Evaluation of an Antigen Capture Enzyme-Linked Immunosorbent Assay for Detection of *Cryptosporidium* Oocysts

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The diagnosis of the small (4- to 6-μm) *Cryptosporidium* oocysts is labor intensive and relies on stool concentration, with subsequent staining and microscopy. The primary purpose of this study was to evaluate the clinical utility of an antigen capture enzyme-linked immunosorbent assay (ELISA) (LMD Laboratories, Carlsbad, Calif.) in detecting *Cryptosporidium* oocysts in human stools. A total of 591 specimens (76 diarrheal, 515 control) obtained from 213 inhabitants of an urban slum in northeastern Brazil were examined by both ELISA and conventional microscopic examination (CME) of formalin-ethyl acetate-concentrated stool samples stained with modified acid-fast and auramine stains. Forty-eight diarrheal stools (63.2%) were positive for *Cryptosporidium* oocysts by CME, with 40 of these positive by ELISA. Thirty-five control stools (6.8%) had *Cryptosporidium* oocysts detected by CME, with 15 of these also positive by ELISA. All of the 480 nondiarrheal stools and all but one of the diarrheal stools negative by CME were negative by ELISA. The test had an overall sensitivity of 66.3% and a specificity of 99.8% (positive predictive value, 98.2%; negative predictive value, 94.8%). In the evaluation of human diarrheal stool samples, the test sensitivity increased to 83.3%, with a specificity of 96.4%, and, in analysis of samples from individual patients with diarrhea, the sensitivity was 87.9%, with a specificity of 100%. These results indicate that this stool ELISA is sensitive and specific for the detection of *Cryptosporidium* oocysts in human diarrheal stool specimens but has limited use in epidemiologic studies for the diagnosis of asymptomatic *Cryptosporidium* infection.

Cryptosporidium parvum is a protozoan parasite that has been increasingly recognized as a cause of diarrhea in both immunocompromised and immunocompetent individuals (23). Studies in the developing world have repeatedly identified this *Cryptosporidium* sp. as a culprit in acute and persistent (>14 days) diarrhea (11, 13, 15, 26). Infection with this parasite causes severe but self-limited diarrhea in immunocompetent individuals and often lethal diarrhea in immunosuppressed individuals, most notably patients with AIDS (23). Recent data suggest that between 15% (United States) (9, 19) and 49% (Uganda) (16) of patients with AIDS are infected with *C. parvum* and that this parasite is a cause of significant morbidity and mortality for this patient population. To date, no effective pharmacotherapeutic agents against *C. parvum* have been developed (4).

Although there are several methods for the identification of *Cryptosporidium* oocysts in stool specimens, all are time-consuming and require considerable experience to accurately identify the small (4- to 6-μm) oocysts by microscopy. The most commonly used techniques employ a concentration step, usually formalin-ethyl acetate, followed by one of several staining procedures that include modified acid-fast (3), auramine O (a fluorescent stain) (12), and immunofluorescent stains (6, 7, 10, 14, 20, 21). Recently, several groups have described the use of enzyme-linked immunosorbent assays (ELISAs) for the copro-diagnosis of

human infections with *Giardia lamblia* (5, 24) and of human and animal infections with *C. parvum* (1, 2, 17, 22).

The purpose of this study was to evaluate a new, commercially available antigen capture ELISA (LMD Laboratories, Carlsbad, Calif.) by comparing its performance with those of conventional staining methods (modified acid-fast and auramine) and microscopy in identifying *Cryptosporidium* antigens in formalinized human stool specimens.

MATERIALS AND METHODS

Stool specimens. All stool specimens were obtained from inhabitants of several favelas (urban slums) in Fortaleza, a large northeastern Brazilian city and the capital of the state of Ceará. During the course of an ongoing study of the epidemiology of *Cryptosporidium* infection, multiple stool samples were obtained from individuals in households in which an infant (<3 years) had been diagnosed with *Cryptosporidium* infection by conventional microscopy. Stool specimens were collected in plastic cups and transferred to 10% formalin by field personnel. From December 1990 to May 1992, 591 stool specimens (76 diarrheal, 515 control) were collected from 213 individuals living in 31 households.

Microscopic examination. All stool specimens were concentrated by the formalin-ethyl acetate method (27) at a centrifuge speed of 800 × *g* (Fecal Parasite Concentrator, Evergreen Scientific, Los Angeles, Calif.) and stained with modified acid-fast (3) and auramine stains (12). Stool specimens were labelled positive if oocysts between 4 and 6 μm

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(as measured with an eyepiece micrometer) with typical morphology were identified by acid-fast and auramine stains.

