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Improved Stool Concentration Procedure for Detection of *Cryptosporidium* Oocysts in Fecal Specimens

RAINER WEBER,† RALPH T. BRYAN,* AND DENNIS D. JURANEK

Parasitic Diseases Branch, Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

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Epidemiologic and laboratory data suggest that coprodiagnostic methods may fail to detect *Cryptosporidium* oocysts in stool specimens of infected patients. To improve the efficacy of stool concentration procedures, we modified different steps of the Formalin-ethyl acetate (FEA) stool concentration technique and evaluated these modifications by examining stool samples seeded with known numbers of *Cryptosporidium* oocysts. Because these modifications failed to improve oocyst detection, we developed a new stool concentration technique that includes FEA sedimentation followed by layering and flotation over hypertonic sodium chloride solution to separate parasites from stool debris. Compared with the standard FEA procedure, this technique improved *Cryptosporidium* oocyst detection. The sensitivities of the two concentration techniques were similar for diarrheal (watery) stool specimens (100% of watery stool specimens seeded with 5,000 oocysts per g of stool were identified as positive by the new technique, compared with 90% of stools processed by the standard FEA technique). However, the most significant improvement in diagnosis occurred with formed stool specimens that were not fatty; 70 to 90% of formed stool specimens seeded with 5,000 oocysts were identified as positive by the new technique, compared with 0% of specimens processed by the standard FEA technique. One hundred percent of formed specimens seeded with 10,000 oocysts were correctly diagnosed by using the new technique, while 0 to 60% of specimens processed by the standard FEA technique were found positive. Similarly, only 50 to 90% of stool specimens seeded with 50,000 oocysts were identified as positive by using the standard FEA technique, compared with a 100% positive rate by the new technique. The new stool concentration procedure provides enhanced detection of *Cryptosporidium* oocysts in all stool samples.

The coccidian parasite *Cryptosporidium* sp. first gained attention as an etiologic agent of human diarrhea when it was identified in patients infected with the human immunodeficiency virus. Subsequently, this organism has also been recognized as an important cause of diarrhea in immunocompetent persons (1, 7, 8, 13–18, 20, 26, 29–31, 35). Various stool concentration techniques, staining methods, and antigen detection assays for the coprodiagnosis of cryptosporidiosis have been developed (2, 4, 9, 10, 20, 23, 27, 30, 32, 38). The Formalin-ethyl acetate (FEA) procedure to concentrate stool specimens and the modified cold Kinyoun acid-fast (AF) and the fluorescein-tagged monoclonal antibody (immunofluorescence [IF]) techniques to stain *Cryptosporidium* oocysts are among the most popular methods currently in use in diagnostic laboratories.

Recent epidemiologic data suggest that coprodiagnostic methods may fail to detect *Cryptosporidium* oocysts in stool specimens of infected patients: in two studies of human immunodeficiency virus-infected patients with chronic diarrhea and negative stool examinations, *Cryptosporidium* oocysts were detected in 4.5 and 11.6% of patients undergoing small-bowel biopsy (12, 19). Similar research with immunocompetent patients without diarrhea has shown that stool examinations for *Cryptosporidium* oocysts may be negative in up to 53% of patients whose duodenal aspirates are positive for oocysts (26).

By examining human stool samples seeded with known numbers of *Cryptosporidium* oocysts, we have previously shown that the minimum number of oocysts that can be detected by using FEA stool concentration with AF- or IF-staining techniques is unexpectedly high. In addition, by analyzing different steps of the FEA stool concentration technique, we found that rather than actually concentrating oocysts, this procedure resulted in a net loss of detectable oocysts (34).

To develop a stool concentration procedure that would improve the sensitivity of *Cryptosporidium* oocyst detection in stool specimens, we modified different steps of the FEA procedure and evaluated these modifications by examining stool samples seeded with known numbers of *Cryptosporidium* oocysts. These efforts led to the development of a new stool concentration technique, which we then compared with the standard FEA procedure for efficacy of oocyst recovery.

