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Uniform Staining of *Cyclospora* Oocysts in Fecal Smears by a Modified Safranin Technique with Microwave Heating

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***Cyclospora*, a coccidian protist, is increasingly being identified as an important, newly emerging parasite that causes diarrhea, flatulence, fatigue, and abdominal pain leading to weight loss in immunocompetent persons with or without a recent travel history as well as in patients with AIDS. Modified Kinyoun's acid-fast stain is the most commonly used stain to identify the oocyst of this parasite in fecal smears. Oocysts of *Cyclospora* stain variably by the modified acid-fast procedure, resulting in the possible misidentification of this parasite. We examined fecal smears stained by six different procedures that included Giemsa, trichrome, chromotrope, Gram-chromotrope, acid-fast, and safranin stains. We report on a safranin-based stain that uniformly stains oocysts of *Cyclospora* a brilliant reddish orange, provided that the fecal smears are heated in a microwave oven prior to staining. This staining procedure, besides being superior to acid-fast staining, is fast, reliable, and easy to perform in most clinical laboratories.**

Cyclospora is increasingly being identified in the fecal smears of immunocompetent persons with or without international travel histories (3–8, 10, 12–16, 18, 22, 24, 25) as well as in patients with human immunodeficiency virus infection or AIDS (4, 11, 19, 21, 23, 25). *Cyclospora*, a coccidian protist, previously identified as a coccidian or cyanobacterium-like body or as a novel organism, was found in the diarrheic stools of Nepalese citizens, foreigners residing in Nepal, and travelers returning from Southeast Asia and Mexico who complained of nausea, vomiting, anorexia, diarrhea, malabsorption syndrome, and weight loss (3, 4, 7, 12, 15, 24, 25). Since then, several outbreaks have been attributed to this parasite (5, 6, 16). Ortega et al. (18) were the first to identify the true coccidian nature of the cyanobacterium-like bodies and describe the sporulation of these organisms. They created a new taxon, *Cyclospora cayetanensis* (18), for this parasite. Although a complete life cycle of this organism has not been elucidated thus far, a recent electron microscopic study indicates that different stages of its life cycle can be identified in the duodenal biopsy specimens of infected patients. It is also believed that the entire life cycle of *Cyclospora* may occur within the enterocytes of the infected patient (23).

The stage that is found in the fecal smears of patients infected with *Cyclospora* is the unsporulated oocyst stage, which measures 8 to 10 μm. The oocysts excreted in the feces sporulate outside the host in about 2 weeks, resulting in two sporocysts, each with two sporozoites (18). The stain most commonly used to identify the oocysts in fecal smears is modified Kinyoun's acid-fast stain, which has also been used extensively for the identification of the oocysts of another coccidian parasite, *Cryptosporidium* (1, 2, 8). Published reports indicate that the oocysts of *Cyclospora* are variably acid fast, with staining patterns ranging from no staining or colorless to pink or deep purple (8). This variability in the staining pattern may result in the misidentification of the parasite and may thus hamper timely treatment of the patient, especially now that we know that the parasite can be eradicated with the administration of trimethoprim-sulfamethoxazole (13, 22). While staining fecal smears with the modified acid-fast stain from patients with diarrhea involved in the newest *Cyclospora* outbreak, involving patients in as many as 14 states and Canada (5, 6), we also observed tremendous variations in the staining properties of the oocysts of these organisms. Although a number of the oocysts in a given fecal preparation stained pinkish red, a large number of the oocysts did not take up any stain at all. Furthermore, we encountered some slides in which none of the oocysts took up the stain. Therefore, we undertook a study to see if we could identify a staining technique that would uniformly stain greater than 90% of oocysts in the fecal smear. We used many different types of chemical stains that have been used previously in clinical laboratories for the identification of *Cryptosporidium* and microsporidia in fecal samples. All except one of the stains that we tested stained the oocysts poorly. The safranin staining procedure developed by Baxby et al. (2) for identifying *Cryptosporidium* oocysts in fecal smears stained *Cyclospora* oocysts pinkish or reddish orange. The safranin method has previously been used to stain oocysts of *Cyclospora* (19), but the sensitivity of the technique was reported to be low (30%). In our hands, however, approximately 70 to 75% of the oocysts stained pinkish orange, while the rest of the oocysts remained colorless or stained variably when we stained the smears by the safranin procedure of Baxby et al. (2). Our modification of the procedure of Baxby et al. (2) resulted in a technique that uniformly stained almost all of the oocysts of *Cyclospora* a brilliant reddish orange. Occasionally, however, an oocyst did not take up the stain, but it could easily be distinguished by its collapsed or wrinkled cyst wall.

