JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1993, p. 198–202
0095-1137/93/020198-05$02.00/0

Vol. 31, No. 2

Cryptosporidiosis: Multiattribute Evaluation of Six Diagnostic Methods

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Received 14 August 1992/Accepted 26 October 1992

Six diagnostic methods (Giemsa staining, Ziehl-Neelsen staining, auramine-rhodamine staining, Sheather's sugar flotation, an indirect immunofluorescence procedure, and a modified concentration-sugar flotation method) for the detection of *Cryptosporidium* spp. in stool specimens were compared on the following attributes: diagnostic yield, cost to perform each test, ease of handling, and ability to process large numbers of specimens for screening purposes by batching. A rank ordering from least desirable to most desirable was then established for each method by using the study attributes. The process of decision analysis with respect to the laboratory diagnosis of cryptosporidiosis is discussed through the application of multiattribute utility theory to the rank ordering of the study criteria. Within a specific health care setting, a diagnostic facility will be able to calculate its own utility scores for our study attributes. Multiattribute evaluation and analysis are potentially powerful tools in the allocation of resources in the laboratory.

The genus *Cryptosporidium* was described by Tyzzer (46) in 1907, but it was not until 1976 (33) that human infection was first reported. Although originally described as a zoonotic infection, cryptosporidiosis is a cosmopolitan and well-recognized cause of diarrhea with abdominal pain (12) in immunocompromised humans (14, 16, 43), immunocompetent children living in the tropics and the developed world (9, 24, 25, 31), international travelers (35), and animal handlers (40).

The complex life cycle of *Cryptosporidium* spp., its small size, and subtle staining characteristics have contributed in the past to difficulty in being able to identify this parasite in routine stool parasitology preparations (5, 10, 15). Many parasitology techniques and modifications of those techniques for the identification of oocysts in stool specimens have been described. These include use of acid-fast (8, 19, 20, 22) and Kinyoun (30) stains, safranin staining (4), dimethyl sulfoxide staining (6), Giemsa staining (GS) (2, 42), sugar flotation (2, 20, 32, 36, 49), auramine and rhodamine (AR) staining (8, 37), and immunofluorescence (3, 18, 44).

The purpose of the present study was to compare six of these methods, taking into account several attributes of diagnostic testing. The six procedures evaluated were GS, the Ziehl-Neelsen (ZN) staining, AR staining, Sheather's sugar flotation method (SSF), the indirect immunofluorescence (IIF) procedure, and a modified concentration-sugar flotation (MCSF) method. The attributes of each of the tests evaluated were diagnostic yield, cost of performing the test, ease of handling, and ability to process large numbers of specimens for screening purposes by batching.

From a laboratory diagnosis point of view, the overall utility of testing for cryptosporidiosis may be done by generating an individual utility for each of these factors. In establishing priorities for health care resources, it must be taken into account that this infection is self-limited in immunocompetent hosts (11, 12) and that no specific therapy has been shown to be effective at clearing the parasite (48) or influencing the ultimate clinical course of the infection. Although gastrointestinal secretory inhibitors (17, 26) can reduce the volume of cryptosporidiosis-associated diarrhea in patients with AIDS, these drugs are expensive and not readily available in the developing world.

A diagnostic method which could be used in the developing world or in field epidemiology in the tropics may have very different values assigned to its attributes than a method applied in a university teaching hospital or other medical setting in the developing world. In all settings, a diagnosis of cryptosporidiosis may have direct benefits to the patient and physician by providing a clinical diagnosis and limiting extensive diagnostic evaluations. It may also reduce the use of empirical therapy for gastroenteritis, which could be ineffective and potentially harmful.

MATERIALS AND METHODS

Specimens. The patient specimens used in this study were preserved in sodium acetate-acetic acid-formaldehyde (SAF). The samples were chosen for the present study on the basis of variation in the number of *Cryptosporidium* oocysts, the presence of other parasites or substances such as pus or mucus, and the presence of sufficient material to complete all components of the study.