MATERIALS AND METHODS

***Cryptosporidium* oocysts.** Viable *Cryptosporidium parvum* oocysts were recovered from fecal material of calves (3) and fixed with 10% Formalin. The number of oocysts in the Formalin stock solution was determined by using a cell-counting chamber (Spencer Bright Line hemocytometer; American Optical Co., Buffalo, N.Y.). All human stool specimens were seeded with 0.5 ml of the oocyst suspension. The number of *Cryptosporidium* oocysts in the dosage suspension was prepared by adjusting the stock solution by adding or removing Formalin.

Human stool specimens. Feces-Formalin suspensions that were free of ova and parasites were prepared by fixing fresh fecal material with 10% Formalin in a 1:3 stool-Formalin ratio. For experiments attempting to improve the FEA

* Corresponding author.
† Present address: Division of Infectious Diseases, Department of Medicine, University Hospital, CH-8091, Zurich, Switzerland.

concentration procedure, specimens from one volunteer with formed stools were used. For experiments evaluating the new stool concentration technique, samples (150 g each) of fresh fecal material from four volunteers with formed stools and of one fresh watery diarrheal specimen from a patient with AIDS were separately prepared. Portions of 20-ml stool-Formalin suspensions containing 5 g of fecal material were placed in stool collection vials and individually seeded with a 0.5-ml suspension containing the number of *Cryptosporidium* oocysts necessary to yield the following concentrations: 5,000, 10,000, 50,000, and 1,000,000 oocysts per g of stool.

Standard stool concentration procedures. The modified Ritchie FEA stool concentration procedure was performed as previously described (25, 36). Briefly, 4 ml of the Formalin-fixed stool suspension was washed with water through wet gauze into 15-ml conical centrifuge tubes. The sediment, which was collected by centrifugation at 500 × *g* for 2 min, was resuspended in 9 ml of 10% Formalin and 3 ml of ethyl acetate and shaken vigorously for 30 s. The second centrifugation step at 500 × *g* for 2 min resulted in the following four layers (from top to bottom): ethyl acetate, plug of debris, Formalin, and sediment. The top three layers were decanted, and slides were prepared by using 10-μl aliquots of the sediment.

Sheather's sugar and zinc sulfate centrifugal flotation techniques were also performed as previously described (22, 28).

Modification of the FEA stool concentration procedure. We evaluated the following modifications of the FEA stool concentration procedure.

(i) Variable centrifugation times. The first and second centrifugation steps were varied between 1 and 10 min and compared with the standard centrifugation time of 2 min.

(ii) Single centrifugation step with variation in centrifugation times. Only one centrifugation step was performed. Four milliliters of the Formalin-fixed stool suspension was washed with approximately 6 ml of 10% Formalin through wet gauze into 15-ml conical centrifuge tubes, mixed with 3 ml of ethyl acetate by shaking for 30 s, and centrifuged at 500 × *g*. Centrifugation times of 2, 5, and 10 min were compared.

(iii) Nongauze filters. We used a 600-μm metallic filter and a commercially available plastic filter (fecal concentrator kit; Remel, Lenexa, Kans.) instead of wet gauze in the crude filtration of the stool suspension.

(iv) Use of a surfactant. Three drops of 20% Triton X-100 were added to the centrifugation tube and mixed with the Formalin-fixed stool suspension before the first centrifugation step in an attempt to reduce the adherence of stool particles.

(v) Variable *g* forces. Centrifugation at the relative centrifugal force of 100 × *g* or 1,000 × *g* was compared with standard centrifugation at 500 × *g*.

Slides were prepared by using 10-μl aliquots of the resulting sediments. *Cryptosporidium* oocysts were counted in 50 random microscopic fields at a magnification of ×400. Three different specimens seeded with 1,000,000 oocysts per g of stool were examined for each experiment. The same specimens, processed by the standard FEA concentration method, served as controls.