Stool ELISA. The ELISA was performed on unconcentrated formalinized stools with a commercially available kit (LMD Laboratories) according to the packaged instructions. For each test procedure, 100 μl of positive or negative control was added to each of the first two wells. One hundred microliters of wash/dilution buffer was then added to each of the remaining wells. Fifty microliters of supernatant from each sample was added to each of these wells, mixed, and incubated at 37°C for 45 min. After three washes, 2 drops of anti-*Cryptosporidium* antibody were added to each well and incubated at 37°C for 20 min. The plates were washed three times, and 2 drops of anti-immunoglobulin G antibody conjugated to biotin was added to each well; the addition was followed by incubation at 37°C for 10 min. After three washes, 2 drops of streptavidin-horseradish peroxidase were added to each well; the addition was followed by incubation at 37°C for 10 min. After three final washes, all wells were washed once gently with deionized water. One drop each of peroxide solution and chromogen was then added to each well, mixed, and incubated at room temperature for 10 min. The reaction was stopped by placing 2 drops of 1 M phosphoric acid in each well.

The results were read visually in accordance with kit instructions, and an assay was considered valid if the control wells were appropriately positive and negative. Any samples with ELISA results discrepant from microscopy results were subjected to repeat ELISA. If the two ELISA results differed, a third run was performed (see "Evaluation of interassay variability" below). In addition, ELISAs testing 118 of 591 (20%) stool samples were read with a spectrophotometer (Molecular Devices Corp., Menlo Park, Calif.) for determination of their optical densities (OD) at 450 nm.

Statistics. Statistical analysis was performed by the unpaired Student t test.

RESULTS

Standardization of visual readings. The kit is designed to be read visually, without the need for a spectrophotometer. To evaluate the possibility of interobserver differences, one run of 46 samples and 2 controls was independently rated by two observers and then compared with the spectrophotometric OD. Each well was rated as "negative," "trace," "significant color but not equal to positive control," or "positive." The observers agreed on 43 of 46 (93.5%) samples, calling 25 negative, 7 trace, 2 significant color, and 9 positive. In two instances, wells that observer 1 labelled as significant color were labelled trace by observer 2. In another instance, a well labelled trace by observer 1 was seen as negative by observer 2.

On the basis of data available from the kit manufacturer, OD readings of 0.250 or higher identify positive samples (8). When analyzed by spectrophotometry, the OD readings of the positive and negative controls of the above-mentioned test run were 0.334 and 0.050, respectively. The 25 visually negative samples had a median OD reading of 0.073 (range, 0.050 to 0.119), and the 7 samples read as trace had a median OD reading of 0.130 (range, 0.115 to 0.155). The samples read as having significant color had OD readings of 0.202 and 0.243, and the visually positive samples had a median OD reading of 0.576 (range, 0.276 to 0.849). The two samples read by one observer as trace and by the other as significant color had OD readings of 0.201 and 0.188, and the sample read as trace by observer 1 and negative by observer 2 had

TABLE 1. Overall correlation between ELISA[a] and microscopic examination in detection of *Cryptosporidium* oocysts in human stool specimens

ELISA result	Microscopy result		
	No. positive	No. negative	Total no.
No. positive	55	1	56
No. negative	28	507	535
Total no.	83	508	591

[a] Sensitivity, 66.3%; specificity, 99.8%; positive predictive value, 98.2%; negative predictive value, 94.8%.

an OD reading of 0.128. Therefore, only those samples that were equal in color to the positive control had positive OD readings, confirming that OD readings are not necessary to determine the positivity of a specimen. In addition, our results indicate that color development equal to that of the positive control contained in the kit is necessary to identify a specimen as positive.

Evaluation of interassay variability. One hundred four human stool samples (including samples that were both concordant and discordant with the results of microscopy) were evaluated multiple times to assess run-to-run variability. Sixty-three of 104 (60.6%) were positive multiple times, 34 of 104 (32.7%) were negative multiple times, and 7 of 104 (6.7%) were discrepant. Five of these seven discrepant samples were evaluated a third time (tiebreaking run). Two of these samples were designated false negatives following the third ELISA; one was designated a false positive; and two (one from a patient recently ill with cryptosporidiosis and one from the father of a child ill with cryptosporidiosis) were given a final result of negative, which was in agreement with microscopy. Two samples never had third ELISAs performed and were dropped from the final analysis.

Evaluation of stool specimens. A summary of the ELISA and microscopy results for all 591 human stool specimens is shown in Table 1. Of the 83 specimens positive by acid-fast- and auramine-stained slides, 55 were positive by ELISA. Of the 508 microscopically negative samples, only 1 was positive by ELISA. The ELISA had an overall sensitivity of 66.3% and a specificity of 99.8% (positive predictive value, 98.2%; negative predictive value, 94.8%).