The characteristics of the stool specimens are given in Table 1. Of the 105 specimens, 53 were previously positive for *Cryptosporidium* spp. by the MCSF method and confirmed by modified ZN staining as described previously (22). These specimens were from children under 16 years of age, except that one specimen was from a patient with AIDS. The remaining 52 specimens were chosen to challenge the capabilities of various current methods used to differentiate *Cryptosporidium* oocysts from other parasites, yeasts, and other substances commonly found in stool specimens. The specimens were processed in appropriately sized batches for each procedure. The study was completed over the period of 1 month to minimize any effect of specimen storage. All

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TABLE 1. Description of stool specimens previously identified as positive or negative for the presence of *Cryptosporidium* oocysts

Characteristic	No. of stool specimens	
	Positive	Negative
Population		
Children[a]	52	22
Adults (immunocompromised)	1 (1)	30 (4)
Total	53	52
Stool description		
Normal color	40	45
Green color	10	3
Presence of mucus	6	0
Presence of pus cells	3	9
Parasites and other findings		
Ascaris lumbricoides	0	1
Chilomastix mesnilii	0	2
Clonorchis sinensis	0	1
Dientamoeba fragilis	1	6
Endolimax nana	1	7
Entamoeba coli	2	1
Entamoeba hartmanni	0	4
Entamoeba histolytica	0	2
Enteromonas hominis	0	2
Giardia lamblia	7	4
Hookworm	0	3
Iodamoeba butschlii	0	2
Other (fungal spores, yeasts, *Blastocystis hominis*)	0	8

[a] Children consisted of those subjects under 16 years of age.

slides were randomly numbered by a second technologist, to ensure blinding by the single technologist interpreting the slide. Examination of the slide and enumeration of the parasites were performed as described previously (38). Briefly, rare was less than 5 protozoa per coverslip, few was 6 to 25 protozoa per coverslip, moderate was 1 protozoon per low-power field, many was 1 protozoon per high-power field, and numerous was 1 or more protozoa per oil immersion field.

Positive control slides for *Cryptosporidium* oocysts were obtained from a patient with AIDS and a histologically confirmed infection on bowel biopsy, an SAF-preserved specimen from the Laboratory Proficiency Testing Program (specimen 8509-1), and a calf stool specimen preserved in 2.5% potassium dichromate (generously donated by P. Lautenslager, Veterinary Laboratory Services, Ministry of Agriculture and Food, University of Guelph, Guelph, Ontario, Canada).

Procedures. All procedures were performed as reported previously or as recommended by the manufacturer except, as described below, where significant modifications were done by the investigators. All specimens were processed by using the formalin-ether concentration (FEC) method of Ritchie (41). ZN acid-fast staining was performed as described by Henriksen and Pohlenz (22). The modification of Haddad (21) of the AR staining method of Truant et al. (45) was used. Briefly, the modification of Haddad (21) was as follows. One drop of FEC (approximately 30 μl) was placed on a clean microscope slide, air dried, fixed in absolute methanol for 30 s, and air dried. The preparation was stained in AR solution for 5 min, washed in cold running tap water for 1 min, decolorized in a 0.5% acid-alcohol bath for 30 s, washed in cold running tap water for 1 min, decolorized in two baths of 0.5% acid-alcohol for 30 s each, and then washed in two baths of absolute acetone for 30 s each. The preparation was then counterstained in 0.5% potassium permanganate for 10 s, washed in cold running tap water for 1 min, and air dried. The stained preparation was screened under low-power magnification and confirmed under high-power magnification by using a fluorescence microscope. The oocysts are orange-pink fluorescent round bodies. GS was done as described by Sherwood et al. (42). The IIF technique (Merifluor Cryptosporidium kit; Meridan Diagnostics) was performed as described by the manufacturer. The method of Zierdt (49) was used for the SSF technique. The MCSF procedure was adapted from the method of Remmler (28). Briefly, by using a 230-mm Pasteur pipette, 1 drop (approximately 30 μl) of sediment from the FEC was added to 200 μl of commercial corn syrup preparation (specific gravity, 1.35; Crown corn syrup; Best Foods Division, Canada Starch Co. Inc., Pt. Claire, Quebec, Canada). The specimen was mixed on a standard microscope slide, and a glass coverslip (24 by 40 mm) was placed over it to give a uniform thin viewing plane and also to protect the microscope lens from the corn syrup preparation. The entire coverslip was scanned at an initial magnification of ×125, and the result was confirmed at a magnification of ×500 by using a bright-field microscope. The 4- to 6-μm oocysts appeared as refractile, pink, round tori.

