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A New Acid-Fast Trichrome Stain for Simultaneous Detection of *Cryptosporidium parvum* and Microsporidial Species in Stool Specimens

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The detection in stool specimens of *Cryptosporidium parvum* and microsporidia, the most frequent parasitic pathogens causing diarrhea in AIDS patients, until now has depended on two different staining methods. However, since double infections occur and minimization of laboratory costs is mandatory, development of a method for simultaneous detection of these parasites appeared desirable. We report on a new, inexpensive, and easy-to-perform staining procedure to demonstrate both acid-fast oocysts of *C. parvum* and other coccidia, as well as microsporidial spores. This acid-fast trichrome stain yields results comparable to those obtained by the Kinyoun and modified trichrome methods and considerably reduces the time necessary for microscopic examination.

AIDS patients frequently suffer from gastroenteritis which can be due to a great number of pathogens, including bacteria, protozoa, and viruses. Commonly occurring protozoan genera include coccidia (*Cryptosporidium* and *Isospora*), at least two different microsporidial genera (*Enterocytozoon* and *Encephalitozoon*, formerly *Septata*), *Giardia*, and *Entamoeba*. More recently, infections with *Cyclospora cayetanensis* coccidia have also been reported (10, 20). Whereas giardiasis and amebiasis are associated mainly with distinct risk factors, such as travel or sexual practices, cryptosporidiosis and microsporidiosis are widespread and typical late complications of human immunodeficiency virus disease.

While there is an estimated prevalence of infection with *Cryptosporidium parvum* of about 2% in immunocompetent patients with diarrhea in industrial countries, the average is about 22% in AIDS patients in developing countries (1). In immunocompromised patients, the obligate intracellular parasite *C. parvum* may cause severe chronic and progressive gastroenteritis with watery diarrhea which may lead to excessive loss of body fluid and death (4). Infections at other body sites, such as the respiratory tract (11) and the gallbladder (12), have also been described. In immunocompetent patients, on the other hand, diarrhea generally is self-limited (4). The diagnosis of cryptosporidiosis is based mainly on detection of the typical oocysts in stool specimens, either by use of an acid-fast stain (8) or by an indirect immunofluorescence assay (IFA) procedure with monoclonal or polyclonal antibodies (7, 15).

Microsporidia, as well as other obligate intracellular protozoa, have long been known as parasites of invertebrate animals. Recently, two microsporidial species, i.e., *Enterocytozoon bieneusi* and *Encephalitozoon* (formerly *Septata*) *intestinalis*, have also been described as causative agents of gastroenteritis in immunocompromised patients. Similar to *C. parvum* infections, these infections may result in severe chronic diarrhea, and *E. intestinalis* may also affect other organ systems, such as the kidneys, liver, or bronchial system (3, 19). A modified trichrome stain with subsequent alterations has been described for diagnosis of intestinal microsporidiosis in stool specimens (5, 13, 17). The microsporidial spores here can be distinguished from bacterial spores by a pale diagonal or horizontal belt, reflecting the typical polar filament. However, spore sizes of 1 to 2 μm and the occasional absence of the typical pale line make identification difficult. Additionally, a method of employing Uvitex, a fluorescence brightener (16), and staining methods using monoclonal or polyclonal antibodies (2, 22) have been developed.

Until now, both parasites have been detectable only by use of different stains (microsporidia are not acid fast, and oocysts of *C. parvum* fail to stain with trichrome). However, double infections occur (9, 18), and to minimize laboratory costs, we have been interested in developing a new staining method for the simultaneous detection of these two parasites. The method should be easy to perform and inexpensive, in light of the importance of these opportunistic parasites in AIDS patients in developing countries. Therefore, we worked out a procedure which is based on the combination of an acid-fast step with a modified trichrome stain. This acid-fast trichrome (AFT) stain allows the detection of oocysts of *C. parvum*, as well as those from other coccidia, by a bright pink or violet color easily distinguished from fungi and fecal elements, whereas microsporidia appear the same as with the modified trichrome staining method.

MATERIALS AND METHODS

Patients. Fecal specimens from AIDS patients routinely submitted to our laboratory and containing oocysts of *C. parvum* and microsporidial spores, as well as negative stool samples, were collected and preserved in SAF stock solution (one part stool and three parts SAF solution) (14, 21). SAF stock solution consisted of 15 g of sodium acetate, 20 ml of acetic acid, and 40 ml of formalin in 925 ml of distilled water. Cryptosporidiosis and microsporidiosis had been previously diagnosed in these patients by detecting *C. parvum* with a modified Kinyoun stain and the microsporidial spores by the modified trichrome method. Cases of microsporidial infection had been additionally confirmed by examination of intestinal biopsies (five of seven cases). To examine different staining

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TABLE 1. Detection of *C. parvum* and microsporidia by AFT staining[a]

Stool specimen content (no. of specimens)	No. of specimens positive by:		
	AFT stain	Trichrome stain	Kinyoun stain
C. parvum (12)	12		12
Microsporidia (7)	7	7	
No *C. parvum*, no microsporidia (25)	0	0	0

