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Comparison of Four Different Methods for Detection of *Cryptosporidium* Species

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Newly available assays offer alternatives to conventional microscopic examination for *Cryptosporidium* spp. We compared two enzyme immunoassays, ProSpect *Cryptosporidium* microtiter assay (Alexon, Inc., Mountain View, Calif.) and Color Vue *Cryptosporidium* assay (Seradyn, Indianapolis, Ind.), and a direct immunofluorescent assay, Merifluor *Cryptosporidium* kit (Meridian Diagnostics, Cincinnati, Ohio), with acid-fast Kinyoun-staining for the detection of *Cryptosporidium* spp. Examinations were performed on 129 stool specimens received from patients during a recent waterborne outbreak. A specimen was considered positive when organisms could be identified visually by acid-fast and immunofluorescent stains or if organisms could be visualized by either acid-fast or immunofluorescent stain and detected by both enzyme immunoassays. The final number of positive specimens was 55. No single procedure detected all 55 positive specimens. Of these, ProSpect and Color Vue detected 52 (sensitivity, 94.5%), and the Kinyoun stain and Merifluor detected 53 (sensitivity, 96.4%). The final number of negative specimens was 74. One false-positive result was seen with both the Kinyoun stain and the ProSpect assay. The Color Vue and ProSpect assays required the most hands-on technologist time. The ProSpect assay and Merifluor kit were easiest to perform. The acid-fast stain was difficult to interpret. The Merifluor kit was easiest to read and was adaptable to both batch and single testing. Overall, the Kinyoun stain and the Merifluor test were preferable to both of the enzyme immunoassays because of the high reagent cost and hands-on time required for the enzyme immunoassays. The difficult interpretation of the Kinyoun stain smears made the Merifluor a more desirable test despite its higher cost. We conclude that all methods tested were equally sensitive and specific for the detection of *Cryptosporidium* spp. Ease of use, adaptability to batch testing, and cost are important criteria in determining the method of choice.

Cryptosporidia are protozoan parasites that cause acute, severe, self-limited disease in immunocompetent individuals. In immunocompromised individuals, they cause a severe, intractable, sometimes fatal diarrhea. Prevalence varies seasonally and geographically. In industrialized countries, the prevalence is 1 to 3%; in underdeveloped countries, the prevalence is 5 to 10% (4). In children and adults with diarrhea or other gastrointestinal symptoms, cryptosporidia are often the most common parasites recovered and are frequently the most common enteric pathogens recovered (4). Worldwide, there is a much higher prevalence in children than in adults. Prevalence is higher during warm wet months (2, 4).

Cryptosporidia are transmitted in feces, and infections are spread from animal to human as well as human to human. Outbreaks in day care centers (3) and hospitals (9, 11) have been reported. Several recent outbreaks have demonstrated contaminated water as a source of the parasites in England, Scotland, Texas, Georgia, Oregon, and Wisconsin (7, 8, 15, 17, 19, 22).

Conventional methods for identification include examination of fecal smears or concentrates using acid-fast stains or auramine-rhodamine stains. These methods are time-consuming and tedious and require an experienced microscopist to identify the organisms (4, 6). Because of the low prevalence of infection in many areas (13, 16), the lack of appreciation of the importance of cryptosporidia as etiologic agents of disease in immunocompetent hosts, and the difficulty of testing for the presence of the parasites, examination for these particular parasites is often not routinely performed (1, 12). A test that is quick, easy to perform, easy to interpret, and not costly would facilitate routine testing.

During a recent waterborne outbreak, we evaluated several commercial kits for the detection of *Cryptosporidium* spp. to determine if any of these methods could more easily be incorporated into the routine procedures for examination of stool specimens from patients with diarrheal illness. These kits were compared with conventional acid-fast staining methods for performance, ease of use, and cost.

MATERIALS AND METHODS

Specimens. Stool specimens were collected from patients who presented with diarrhea during a recent waterborne *Cryptosporidium* outbreak. Stool specimens (129) were collected from 9 April 1993 to 4 June 1993 and preserved in sodium acetate-acetic acid-formaldehyde (Meridian Diagnostics, Cincinnati, Ohio). This preservative was acceptable for all methods evaluated. The specimens were stored at 2 to 6°C until tested. The specimens were tested blindly and processed in appropriately sized batches for each procedure.

Stains. Stool in sodium acetate-acetic acid-formaldehyde was sedimented by centrifugation at 500 × *g* for 10 min. A thin fecal smear 14.5 mm in diameter was prepared from stool sediment on each of two slides and air dried. One slide was heat fixed and stained with the cold acid-fast Kinyoun stain (Difco, Detroit, Mich.). Smears were initially scanned at a magnification of ×400 with confirmation at a magnification of ×1,000 (21). The second slide was stained by a direct immunofluorescence technique (Merifluor *Cryptosporidium* kit; Meridian Diagnostics) and initially scanned at a magnification of ×100 with confirmation at a magnification of ×400, according to the manufacturer's instructions.