New stool concentration method (Fig. 1). Four milliliters of the Formalin-fixed stool suspension, 6 ml of 10% Formalin, and 3 ml of ethyl acetate were placed into a 15-ml conical glass centrifuge tube, mixed by shaking vigorously for 30 s, and centrifuged at 500 × *g* for 5 min. This centrifugation resulted in the following four layers (from top to bottom):

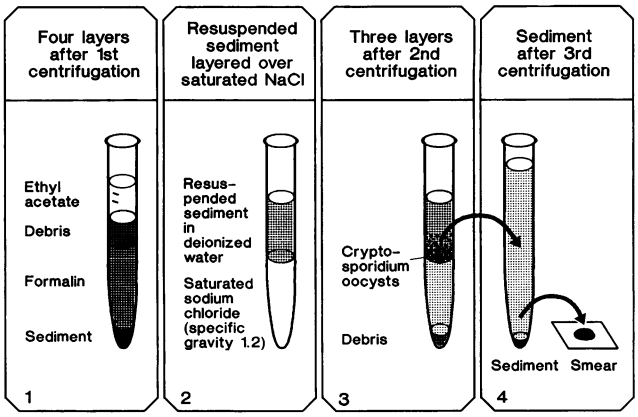

FIG. 1. Flow diagram of the new stool concentration method.

ethyl acetate, plug of debris, Formalin, and sediment. The top three layers were decanted. The sediment was resuspended in 5 ml of deionized water, layered over 5 ml of saturated sodium chloride (specific gravity, 1.20) by using a disposable plastic bulb pipette, and centrifuged at 500 × *g* for 10 min. This second centrifugation step resulted in the following three layers (from top to bottom): deionized water containing a small amount of fecal debris and *Cryptosporidium* oocysts, which were just above the surface of the sodium chloride layer; saturated sodium chloride; and a pellet containing most of the fecal debris. The uppermost 3.5 to 4 ml of the top layer was removed by using a disposable plastic bulb pipette and discarded. The remainder of the top layer (containing the parasites) and approximately 0.5 ml of the top portion of the sodium chloride layer were removed with the same pipette and washed in approximately 13 ml of deionized water by centrifugation at 500 × *g* for 10 min. Slides were prepared by using 10-μl aliquots of the resulting sediment.

Ten samples per oocyst concentration per person were processed by the new stool concentration procedure and by the standard FEA technique. Slides were stained and completely scanned by using fluorescent microscopy at a magnification of ×400.

Staining of slides. All slides were stained by using the indirect immunofluorescence detection procedure (Merifluor *Cryptosporidium*; Meridian Diagnostics, Cincinnati, Ohio) (4, 9, 27) according to the instructions of the manufacturer.

RESULTS

Modifications of the FEA stool concentration procedure. Modifications of the FEA concentration technique offered no improvement in *Cryptosporidium* oocyst recovery compared with that by the standard FEA procedure.

Increasing the centrifugation time did not result in higher numbers of detected oocysts (Table 1) and made slides more difficult to read than those prepared from sediments obtained by shorter centrifugation times. Slides prepared from specimens centrifuged for longer times had fecal debris that was more compact than that of control slides; the compact nature of the material on the slide resulted in increased background fluorescence, which reduced contrast between the apple-green fluorescence of *Cryptosporidium* oocysts and fecal debris.

The single-step centrifugation procedure was significantly

TABLE 1. Comparison of different centrifugation times in FEA stool concentration procedure

Centrifugation time (min)		Mean no. (range) of detected oocysts[a]
Step 1	Step 2	
2	2[b]	69 (58–86)
2[c]	ND	31 (24–40)
5	ND	29 (25–31)
10	ND	32 (21–52)
1	1	27 (26–31)
2	5	54 (45–59)
2	10	72 (59–86)
10	2	65 (53–74)
10	10	43 (39–48)

[a] Oocysts were counted in 50 random microscopic fields at a magnification of ×400. Three specimens seeded with 1,000,000 *Cryptosporidium* oocysts per g of stool were examined for each combination of centrifugation times.
[b] The standard FEA technique was used.
[c] The single centrifugation stool concentration procedure as described in Materials and Methods was used.

less efficacious than the standard two-step FEA technique (P, <0.001).

Neither using metallic or plastic filters instead of wet gauze to filter stool suspensions nor adding a surfactant to the stool suspension improved oocyst recovery.

Centrifugation at a relative centrifugal force of 100 × g led to less concentration of oocysts, and centrifugation at 1,000 × g resulted in oocyst detection similar to that obtained with standard centrifugation at 500 × g. However, increasing the relative centrifugal force adversely affected the reading of slides in a manner similar to that of increasing the centrifugation times; compaction of fecal elements by high-speed centrifugation resulted in more background fluorescence.