[a] The testing reliability of the AFT stain was compared with the combined results of the Kinyoun and trichrome stains. Of the 44 stool specimens tested, 12 contained oocysts of *C. parvum* and 7 contained microsporidia.

procedures, two stool specimens known to contain microsporidia (microsporidiosis confirmed by intestinal biopsy) and oocysts of *C. parvum*, respectively, were mixed in a 1:1 volume ratio. For reliability assays, 44 stool specimens from different AIDS patients were fixed as described previously. Of these, 7 contained microsporidia and 12 contained cryptosporidia. The specimens were coded and randomized before being tested. To examine staining characteristics of oocysts of other coccidia, one routinely submitted stool specimen containing *Isospora belli* was used.

Specimen preparation. For concentration of *Cryptosporidium* oocysts, SAF-preserved stool specimens were concentrated by using the formalin-ethyl acetate sedimentation procedure with centrifugation at 650 × *g* for 10 min. A 0.01-ml sediment sample was thinly spread on a glass slide and air dried. Slides were immediately fixed in methanol for 5 min.

AFT stain. A number of variations of both the acid-fast step and the following trichrome stain were tried, and optimal results were achieved with the following procedure. Fixed slides were covered with a carbol-fuchsin solution (25.0 g of phenol, 500 ml of distilled water, and 25.0 ml of a saturated alcoholic fuchsin solution consisting of 2.0 g of basic fuchsin in 25 ml of 96% ethanol) for 10 min without heating, washed briefly with tap water, decolorized with 0.5% HCl-alcohol, and again washed with tap water. Subsequently, slides were immediately stained for 30 min at 37°C with Didier's trichrome solution (5). The latter had been prepared by dissolving 6.0 g of Chromotrope 2R (Sigma), 0.5 g of aniline blue (Merck), and 0.7 g of phosphotungstic acid (Sigma) in 3 ml of acetic acid at room temperature for 30 min, addition of 100 ml of distilled water, and adjustment to pH 2.5 by addition of 2 N HCl. Slides were then rinsed for 10 s with acid alcohol (4.5 ml of acetic acid in 995.5 ml of 90% ethanol), washed for 30 s with 95% ethanol, and examined directly after air drying with oil immersion (magnification, ×1,000), requiring approximately 10 min for each slide.

RESULTS

Staining characteristics and simultaneous detection of *C. parvum* and microsporidia. As shown in Fig. 1, the AFT stain allows the simultaneous detection of both oocysts of *C. parvum* and spores of microsporidia. Oocysts of *C. parvum* appear bright pink or violet, and sporozoites can sometimes be seen. As in Kinyoun staining, oocysts are often only partly stained. In contrast, yeast cells never show a bright appearance. As described by Didier et al. (5), microsporidia appear pink. Most of them show a vacuole or a pale diagonal or horizontal stripe. Both organisms can easily be distinguished from fungi, bacteria, or fecal elements.

Reliability studies. The AFT stain was compared for reliability with the Kinyoun and trichrome stains, respectively. Therefore, 44 stool specimens were processed as described above. Of these, 12 contained oocysts of *C. parvum* and 7 contained microsporidia. From each sediment, three slides were prepared and stained with the Kinyoun, trichrome, and AFT stains, respectively. As shown in Table 1, the new staining method allowed the detection of all microsporidia, as well as all *C. parvum* oocysts, compared with the respective single staining method.

Staining characteristics of *I. belli*. Because we were interested in whether other coccidial oocysts stain when the new protocol is followed, we processed and stained one stool specimen known to contain *I. belli*. The staining characteristics of immature and mature oocysts of *I. belli* are shown in Fig. 2.

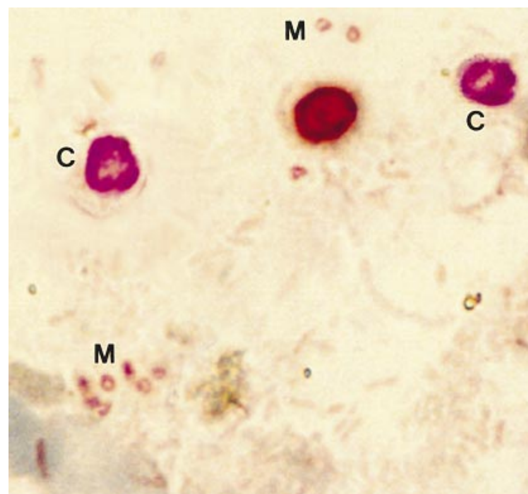

FIG. 1. Simultaneous detection and staining characteristics of *C. parvum* (C) and microsporidia (M). Oocysts of *C. parvum* appear bright pink or violet. Microsporidial spores stain pink with a pale stripe or vacuole (magnification, ×1,000).

They appear shining violet and ellipsoid, with a single granular mass representing the unsegmented zygote or the typical two sporocysts within, and can readily be detected at lower magnification, e.g., ×200 to 250.