Table 2 summarizes the performance of the stool ELISA for diarrheal stool samples. Of the 48 diarrheal stools with *Cryptosporidium* oocysts identified by microscopy, 40 were positive for *Cryptosporidium* oocysts by ELISA. Of the 28 microscopically negative samples, only 1 was positive by ELISA. The sensitivity for the detection of *Cryptosporidium* oocysts in diarrheal stools is 83.3%, and the specificity

TABLE 2. Correlation between ELISA[a] and microscopic examination in detection of *Cryptosporidium* oocysts in human diarrheal stool specimens

ELISA result	Microscopy result		
	No. positive	No. negative	Total no.
No. positive	40	1	41
No. negative	8	27	35
Total no.	48	28	76

[a] Sensitivity, 83.3%; specificity, 96.4%; positive predictive value, 97.6%; negative predictive value, 77.1%.

TABLE 3. Correlation between ELISA[a] and microscopic examination in detection of *Cryptosporidium* oocysts in patients with diarrhea[b]

ELISA result	Microscopy result		
	No. positive	No. negative	Total no.
No. positive	29	0	29
No. negative	4	17	21
Total no.	33	17	50

[a] Sensitivity, 87.9%; specificity, 100%; positive predictive value, 100%; negative predictive value, 81.0%.
[b] Patients with one to three stool examinations.

96.4% (positive predictive value, 97.6%; negative predictive value, 77.1%).

Of the 213 patients evaluated in this study with one to three stool examinations, 48 (22.5%) were identified with *Cryptosporidium* infection by conventional microscopic evaluation (CME). Twenty-nine of these 48 were also positive by ELISA. Of the 165 patients negative for *Cryptosporidium* infection by CME, all were negative by ELISA as well. In the evaluation of all patients, the ELISA had a sensitivity of 60.4% and a specificity of 100% (positive predictive value, 100%; negative predictive value, 89.7%). As shown in Table 3, in the evaluation of only those patients with diarrhea (n = 50), CME identified 33 individuals with *Cryptosporidium* infection, of which the ELISA identified 29. Of the 17 patients with diarrhea negative for *Cryptosporidium* oocysts by CME, all were negative by ELISA as well. In the evaluation of patients with diarrhea, the ELISA had a sensitivity of 87.9% and a specificity of 100% (positive predictive value, 100%; negative predictive value, 81.0%). Therefore, of the 19 individuals falsely negative for *Cryptosporidium* infection by ELISA, 15 (78.9%) were asymptomatic individuals.

The 28 stool samples (8 diarrheal stools, 20 formed stools) positive by CME for *Cryptosporidium* oocysts but negative by stool ELISA were examined further. Six formed samples (19.7%) were from children convalescing from cryptosporidial diarrhea, 14 (50%) were from asymptomatic children (n = 9) or adults (n = 5), and 8 (28.6%) were from individuals with diarrhea. We hypothesized that the stools falsely negative by ELISA would have fewer oocysts than those positive by ELISA. Thus, the oocyst number in 100 high-power oil immersion fields was counted for the 28 samples falsely negative by ELISA and for 23 stool samples positive by ELISA. These slides were originally examined by complete scanning of a 10-mm-diameter etched circle. Therefore, some slides labelled positive in initial readings revealed no oocysts in 100 fields, and these have been recorded as 0 of 100 high-power fields (hpf). Of the 28 false negatives, 24 (85.7%) were specimens in which fewer than 10 oocysts were visible in 100 hpf. Among the 8 false negatives from the diarrheal group, there was a median of 4 oocysts per 100 hpf (range, 2 to 59), as opposed to a median of 0 per 100 hpf (range, 0 to 61) among the stools from asymptomatic patients with *Cryptosporidium* infections. In contrast, of 23 slides in which the ELISA was positive, 17 (74%) had more than 10 oocysts per 100 hpf (median, 25; range, 1 to 614). A comparison of the mean number of oocysts per 100 hpf for the false-negative samples (n = 28) and for samples positive by ELISA and CME (n = 23) yielded a P value of $\leq$0.01.

The one false-positive test result was for a stool sample from a child recovering from cryptosporidial diarrhea whose two previous samples (11 and 15 days previously) had been positive by both CME and ELISA.