MATERIALS AND METHODS

Fecal smears from a total of 71 Formalin-fixed stool samples as well as 4 samples stored in potassium dichromate originating from multiple states including Florida, Massachusetts, Georgia, and Texas were examined for the presence of *Cyclospora* oocysts. Of these 71 Formalin-fixed samples, 14 samples had previously been found to be positive for *Cyclospora* oocysts by acid-fast staining. Additionally, three of the Formalin-fixed samples that were positive for *Cyclospora* oocysts were also kept frozen at −20°C for 1 week before staining. Smears were prepared by placing a drop (about 50 μl) of fecal suspension on microscope

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slides, and the suspension was spread with an applicator stick to make a thin smear and dried on a slide warmer set at 60°C. The smears were stained with one of the following: acid-fast, Giemsa, trichrome, chromotrope (8), and the recently developed Gram-chromotrope (17) stains, and, by the safranin staining method of Baxby et al. (2), several different concentrations (0.5, 1, 2.5, and 5%) of aqueous solution of safranin. We also used aqueous safranin solutions whose pHs were adjusted to 2.5 and 6.5 as well as safranin solution made up in citrate buffer (pH 5.2). Furthermore, we examined the smears, both stained and unstained, under a microscope equipped with differential interference contrast (DIC) and epifluorescence optics.

The method of Baxby et al. (2) requires heating, over a flame, of the safranin solution that covers the smear to the point of boiling during staining. Heating over a flame may not always heat the smears uniformly, and sometimes, the stain covering the smear may dry due to evaporation and leave behind crystalline residues (9) which may interfere with proper staining of the oocysts. In order to be more precise, we heated the safranin-covered smears at specified time intervals, as follows: (i) in a 60°C water bath for 1, 5, and 10 min and (ii) in a microwave oven at full power (650 W) for 30, 60, and 120 s. The smears were rinsed with tap water for 30 s and were counterstained with either 1% aqueous methylene blue or 1% aqueous malachite green for 1 min, rinsed in water, air dried, and mounted with a no. 1 coverslip by using Cytoseal 60 (Stephens Scientific, Riverdale, N.J.). The slides were examined and photographed with either an Olympus BH2 or a Zeiss Axioscope microscope.

RESULTS AND DISCUSSION

Cyclospora oocysts uniformly stained a brilliant reddish orange in 14 of the 71 samples tested when they were stained with the safranin stain.

Figure 1 illustrates the staining characteristics of *Cyclospora* oocysts by the different staining techniques. Figure 1a shows oocysts stained with the modified Kinyoun's acid-fast stain. Note the great variability in the staining of oocysts from dark red to colorless. In the colorless oocysts, the wrinkled appearance of the oocyst wall was used to help identify the parasite. Such variability has been demonstrated for *Cryptosporidium* oocysts by Baxby et al. (2) and others when they are stained with acid-fast stain. Because of this variability, Baxby et al. (2) developed the safranin technique for the identification of *Cryptosporidium* oocysts. The original procedure of Baxby et al. (2) did not stain the oocysts of *Cyclospora* uniformly. Heating the smears in a water bath at 60°C for specified times also did not give good results. However, when the smears were heated in a microwave oven, after the smears were covered with the aqueous safranin solution (either pH 2.5 or pH 6.5 or in citrate buffer), remarkably, uniform staining of the oocysts was seen. Heating of the smears in the microwave oven for 30 or 60 s while they were immersed in 1% aqueous safranin (pH 6.5) gave the best results, with more than 98% of the oocysts staining a brilliant reddish orange. Heating of the smears for 2.5 and 5 min, however, did not improve the staining quality. Results for smears stained with safranin-methylene blue are presented in Fig. 1b, and the results for smears stained with safranin-malachite green are presented in Fig. 1c. In both cases the oocysts stain brilliant reddish orange on either a blue (methylene blue) or a green (malachite green) background.

Giemsa staining has been used to stain oocysts of *Cryptosporidium* and has been found to be of limited use. When we stained fecal smears with the Giemsa stain, many oocysts of *Cyclospora* stained faintly bluish and others did not take up the stain (Fig. 1d). Yeasts and other particulate materials in the feces also stained bluish, thus making it very difficult to distinguish oocysts from these structures. *Cyclospora* oocysts also remained colorless when stained with the trichrome stain (Fig. 1e). Neither the chromotrope (Fig. 1f) nor the Gram-chromotrope (Fig. 1g) staining method differentially stained oocysts of *Cyclospora*; hence, neither method was useful for the identification of these parasites in fecal smears.

