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Comparison of Sedimentation and Flotation Techniques for Identification of *Cryptosporidium* sp. Oocysts in a Large Outbreak of Human Diarrhea

SCOTT J. N. McNABB,[1]* DIANE M. HENSEL,[2] DAVID F. WELCH,[2,3] HARALD HEIJBEL,[4] GARRY L. McKEE,[5,6] AND GREGORY R. ISTRE[7]

Diagnostic Microbiology Division[1] and Public Health Laboratory Services,[5] Oklahoma State Department of Health, Oklahoma City, Oklahoma 73124; Microbiology Laboratory, Oklahoma Children's Memorial Hospital, Oklahoma City, Oklahoma 73126[2]; Division of Pediatric Infectious Diseases[3] and Department of Microbiology and Immunology,[6] University of Oklahoma College of Medicine, Oklahoma City, Oklahoma 73190; Division of Field Services, Epidemiology Program Office, Centers for Disease Control, Atlanta, Georgia 30333[4]; and Epidemiology Service, Oklahoma State Department of Health, Oklahoma City, Oklahoma 73152[7]

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Cryptosporidiosis, previously seen mostly among immunocompromised patients, is now recognized among immunocompetent patients. During a large outbreak of cryptosporidiosis in two day-care centers, we compared two procedures for the demonstration of the organism in preserved stool specimens. Of 703 stool specimens tested by both techniques, Sheather sucrose flotation (SSF) identified 127 (18.1%) as positive for *Cryptosporidium* sp. oocysts. Ritchie Formalin-ethyl acetate sedimentation (F/EA) plus a modified cold Kinyoun acid-fast stain (MCK) of the sediment identified 129 (18.4%) as positive for *Cryptosporidium* sp. oocysts. The degree of agreement between the two tests was statistically highly significant ($P < 0.0001$). A total of 161 (22.9%) were positive by one technique or the other; 95 (13.5%) were positive by both techniques. A total of 32 specimens were positive by SSF but negative by F/EA plus MCK, and 34 specimens were positive by F/EA plus MCK but negative by SSF. The discrepancies between the two techniques occurred in stool specimens that contained rare to a few oocysts. Other parasitic forms were found by both techniques. F/EA plus trichrome staining recovered 126 (17.9%) specimens with *Giardia lamblia*, whereas SSF recovered only 42 (6.0%) specimens with *G. lamblia*. No association ($\chi^2 = 0.02$, $P = 0.89$) was observed between the presence of *G. lamblia* and *Cryptosporidium* sp. in these stool specimens. We concluded that F/EA plus MCK of the sediment was as effective in the concentration and identification of *Cryptosporidium* sp. oocysts as SSF. F/EA plus MCK may be advantageous as a single concentration method for general parasitology when *Cryptosporidium* sp. is also being sought.

Until recently, human infections by the coccidian parasite *Cryptosporidium* sp. have been reported infrequently. The first two documented cases of cryptosporidiosis were reported in 1976 (11, 13). Case studies of this disease link its occurrence with exposure to infected animals (5, 14). Immunosuppression (17) or immunodeficiency (9), including acquired immunodeficiency syndrome (3), are other predisposing conditions. *Cryptosporidium* sp. oocysts were implicated recently in an outbreak of diarrhea among immunocompetent children in a Philadelphia day-care center (1). The Centers for Disease Control has reported several investigations of a similar nature in normal children (4).

The diagnosis of cryptosporidiosis was based originally on finding the organism in histological sections of intestinal tissue obtained by biopsy. Unstained and iodine-stained wet mounts of directly prepared or concentrated fecal material or permanent staining techniques (e.g. trichrome or iron hematoxylin) failed to demonstrate the organism. *Cryptosporidium* sp. oocysts are small (4 to 6 μm) and may easily be confused with yeasts or artifacts.

In 1980 oocysts were found in human stool specimens (16). In 1981 the acid-fast nature of the organism was demonstrated (8). Since this time, new and varied concentration and staining techniques (2, 6, 10) for demonstrating the organism in stools have been reported, eliminating the necessity for tissue biopsy techniques and allowing routine diagnosis by examination of freshly passed or preserved feces. However, these studies dealt with only a small number of positive cases, i.e., 10 confirmed cases in one study (10) and 45 confirmed or suspected cases in another (6). Little data are available as to the most effective routine techniques for the recovery and identification of *Cryptosporidium* sp. oocysts, especially in terms of sensitivity, specificity, technical ease, and reproducibility.