Flotation methods. Using Sheather's sugar or zinc sulfate centrifugal flotation techniques, we detected no oocysts in 10 specimens of formed stool seeded with 50,000 *Cryptosporidium* oocysts per g of stool, whereas the standard FEA procedure showed 9 oocyst-positive slides of 10.

New stool concentration method. *Cryptosporidium* oocyst detection was improved by our new stool concentration method compared with the FEA procedure (Tables 2 and 3). The threshold of detection of *Cryptosporidium* oocysts differed from one specimen to the next because stool specimens from different volunteers had different characteristics. Scanning smears from sediments obtained by the new stool concentration technique, we observed a substantially better separation of fecal debris and parasites than we obtained with the standard FEA technique. The slides had considerably less fecal debris and background fluorescence. Fat in the stool reduced recovery of oocysts by both the new technique and the standard FEA technique; nevertheless, the new technique gave better results than the standard FEA technique with the fatty stool.

DISCUSSION

We sought to improve stool concentration procedures on the basis of our previous finding that the threshold of detection of *Cryptosporidium* oocysts (i.e., the minimum number of oocysts that can be detected in stool specimens) with the FEA concentration technique and AF- and IF-staining methods is unexpectedly high (34). Others also have reported that the standard stool concentration method results in the loss of large numbers of oocysts (5, 6). In addition, epidemiologic studies reporting the presence of *Cryptosporidium* oocysts in small-bowel biopsies or duodenal aspirates of patients whose stool examinations were negative indicated that new or modified coprodiagnostic techniques were needed (12, 19).

On the basis of coprodiagnostic experiences with *Giardia* spp., one might expect that nonmicroscopic methods for detecting *Cryptosporidium* antigen in stool specimens would be more sensitive than traditional microscopic techniques for oocyst detection. However, recent reports indicate that available *Cryptosporidium* antigen detection methods are not more sensitive than the combination of FEA processing and microscopy for oocyst detection (2, 32). Anusz et al. found that the monoclonal antibody capture enzyme-linked immunosorbent assay was less sensitive than the IF procedure for detecting *Cryptosporidium* oocysts (2). Similarly, the indirect, double-antibody enzyme-linked immunosorbent assay described by Ungar (32) failed to detect *Cryptosporidium* antigen in all stool specimens in which oocysts were detected by light microscopy. Until the sensitivity of antigen detection methods improves, better stool concentration methods may provide a useful adjunct to existing techniques for the diagnosis of cryptosporidiosis.

On the basis of the results of others who have found the IF detection procedure to have better sensitivity than the AF-staining procedure in epidemiologic studies (4, 9, 27), and on the basis of our laboratory findings showing that the yield by the AF-staining technique was significantly worse than that by the IF technique (34), we exclusively used the IF technique to evaluate the stool concentration methods.

Because our analysis of the FEA stool concentration technique showed high numbers of *Cryptosporidium* oocysts in the discarded elements of the FEA procedure (i.e., in the

TABLE 2. Comparison between new stool concentration technique and standard FEA stool concentration procedure

Oocyst concn[b]	No. of specimens positive/no. examined[a]									
	Stool 1 (formed)		Stool 2 (formed)		Stool 3 (formed)		Stool 4 (formed/fatty)		Stool 5 (watery)	
	FEA[c]	New[d]	FEA	New	FEA	New	FEA	New	FEA	New
1,000	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
5,000	0/10	9/10	0/10	8/10	0/10	7/10	0/10	0/10	9/10	10/10
10,000	6/10	10/10	0/10	10/10	4/10	10/10	0/10	3/10	10/10	10/10
50,000	9/10	10/10	5/10	10/10	6/10	10/10	2/10	7/10	10/10	10/10

[a] Slides were scanned completely.
[b] Number of oocysts per gram of stool.
[c] FEA, FEA stool concentration procedure.
[d] New, new stool concentration procedure.

