) as well as 40 negative slides were prepared, coded, and included in the routine laboratory program for 1 month. Whereas the MTS method failed to detect two positive slides, which were also negative upon review, the U2B method did not yield false-negative results. Both methods identified all negative slides. The data obtained demonstrate that the detection rates of both methods were not influenced by changes in the people who carried out the diagnostic procedures. Overall sensitivities (95% CIs, 0.84 to 1.00 for the MTS method versus 0.91 to 1.00 for the U2B method), specificities (95% CIs, 0.91 to 1.00), positive predictive values (95% CIs, 0.91 to 1.00), and negative predictive values (95% CIs, 0.84 to 1.00 for the MTS method versus 0.91 to 1.00 for the U2B method) were not statistically different between the two methods under routine laboratory conditions.

DISCUSSION

Intestinal microsporidial infections in AIDS patients may lead to severe and chronic diarrhea. The diagnosis of microsporidiosis was first established by electron or light microscopic examination of duodenal biopsy specimens, retrieval of which requires invasive procedures, but diagnosis is now mainly based on the detection of microsporidial spores in stool specimens. Two basic staining procedures are now widely used: staining with an MTS or staining with fluorochromes. Methods that use monoclonal or polyclonal antibodies have also been developed (1, 9, 29), but they are not commercially available yet and yielded results inferior to that of MTS or Calcofluor White staining in one study (9). Therefore, the present study was designed to compare the sensitivities and specificities of the MTS and U2B methods, especially with respect to low numbers of microsporidial spores, as well as to examine their handling under routine laboratory conditions.

The detection thresholds determined for the U2B and MTS

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<tr>
<th>Specimen no.</th>
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a Values are the titer of the last dilution of stool specimens yielding a positive test result.
b Method did not yield positive test result with undiluted specimen.
methods differed in all 14 stool specimens, and the U2B and MTS methods failed to detect microsporidia in 2 and 1 of 17 stool specimens, respectively, indicating that substances in the stool specimens might differently influence the staining results of both procedures. Thus, in the case of extremely rare microsporidial spores in stools, performance of the combination of both methods may yield more accurate results than performance of only one method. In addition, two positive slides from the final experiment were false negative by the MTS method, which was probably due to incorrect decolorization of the slides. Our results indicate that both methods are equally sensitive and specific, even when low numbers of microsporidial spores are present.

Our data are in accordance with findings of DeGirolami and coworkers (6). Those investigators had examined 305 specimens by using the MTS method and subsequently a subset of these samples by using the U2B method. For the latter 108 samples they could confirm all previous MTS method-positive samples by the U2B method and a few specimens initially only positive by the U2B method were positive by the MTS method upon review. For all patients with biopsy results, both the MTS and the U2B methods exhibited a sensitivity of 100%. However, specificity was not assessed in this study. In contrast, Didier et al. (9) showed that fluorochrome staining was the most sensitive method but yielded false-positive results, leading to reduced specificity. However, they used Calcofluor White instead of U2B, which might be more sensitive but less specific because of increased nonselective staining, e.g., of small fungi. It is known that the staining characteristics of the various fluorochromes, which bind to chitin layers in the cell wall, differ with regard to fluorescence intensity and selectivity (26). Furthermore, U2B does not stain *Pneumocystis carinii* (19a), while staining of this microorganism with Cellulfluor White, which resembles Calcofluor White, is possible (3). However, Luna et al. (15) found Calcofluor White to be more sensitive than but as specific as MTS, and Conteas et al. (5) observed that background staining leading to false-positive results may be reduced by using different UV wavelengths. In addition, Vavra et al. (24) reported that Calcofluor White and U2B are equally efficient in labelling spores from various microsporidial species and recommended alkali treatment of samples, particularly materials with unknown storage and fixation histories. The treatment with 1 N NaOH led to full restoration of the fluorescence of old spores and also decreased the background staining in some samples.

Comparing the laboratory handling required for both methods, for the U2B method less time is required for staining and microscopic evaluation than is required for the MTS method. However, the performance of the U2B method strictly depends on the use of non-formalin-fixed specimens (22; unpublished data), whereas other fluorochromes such as Fungifluor, Calcofluor White, and Fungiquel A can be used for formalin-fixed specimens (5). In addition, a fluorescence microscope must be available, and in our experience, the fluorescence intensities of microsporidial spores may be considerably variable with U2B.

With respect to the MTS method, we used the procedure originally described by Weber and coworkers (27) in this study. However, a number of modifications have recently been reported. Ryan et al. (20) varied the counterstain by using aniline blue instead of fast green, and Kokoskin et al. (13) reported more intense staining of spores by incubating the slides at a higher temperature. The latter procedure allowed the shortening of the incubation time to 10 min, whereas the originally described staining with chromotrope solution requires 90 min. In our experience, the most convenient MTS method has been developed and recently described by Didier et al. (9), who shortened the incubation time to 30 min by using 37°C as the incubation temperature and aniline blue as the counterstain. Similar to U2B, MTS may also stain nonmicrosporidial structures such as bacterial spores or fungi, and the differentiation also strongly depends on the detection of the microsporidial vacuoles or the typical belt-like stripe inside of the spores representing the tubular filament.

Whereas the U2B method may additionally be used in the diagnosis of other microorganisms, such as the detection of hyphae of *Aspergillus* spp. in bronchial materials, the MTS method stains other microorganisms rather suboptimally (20; unpublished data). Because in this form it is only useful for the diagnosis of microsporidial infections, we recently developed an MTS combined with an acid-fast step for the simultaneous detection of microsporidia and cryptosporidia, thus detecting in AIDS patients two of the most important intestinal pathogens, neither of which can be diagnosed by using a wet mount (12).

In conclusion, both the U2B and the MTS methods are equally sensitive, specific, and robust in the laboratory diagnosis of intestinal microsporidiosis, and the decision to use either one may be based on technical criteria such as the availability of a fluorescence microscope. Nevertheless, both methods may stain nonmicrosporidial structures as well, and the detection of the typical stripes or vacuoles appears to be absolutely essential to establishing the diagnosis. These difficulties in differentiation and the extremely small size of the microsporidial spores demand well-trained and experienced personnel for interpretation of staining results by both methods. Finally, the performance of both methods may yield more positive results than the performance of only one method in the case of extremely rare microsporidial spores in stool specimens. Therefore, the development of molecular biologic methods such as PCR (25) or better immunosassays seems desirable, particularly for studies in which larger numbers of samples are to be examined, e.g., in epidemiological surveys.

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