Enzyme immunoassays. Stool in sodium acetate-acetic acid-formaldehyde was also tested by using two enzyme immunoassays. The ProSpect *Cryptosporidium* microtiter assay (Alexon, Inc.) and the Color Vue *Cryptosporidium* assay (Seradyn) were performed as described by the manufacturers. The results were read visually and spectrophotometrically at 450 nm.

Method evaluation. These four methods were compared for their ability to detect *Cryptosporidium* spp. in fecal specimens. Since there is no gold standard

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for the detection of *Cryptosporidium* spp., a specimen was considered to be positive if the organism could be visualized by both acid-fast and immunofluorescent methods. Specimens were also considered positive if organisms could be visualized by either acid-fast or immunofluorescent stain and detected by both enzyme immunoassays. If discrepant results were obtained, testing was repeated. Only the original assay result was used in calculating performance characteristics of the assay. Sensitivity, specificity, and positive and negative predictive values were calculated for each method.

Each test was evaluated by using the method of multiattribute evaluation described by MacPherson and McQueen (10) for the comparative study of diagnostic tests. First, attributes important to the use or interpretation of diagnostic tests were identified. Then, the tests were assigned a comparative score for each attribute. This allowed for ranking of the diagnostic tests based on performance in each attribute. The relevant attributes identified were performance, cost, ease of use, and the ability to perform batch testing. The evaluation of performance included both sensitivity and specificity; the above-described criteria were used for determining a true positive. Cost included both reagent costs and technologist time. No capital equipment costs were included. Ease of use and ease of interpretation were subjective evaluations based on the number of reagent steps and the ease with which decisions were made regarding positive and negative results. The ability to perform the test in large batches was also evaluated. Each attribute was ranked from 1 to 4, with 4 being the highest rank.

TABLE 1. Sensitivity, specificity, and predictive values for *Cryptosporidium* detection methods[a]

Method	Sensitivity[b]	Specificity[c]	Predictive value	
			Positive[d]	Negative[e]
Merifluor	96	100	100	97
Kinyoun	96	99	98	97
Color Vue	94	100	100	96
ProSpect	94	99	98	96

[a] The total number of positive specimens was 55, and the total number of negative specimens was 74.
[b] Calculated as follows: (number of true positives/number of true positives + number of false negatives) × 100.
[c] Calculated as follows: (number of true negatives/number of true negatives + number of false positives) × 100.
[d] Calculated as follows: (number of true positives/number of true positives + number of false positives) × 100.
[e] Calculated as follows: (number of true negatives/number of true negatives + number of false negatives) × 100.

RESULTS

The final number of positive specimens was 55; the Kinyoun stain identified 53 of these, and 2 were identified as positive by other tests. Fifty-four specimens were positive by initial Kinyoun acid-fast staining. One acid-fast positive specimen was negative by all other tests and was considered a false-positive Kinyoun stain result. Repeat Kinyoun testing of that specimen and a second specimen collected the same day from the same patient was negative. Two specimens were positive by all other tests but negative by Kinyoun staining. Repeat acid-fast staining of both specimens resulted in organisms being seen in one specimen. Both of these specimens were considered false-negative Kinyoun stain results.

No single procedure detected all 55 positive specimens. ProSpect and Color Vue detected 52 each (sensitivity, 94.5%) and the Kinyoun stain and Merifluor detected 53 (sensitivity, 96.4%). Repeat testing of the two false-negative ProSpect specimens tested, one of the two false-negative Kinyoun specimens, and the one false-negative Color Vue specimen tested yielded positive results. The two false-negative Merifluor specimens and one of the false-negative Kinyoun specimens did not yield positive results upon repeat testing.

The final number of negative specimens was 74. There were only two false-positive specimens: one Kinyoun stain and one ProSpect specimen. All four methods demonstrated high specificity (98.6 to 100%). Sensitivity, specificity, and positive and negative predictive values were calculated for each method on the basis of the results of the first test applied to the specimen (Table 1). Assay performance rank was based on sensitivity and specificity.

The preparation of the slide and the performance of the acid-fast stain procedure required about 7 min of technologist time. In our laboratory, the reading and interpretation of the smear required an additional 5 min. The Merifluor stain procedure required a total time of 35 min, with only 6 min of that as hands-on time. Interpretation of the Merifluor smears was straightforward and required less than 1 min per specimen. The ProSpect and Color Vue assays each required a total of 2 h; however, hands-on time was 17 min for the ProSpect assay and 25 min for the Color Vue assay. Reagent costs (including controls) for each assay range from $0.47 per test to $18.60 per test, depending on the number of tests performed per batch and the method employed. Technologist time added significantly to these costs, as summarized in Table 2.

Ease of use and ease of interpretation were subjective evaluations based on the number of reagent steps and the ease with which decisions were made regarding positive and negative results. Both stains were easy to perform, with the Merifluor procedure judged to be slightly better because it had fewer steps. The ProSpect assay required an initial dilution of the stool specimen; however, the Color Vue assay required more reagents and washes, thus requiring more hands-on time. The Color Vue assay was, therefore, ranked lower for ease of use.