The safranin-methylene blue and safranin-malachite green techniques can be used on Formalin-fixed specimens and fecal concentrates. Unlike acid-fast and Giemsa stains, there is no critical destaining stage: it is simple, reliable, and quick. On a few occasions, fecal debris, usually about 4 to 5 μm, but sometimes larger, can also stain reddish pink, but these can easily be distinguished from the oocysts of *Cyclospora*. Baxby et al. (2) also noted such staining when they used this technique to stain *Cryptosporidium* oocysts. Baxby et al. (2) also stated that occasionally bacterial spores may also take up an orange to red color upon staining. We have rarely noticed such staining, and we have no difficulty in identifying these as debris. Drying of the smears on a slide warmer not only reduces the time of drying but also helps to affix the oocysts to the slides. We tried various concentrations of safranin (0.5, 1, 2.5, and 5%) and heated the slides in a microwave oven during the staining process. The best results were obtained when the smears were stained in 1% safranin solution. Higher concentrations of safranin did not improve the staining properties of the oocysts, while 0.5% safranin gave poor results.

Heating in a microwave oven has previously been used to facilitate staining by the Ziehl-Neelsen method of mycobacteria from culture, from sputum, and in tissue sections (9). It has also been shown that heating of Formalin-fixed, paraffin-embedded tissue sections in a microwave oven before reacting the sections with fluorescein isothiocyanate-conjugated monoclonal or polyclonal antibody appears to enhance staining of the antigen (20). The exact mode of action of heating in a microwave oven is not known. It is believed, however, that the cross-linking of proteins may be altered by heating in a microwave oven (21). It is possible that heating in a microwave oven may alter the surface configuration of the oocyst, thus making it permeable so that the safranin stain may bind with it uniformly. It is also known that heating of the sections in a microwave oven can unmask given antigenic sites, enhancing the reactivity of the antibodies with some antigens. Microwave ovens are relatively inexpensive and are available in many clinical laboratories. If a microwave oven is not available, fecal smears immersed in the staining solution may be heated on a hot plate to give relatively fair results.

In our hands smears made from samples stored in dichromate solution did not give uniform results. However, the oocysts stained uniformly if the dichromate was removed by washing the samples in phosphate-buffered saline and the samples were then fixed with Formalin and stained with safranin as described above.

Freezing of the stool sample at −20°C did not adversely affect the staining properties of the oocysts, which stained a brilliant reddish orange, just like those in the unfrozen smears. The effect of long-term storage at −20°C is under investigation.

Baxby et al. (2) found malachite green to be unsatisfactory. In our hands, however, both 1% methylene blue and 1% malachite green worked well. The source of the safranin stain may be important. We have used safranin solution supplied by the Gram stain kit SG100 (Carr-Scarborough, Decatur, Ga.), with good results. We have also prepared safranin (Merck, Darmstadt, Germany) solution directly in water as a 1% suspension. The results obtained with this aqueous solution, although satisfactory, were not very consistent. However, when the 1% solution was made in acidified water adjusted to either pH 2.5 or pH 6.5, the staining of oocysts was consistently uniform. We have used 1.0% safranin at pH 6.5 and with 1.0% malachite green counterstaining of Formalin-fixed fecal concentrates on a regular basis in our laboratory to stain *Cyclospora* oocysts and found it to be quite satisfactory. Individual smears may be covered with the safranin solution and microwaved for 30 s, or groups of 5 to 10 smears can be placed in a Coplin jar con-