TABLE 3. Comparison between new stool concentration technique and standard FEA stool concentration procedure

Oocyst concn[b]	Mean no. (range) of detected oocysts[a] in:									
	Stool 1 (formed)		Stool 2 (formed)		Stool 3 (formed)		Stool 4 (formed/fatty)		Stool 5 (watery)	
	FEA[c]	New[d]	FEA	New	FEA	New	FEA	New	FEA	New
1,000	0	0	0	0	0	0	0	0	0	0
5,000	0	2.3 (0–6)	0	1.5 (0–5)	0	1.2 (0–3)	0	0	2.9 (0–6)	4.1 (1–9)
10,000	1.0 (0–3)	2.6 (1–6)	0	3.3 (1–7)	0.4 (0–1)	4.9 (1–7)	0	0.3 (0–3)	12.2 (4–16)	18.3 (6–22)
50,000	2.8 (0–5)	40.9 (19–64)	1.0 (0–3)	12.6 (2–21)	0.7 (0–2)	19.5 (10–37)	0.3 (0–3)	2.4 (0–5)	—[e]	—[e]

[a] Slides were scanned completely.
[b] Number of oocysts per gram of stool.
[c] FEA, FEA stool concentration procedure.
[d] New, new stool concentration procedure.
[e] —, all 10 specimens examined were positive; oocysts were not counted.

gauze that was used for stool filtration and in the supernatants from both the first and second centrifugation steps), we attempted to modify the FEA procedure (i.e., by using alternative filter material or varying the relative centrifugal force or centrifugation times) to improve oocyst recovery (34). However, these modifications offered no improvement and, in some cases, impaired oocyst detection.

Separation of parasites from other fecal elements is a crucial step in coprodiagnostic procedures and appears to be enhanced by centrifugal flotation techniques, such as Sheather's sugar and zinc sulfate. In our experience, these procedures provide clean preparations of oocysts but are inferior to other stool-processing techniques for concentrating oocysts. Similarly, epidemiologic data do not support the superiority of Sheather's sugar centrifugal flotation for detection of *Cryptosporidium* spp. (21). In our study, Sheather's sugar and zinc sulfate centrifugal flotation techniques had a lower sensitivity for oocyst recovery than did the FEA procedure.

The new stool concentration procedure described here provided excellent separation of parasites from stool debris and enhanced detection of *Cryptosporidium* oocysts in all stool samples. The greatest improvement in oocyst recovery occurred when formed stool specimens were processed. In the first step of the procedure, stool fat and mucus were removed by Formalin-ethyl acetate sedimentation. In the next step, parasites were separated from most fecal debris. In the third step, fecal material containing *Cryptosporidium* oocysts was concentrated to obtain the sediment that was used to prepare the slides. The new method was technically easy to perform but involved longer centrifugation times and additional pipetting. This work load can be reduced, however, by processing stool specimens in batches. Moreover, whereas slides of FEA concentrations required microscopic examination at a magnification of ×400 to distinguish oocysts from fluorescing background fecal debris, we were able to scan slides rapidly at a magnification of ×200, using the new technique without affecting the sensitivity of oocyst detection because these slides had much less background debris.

Improved detection of *Cryptosporidium* oocysts in formed stools has important implications for both clinical and epidemiologic studies when the diagnosis of early or asymptomatic infections is needed. Indeed, asymptomatic carriage of *Cryptosporidium* spp. has been described repeatedly (11, 15, 16, 24, 26, 33, 37), and a high rate of asymptomatic carriage (12.7%) has been documented in one group of immunocompetent patients undergoing endoscopy (26). Sensitive stool detection techniques are also required to monitor responses to new therapeutic agents for cryptosporidiosis. As therapeutic agents become available, early detection could allow for earlier treatment and possible prevention of the life-threatening diarrhea often seen in immunocompromised patients. Available epidemiologic data (12, 19, 26) as well as our findings suggest that in patients who are stool negative but are strongly suspected of harboring a *Cryptosporidium* infection, examination of intestinal biopsies and duodenal aspirates may be indicated since current stool detection methods will not detect infections in patients who are excreting fewer than 5,000 to 10,000 *Cryptosporidium* oocysts per g of stool.

Further validation of the new stool concentration technique as an epidemiologic or clinical tool is needed. In addition, further study is needed to assess the ability of this technique to concentrate helminth eggs and protozoa other than *Cryptosporidium* spp. The absence of a "gold standard" for the diagnosis of *Cryptosporidium* infection continues to be an obstacle to the development and evaluation of new coprodiagnostic techniques. The techniques described here (i.e., with stool specimens seeded with known numbers of *C. parvum* oocysts) maybe useful to others who wish to pursue this objective.

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