DISCUSSION

Diarrhea caused by opportunistic agents such as *C. parvum*, *I. belli*, or microsporidia contributes to morbidity and mortality in AIDS patients. In developing countries, these infections, taken together, may account for a great number of cases of diarrhea in AIDS patients. Therefore, we attempted to develop a staining method which would allow the detection of all of these parasites and could also be performed under simple laboratory conditions. It should therefore be cheap and easy to perform without requiring too many technical aids and yield results comparable to those of presently used staining methods. Therefore, staining methods using fluorescence brighteners were not considered, although we found early on that oocysts of *C. parvum* can also be detected by the Uvitex method (results not shown). Nevertheless, our preliminary results obtained with ethanol-fixed stool specimens had also shown that only part of the oocysts are stained by this method. This might be due to the previously described differences in oocyst wall thickness (4), leading to a lack of sensitivity. We concentrated our efforts on a combination of an acid-fast step with a trichrome stain and examined a number of staining protocols by using a 1:1 volume mixture of two stool specimens containing microsporidia and oocysts of *C. parvum*, respectively. First, we varied the time of staining with carbol-fuchsin and examined the influence of heating of the slide. However, we found that a 10-min period is sufficient to stain the oocysts and heating was not necessary. When the decolorization step was varied, 1 to 2% H_2SO_4-alcohol was not sufficient to decolorize all non-acid-fast fecal elements. Thus, bacterial spores remained red and could be mistaken for microsporidia. We achieved optimal results by using 0.5% HCl-alcohol. Subsequently, we examined the combination with three previously described trichrome stains (5, 13, 17) and achieved optimal results by using the stain described by Didier et al. (5). Comparison of slides with and without the foregoing acid-fast step

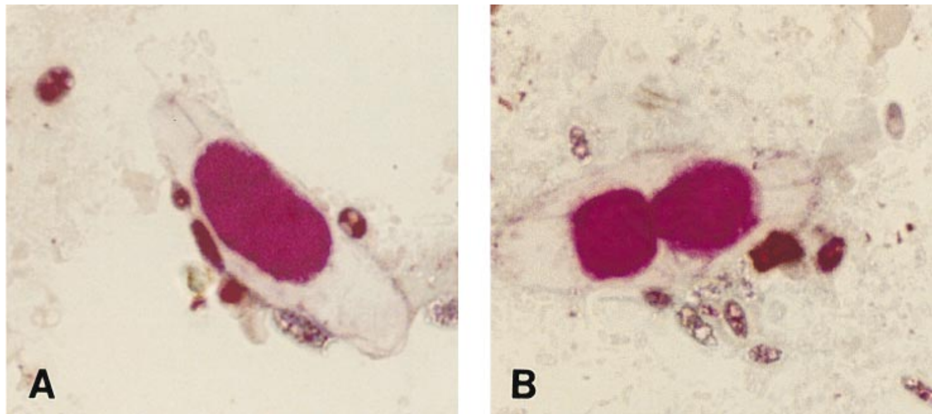

FIG. 2. Staining characteristics of immature (A) and mature (B) oocysts of *I. belli*. They appear ellipsoidal, and the unsegmented zygote or the two sporocysts, respectively, are stained bright pink or violet (magnification, ×1,000).

showed that the following trichrome stain is not influenced by prestaining with carbol-fuchsin and decolorization with acid-alcohol. We then altered the staining procedure of Didier et al. in that the slides were washed after incubation in trichrome solution with 95% ethanol for only 30 s without further incubation in ethanol. Slides were then air dried and examined directly under oil immersion. This shortened the total staining procedure to about 45 min without having any negative influence on the results. In comparison, Didier's trichrome stain took 60 min, the IFA stain took 130 min, and it was necessary to perform an additional acid-fast staining step for detection of cryptosporidia; this resulted in total staining times of 85 and 135 min, respectively.

Additionally, we tested the reliability of this staining method by examination of various stool specimens known to contain microsporidia or *C. parvum*. With the AFT stain, parasites could be detected in all slides from positive stools whereas negative controls did not yield positive results. Finally, we tested whether oocysts of *I. belli* coccidia can also be stained with the AFT stain because it is known that they behave similarly to oocysts of *C. parvum* when an acid-fast staining method is used (6). Oocysts of *I. belli* can easily be distinguished by their size.

In conclusion, this study demonstrated that simultaneous detection in stool specimens of two of the most important parasites in the pathogenesis of diarrhea in AIDS patients is possible by use of one staining procedure. It takes about 45 min, is easy to perform (incubation is the only necessary technical requirement), is low in cost, and is as reliable as standard staining methods, and the time for microscopic examination is considerably reduced. Because of these advantages, the AFT stain might be of interest, particularly in developing countries with a high rate of the described parasitic infections. We are currently comparing the sensitivity and specificity of the AFT stain with those of other, established diagnostic methods, e.g., IFA.

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