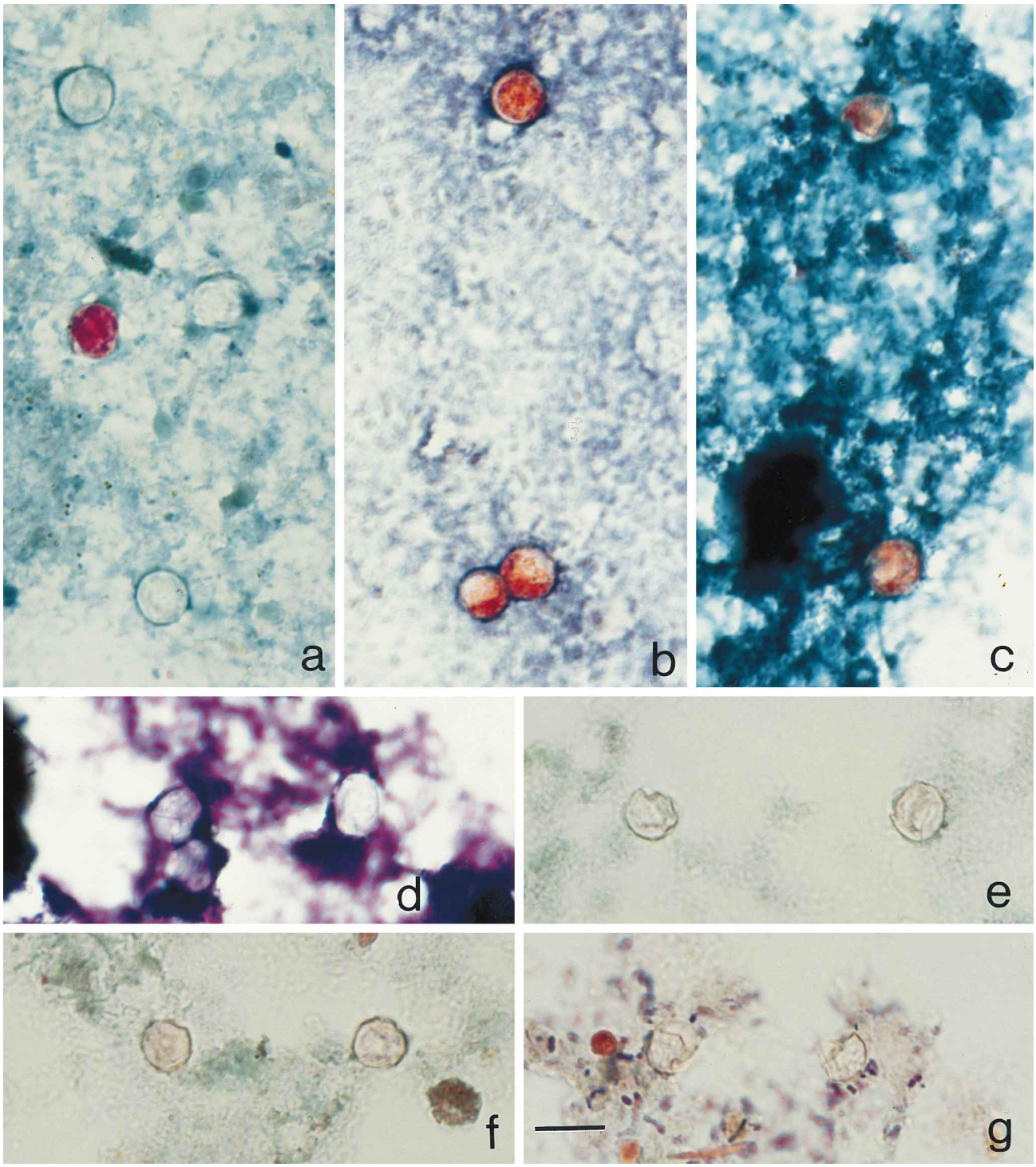

FIG. 1. *Cyclospora* oocysts in fecal smears demonstrated by various staining procedures. (a) Modified Kinyoun's acid-fast stain showing four oocysts, with one stained reddish and the other three unstained; (b) safranin-methylene blue stain, with all three oocysts stained orange; (c) safranin-malachite green stain, with two oocysts stained orange; (d) Giemsa stain, with two oocysts stained bluish and the third unstained; (e) trichrome stain, with two unstained oocysts; (f) chromotrope stain, with two unstained oocysts; (g) Gram-chromotrope stain, with two unstained oocysts. Bar, 10 μm.

taining the safranin solution and microwaved for 1 min. This procedure can also be used to stain *Cryptosporidium* oocysts. The step-by-step procedure used to perform the staining is detailed as follows. (i) Prepare a thin smear of the fecal material (Formalin-preserved stool specimens or fecal concentrates) to be stained. (ii) Dry the smear on a slide warmer at ~60°C. Cool to room temperature before staining. (iii) Place the slide in a Coplin jar containing acidic alcohol (3% [vol/vol] HCl in methanol) and let stand for 5 min. (iv) Wash off excess acidic alcohol with cold tap water. (v) Place the slide(s) in a

Coplin jar containing safranin (1% [wt/vol]) solution in acidified water (pH 6.5) and microwave at full power (650 W) for 1 min. (It is recommended that the Coplin jar be placed in a glass or plastic tray so that the overflowing stain [because of boiling] may be caught in the tray and not on the microwave oven floor.) (vi) Wash off excess stain with tap water. (vii) Place the slide(s) in a Coplin jar containing an aqueous solution of either 1% (wt/vol) methylene blue or 1% (wt/vol) malachite green and let stand for 1 min. (viii) Rinse gently with tap water, let dry, and mount a no. 1 coverslip by using Cytoseal 60 or a suitable mounting medium and examine in a microscope equipped with bright-field optics. Note that for consistent results fresh staining solution should be used after a cycle of 10 microwave heatings.

Oocyst walls in the safranin-stained smears exhibited weak to moderate autofluorescence when they were examined with epifluorescence optics. Furthermore, the oocyst walls in the stained smears were sharp and clearly visible when they were examined with the DIC optics.

The features used to identify *Cyclospora* oocysts in safranin-stained slides are as follows: (i) size, 8 to 10 μm; (ii) crinkled or wrinkled oocyst wall still evident in the safranin-stained smears; (iii) staining characteristics of color (reddish orange); (iv) oocyst wall in the safranin-stained smear still autofluoresces weakly or moderately; and (v) stained smears reveal the cyst wall more clearly when they are examined by DIC (Nomarsky) microscopy.

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