A large-scale outbreak of cryptosporidiosis in two Oklahoma day-care centers recently provided us with the opportunity to compare two concentration techniques currently recommended for use in the diagnosis of *Cryptosporidium* sp. infections: (i) The Ritchie Formalin-ethyl acetate (F/EA) (7, 12) concentration procedure combined with a modified cold Kinyoun acid-fast stain (MCK) of the sediment and (ii) Sheather sucrose flotation (SSF) (15), a specialized procedure adapted from veterinary science to clinical microbiology laboratories (14) and currently recommended for the diagnosis of cryptosporidiosis (10).

MATERIALS AND METHODS

Specimen collection and handling. Of over 1,100 stools collected in this outbreak, the first 703 were chosen to evaluate the two methods. The fresh stool specimens were divided and placed into 10% buffered Formalin or polyvinyl alcohol preservative. The specimens in Formalin were

* Corresponding author.

TABLE 1. Recovery and quantitation of *Cryptosporidium* sp. oocysts by SSF and F/EA plus MCK in 703 specimens obtained in Oklahoma in 1984

No. of oocysts/no. of oil immersion fields detected by F/EA plus MCK	No. of specimens with indicated no. of oocysts/high-power field detected by SSF				
	0	<1	1 to 9	10 to 49	>50
0	542	22	10	0	0
1/10 to 100	26	4	7	1	0
1/1 to 10	5	11	14	2	0
1 to 5/1	3	3	12	5	2
>5/1	0	2	8	15	9

subdivided into two parts. One part was processed by F/EA sedimentation, and the other part was processed by SSF. The two concentration procedures and examination of slides were performed independently in two laboratories by experienced parasitologists who were unaware of the results obtained in the other laboratory until the end of the study. A permanent trichrome stain was made from the polyvinyl alcohol-preserved specimens and examined under oil immersion magnification for the presence of other parasites.

F/EA and MCK. The F/EA concentration procedure was performed as described previously (7, 12). Briefly, 4 to 5 ml of the Formalin-treated stool specimen was washed in 10% Formalin–saline, and the sediment, collected by centrifugation at 650 × *g* for 5 min, was suspended in 8 ml of Formalin-saline–3 ml of ethyl acetate (certified American Chemical Society grade; Fisher Scientific Co.). This mixture was vortexed vigorously for 3 min and centrifuged at 500 × *g* for 5 min, resulting in four layers: a layer of ethyl acetate, a plug of debris, a layer of Formalin-saline, and sediment. The plug was rimmed with an applicator stick, and the top three layers were decanted. One portion of the sediment was placed on a microscope slide and dried for the acid-fast stain. The remainder of the sediment was examined at 100 and 450× for eggs and cysts in saline and iodine wet mounts.

The protocol for MCK has been described previously (10). Smears were fixed in methanol for 1 min, stained in the primary dye (Kinyoun carbolfuschin) for 15 min, and then decolorized for 1 min in 10% H_2SO_4. The counterstain was light-green SF yellowish stain. Each MCK-stained slide was examined under oil immersion power for 15 min. Results were recorded as the average number of oocysts observed per oil immersion field.

SSF. Approximately 2 ml of the Formalin-treated stool suspension was strained through two layers of gauze into a conical tube. Eight milliliters of Sheather sugar solution (500 g of sucrose, 6.5 g of phenol, 320 ml of distilled water) was added, and the suspension was mixed thoroughly by inversion. The tube was capped and centrifuged at 500 × *g* for 15 min. After centrifugation, material at the surface of the suspension was removed with a loop bent at a 90° angle. Three loopfuls were placed on a slide and covered with a cover slip (22 by 22 mm). Each slide was examined for approximately 5 min for pink, refractile oocysts by bright-field microscopy and with the 45× objective lens of an American Optical Corp. microscope. Results were recorded as the average number of oocysts observed per high-power dry field.

Statistical analysis. The McNemar test for matched pairs was used for statistical analysis of the data for the presence of *Cryptosporidium* sp. oocysts and *G. lamblia* in the stool specimens. The Kappa statistic was used to evaluate the data.