Interpretation of the acid-fast stain was difficult, while the immunofluorescent stain was easy to read. Thus, the Merifluor immunofluorescent stain was ranked highest for this attribute. The development of a faint yellow color in the ProSpect assay was difficult to interpret visually on six separate occasions. Spectrophotometric readings identified four negative and two positive specimens. The Color Vue assay was easy to interpret visually. The Color Vue and ProSpect assays were ranked 2nd and 3rd for interpretation, respectively, with the acid-fast stain determined to be the most difficult to interpret.

Both the acid-fast stain and the Merifluor stain were adaptable to batch and single tests. The Merifluor assay was judged to be slightly more adaptable to batch testing because of the simple stain procedure. Both of the enzyme immunoassays were well suited to large-volume batch testing. The Color Vue assay was judged to be slightly better suited for batch testing because it did not require a predilution step.

Each method's ranks for performance, cost (including reagent and technologist time), ease of use, ease of interpretation, and ability to batch specimens are listed in Table 3.

DISCUSSION

We compared three methods for the detection of *Cryptosporidium* spp. in clinical specimens with conventional techniques and found all methods to be of comparable sensitivity and specificity. The assays which detect *Cryptosporidium* antigens

TABLE 2. Cost of *Cryptosporidium* detection methods

Method	Reagent costs ($/test) for:		Technologist time (min)[a]
	Single test	Batch[b]	
Kinyoun	1.17	0.47	12
Merifluor	12.60	5.04	7
Color Vue	15.93	6.37	25
ProSpect	18.60	7.55	17

[a] Time is for a single test plus controls.
[b] Ten tests per batch.

TABLE 3. Ranking of *Cryptosporidium* detection methods[a]

Method	Performance	Cost[b]	Ease of:		Batch ability
			Use	Interpretation	
Merifluor	4	3	4	4	2
Kinyoun	3	4	3	1	1
ProSpect	1	2	2	2	3
Color Vue	2	1	1	3	4

[a] The evaluation method used is described in reference 10. Tests were ranked from 1 to 4 for each attribute, with 4 being the highest.
[b] Calculated as follows: reagent cost + (hands-on time × $/min).

(ProSpect and Color Vue) were not significantly more sensitive than was visual detection of organisms. We also did not see a significant number of false-positive antigen results, suggesting that detection of antigen without visual organism detection did not occur. Previous studies have found similar results for the Kinyoun acid-fast stain and the Color Vue enzyme immunoassay (5, 14, 18, 20). Garcia et al. (5) found increased sensitivity of the direct immunofluorescent method when compared with the modified acid-fast stain (hot method). This may have been because different concentration methods were employed in the two studies. There is no gold standard for the detection of *Cryptosporidium* spp., and recovery of *Cryptosporidium* spp. from seeded specimens is known to be low (23). The true ability of any of these tests to differentiate patients infected with low numbers of organisms from uninfected patients is unknown.

Since the sensitivity and specificity of the methods are comparable, the choice of method must be based on other criteria. We evaluated the methods for ease of use, ease of interpretation, and cost, in addition to performance. All of the assays were easy to perform. Formalin ethyl acetate concentration or sedimentation by centrifugation (without ethyl acetate) is recommended for initial processing of the specimen prior to testing with the Kinyoun stain or the Merifluor kit (21). These processes are easily incorporated into routine stool processing and yield similar results. A predilution step is required in the ProSpect assay. These pretesting processes add to the complexity of these methods and the time required to perform the tests.

The ease of interpretation of results varied considerably for each assay. The acid-fast stained smears were difficult to interpret, requiring frequent examination at ×1,000 oil magnification to identify the organisms. The Merifluor test was extremely easy to read as the brilliant apple-green-fluorescent organisms with typical morphology were visible at ×100 and could be easily identified at ×400 magnification, thus requiring much less technologist time. The test does require a fluorescent microscope, but this is becoming a standard piece of equipment in many microbiology laboratories. The enzyme immunoassays can be read visually, eliminating the need for a spectrophotometer. However, six borderline specimens in one run were obtained in this study, and the spectrophotometric readings were helpful in determining the results for these specimens.

The assays vary considerably in direct reagent costs, with cost per test dependent upon the number of specimens tested per batch. The hands-on time required to perform the enzyme immunoassays and the interpretation time required for the Kinyoun stain procedure added significantly to the total cost per test.

Ranking of tests for each of the attributes studied allowed for a simple comparative evaluation of methods (10). Overall, the Kinyoun stain and the Merifluor test ranked higher than both of the enzyme immunoassays because of the high reagent cost and hands-on time required for the enzyme immunoassays. The difficulty of interpretation of the Kinyoun stain smears made Merifluor a more desirable test, despite its higher cost.

Despite the reported low recovery of organisms with Kinyoun staining, none of the newer methods evaluated was significantly more sensitive. Ease of use, adaptability to batches, and cost are important criteria in determining the method of choice.

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