DISCUSSION

Because of the difficult and time-consuming nature of CME for the detection of *Cryptosporidium* oocysts, there is a need for a simple, rapid, and objective test for the copro-diagnosis of *Cryptosporidium* infection. This need is further underscored by a recent paper suggesting that all of the currently available microscopic methods have a low sensitivity for the detection of oocysts (25). In addition, both auramine O and immunofluorescence require the use of a fluorescence microscope, equipment not readily available in many developing-world settings in which infection is most prevalent. The existence of a reliable and relatively inexpensive stool ELISA would obviate the current need for intensive training and/or expensive equipment necessary to accurately diagnose *Cryptosporidium* infection.

There are currently three commercially available stool ELISA kits for the detection of *Cryptosporidium* oocysts in stools. One such kit (IDEIA *Cryptosporidium*; Dako Diagnostics, Ltd.) was recently evaluated by Siddons et al. for the ability to detect *Cryptosporidium* oocysts in fresh fecal specimens and was shown to be 100% sensitive and specific compared with immunofluorescence (17). Preliminary data indicate that the second kit (Color-Vue *Cryptosporidium*; Seradyn, Inc.) has a specificity of 92% and a sensitivity of 100% in the evaluation of fresh, frozen, and formalinized stools (18).

The present study represents the first large evaluation of a third kit that is identical to the Seradyn kit but marketed under a different name (LMD Laboratories) and designed for the evaluation of fresh, frozen, or formalinized diarrheal stools. The data show that this kit performed admirably in the evaluation of diarrheal stools and, in general, failed to detect oocysts only in specimens with very small numbers of oocysts: specimens in which *Cryptosporidium* oocysts were detected only following complete examination of the material contained within a 10-mm-diameter etched glass circle. In addition, the kit was specific and performed very well in the evaluation of samples from patients with diarrhea, with a sensitivity of nearly 88% in identifying individuals with symptomatic cryptosporidiosis.

The stools for this study were those collected during epidemiologic surveillance of households with an identified case of *Cryptosporidium* infection. Therefore, the percentage of samples with *C. parvum* is higher than would be found in a routine diagnostic laboratory. In this study, the majority of the false negatives occurred with asymptomatic individuals who were household contacts of infants ill with cryptosporidiosis or with children recovering from *Cryptosporidium* infection who no longer had diarrhea. There were, however, two diarrheal specimens that had significant numbers of oocysts by microscopy (39 and 59 per 100 hpf) that were negative by ELISA (false negatives). The one false positive was from a child recovering from cryptosporidiosis and may represent the continued presence of cryptosporidial antigens in the stool without the presence of whole oocysts.

This study did not evaluate or compare the use of fluorescent antibody (FA) in identifying *Cryptosporidium* infection because the cost of performing this procedure with so many

samples was prohibitive. Several studies have indicated that FA is more sensitive than other staining procedures in CME of slides for Cryptosporidium oocysts (6, 7, 14, 25). One recent study, however, found FA to be less sensitive than auramine-rhodamine and Sheather's sugar flotation in identifying Cryptosporidium oocysts and found FA to be the most expensive and difficult of the available staining methods (10). Two of these studies (10, 25) also noted the occasional difficulty in differentiating oocysts from the nonspecific background fluorescence.

One strong advantage that the ELISA kit has over FA and other conventional microscopic techniques is the simplicity and objectivity in reading the results. The concordance between observers was excellent, as was the correlation between visual and spectrophotometric readings. These findings indicate that the kit is suitable for use in developing-world settings in which equipment such as a spectrophotometer is often not available. In addition, the ELISA kit is far less time-consuming than microscopy if more than several samples are to be examined. The ELISA requires approximately 60 min of work and 90 min of incubation time to complete the examination of 24 to 48 samples and does not change appreciably with changes in sample number. Microscopy, on the other hand, requires approximately 45 min of work, which includes concentration, slide making, staining, and slide reading, and about 45 min of waiting time (during drying and staining) per sample, although several slides may be stained simultaneously. Because there is currently no treatment for Cryptosporidium infection and quick diagnosis is therefore not essential, samples batched and run by ELISA may represent significant time savings for a busy parasitology laboratory.

However, given the apparent inability of this assay to detect small numbers of oocysts in stool samples, patients with ELISAs negative for Cryptosporidium oocysts but for whom clinical suspicion for Cryptosporidium infection is high should have stool samples submitted for microscopic examination by one or more staining techniques, which remains the "gold standard" for diagnosis of this parasite. Similarly, this assay appears not to be appropriate for the evaluation of samples from asymptomatic individuals, which is consistent with the fact that this kit is licensed by the Food and Drug Administration only for use with human diarrheal stools. However, for routine diagnosis of Cryptosporidium oocysts in diarrheal stool specimens, this stool ELISA offers a simple, objective, and cost-effective alternative to conventional microscopy.

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