RESULTS

A total of 703 stool specimens were examined by the two procedures (Table 1), and *Cryptosporidium* sp. oocysts were identified in 161 (22.9%) specimens. Using SSF, we identified oocysts in 127 (18.1%) specimens. F/EA sedimentation plus MCK identified oocysts in 129 (18.4%) specimens. A total of 542 specimens were negative for *Cryptosporidium* sp. oocysts by both techniques. A total of 22 specimens that were positive by SSF but negative by F/EA plus MCK contained less than one oocyst per high-power field; 10 such specimens contained one to nine oocysts per high-power field. Of 34 specimens from which oocysts were recovered by only F/EA plus MCK, 26 contained one oocyst per 10 to 100 oil immersion fields, 5 contained one oocyst per 1 to 10 oil immersion fields, and 3 contained one to five oocysts per 1 oil immersion field.

We found no correlation between the presence of *Cryptosporidium* sp. and *G. lamblia* in stool specimens in this study. Of 129 F/EA-concentrated specimens with *Cryptosporidium* sp. present, 21 (16.3%) contained *G. lamblia*, whereas of 574 specimens without *Cryptosporidium* sp. present, 105 (18.3%) contained *G. lamblia* ($\chi^2 = 0.02$, $P = 0.89$).

Parasitic forms other than *Cryptosporidium* sp. were also found in the 703 stool specimens. A total of 126 *G. lamblia*, 9 *Entamoeba coli*, 6 *Endolimax nana*, 8 *Blastocystis hominis*, and 2 *Dientamoeba fragilis* were found by examination of wet mounts of material concentrated in F/EA or of polyvinyl alcohol-preserved material stained with trichrome. Many of these parasites were not observed (e.g., only 42 *G. lamblia*) in material examined by SSF.

DISCUSSION

During an outbreak of diarrheal disease in a day-care center we had the opportunity to investigate the comparative sensitivities of two methods for the recovery of *Cryptosporidium* sp. from stool specimens. A general-purpose technique for parasite concentration, the F/EA concentration procedure, was compared with the more classical method (SSF) for the detection of *Cryptosporidium* sp. There was no significant difference between these two methods in rates of recovery of *Cryptosporidium* sp. oocysts. All disparate results were associated with the presence of very few oocysts. The use of both techniques resulted in a 22.9% positivity rate, as compared with rates of approximately 18% for either technique alone. In view of the large number of specimens included in this study, this difference in positivity rates may appear to warrant the recommendation that both methods be used for the optimal detection of *Cryptosporidium* sp. However, an examination of multiple specimens per patient by either technique may provide a similar increase in sensitivity. As the F/EA concentration procedure is already performed routinely in most clinical microbiology laboratories, preparation of the acid-fast smear for detecting *Cryptosporidium* sp. in the same sediment may reduce cost and technical time. The acid-fast smear provides a permanent record of the results, but the preparation and reading of the smear require some technical expertise. SSF concentration is easy to perform, and the pink, refractile oocysts are easily recognized under high-power magnification, but the wet mounts should be examined within 15 min after preparation or the oocysts may

collapse. This difficulty may prevent batching specimens if this method is used for a large number of stools.

In contrast to previously reported findings (18), we did not find an association between *Cryptosporidium* sp. and *G. lamblia*. Only 21 (3%) of the 703 stool specimens contained both parasites, whereas 213 (30%) of the 703 stool specimens contained one or the other parasite. The occurrence of commensal parasites suggests hand-to-mouth transmission and is probably an indication of ingestion of contaminated feces in this day-care center.

We examined each MCK-stained slide for 15 min under oil immersion magnification to cover approximately 100 fields. This amount was roughly equal to the area covered when we examined each SSF wet mount for 5 min under high-power dry magnification. We chose these two parameters to standardize our procedures and make our data available for comparison. However, because of time constraints and the amount of stool material required, we were unable to perform reproducibility studies of these two techniques during this outbreak.

In conclusion, F/EA sedimentation plus MCK was as sensitive as the specialized SSF technique for the detection of *Cryptosporidium* sp. oocysts. The method selected for use by individual laboratories may depend on the desirability of incorporating an examination for *Cryptosporidium* sp. oocysts into general parasitology and the number of specimens received for examination.

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