The cost calculations for each procedure included material and reagent costs and the cost of the technologist's time. No capital costs were included, although it should be noted that a fluorescence microscope was required for AR staining and IIF (approximate cost, $9,100.00). All values were calculated in 1988 Canadian dollars. The diagnostic yield of the procedure was compared with the yield by any other procedure alone or in combination.

Ease of handling and the ability to process large numbers of specimens were subjectively evaluated on the basis of normal procedures in a diagnostic laboratory performing parasitology examinations only and the ability to test batches of up to 25 specimens in the routine staining procedure.

The process of accepting one diagnostic procedure over another involves a complex decision analysis of several criteria related to those tests. The method of multiattribute evaluation that identifies, characterizes, and then combines these variables is needed to evaluate the true ranking of these diagnostic tests in any particular health care setting. Multiattribute utility theory (27) and the analytical hierarchy process (14) are two such methods. These processes have recently been applied to clinical medicine (7, 13). Following our evaluation, the four attributes were given a rank order from 1 to 6, with 1 being least desirable and 6 being most desirable. The application of multiattribute utility theory is discussed below.

RESULTS

The human and calf oocysts gave similar results (data not shown). The SAF, 2.5% potassium dichromate, or 10% formalin fixatives did not interfere with any of the staining or fluorescence procedures. The relative numbers of oocysts detected by the various procedures are given in Table 2. No single procedure detected all 53 specimens previously positive for *Cryptosporidium* spp. Eighty-one specimens gave results in agreement with those previously obtained in our laboratory (51 negative and 30 positive). There was discor-

TABLE 2. Enumeration of *Cryptosporidium* oocysts by procedure

Procedure	No. of specimens		No. of positive specimens				
	Total negative	Total positive	Rare	Few	Moderate	Many	Numerous
AR staining	54	51	18	8	3	10	12
SSF	58	47	16	8	8	8	7
IIF	60	45	23	6	5	5	6
ZN staining	64	41	13	8	9	7	4
MCSF	69	36	13	5	11	4	3
GS	70	35	10	6	5	6	8

dance in the results for 24 specimens, for which one or more procedures failed to give the expected previous result. The final number of specimens considered positive in the present study was 54. Of the 54 positive specimens, AR staining detected 51 specimens (diagnostic yield, 94.4%), SSF detected 47 specimens (87%), IIF detected 45 specimens (83.3%), ZN staining detected 41 specimens (75.9%), MCSF detected 36 specimens (66.7%), and GS detected 35 specimens (64.8%). The costs of performing each of the six procedures are given in Table 3.

The rank evaluation of diagnostic yield, cost, ease of handling, and ability to batch specimens is shown in Table 4.

DISCUSSION

The AR staining procedure gave the highest diagnostic yield and was rapid, but modestly expensive. It lent itself to the examination of large numbers of specimens with ease, and specimens could be batched for the procedure. The orange-pink fluorescence of the oocysts was easy to detect in the dark background, although if internal detail was not evident, another staining method would be needed to confirm the presence of oocysts. The disadvantages of this technique included its complexity, which required frequent quality control monitoring and technological expertise, and the fact that it is a nonspecific stain. In addition, the staining fluid is potentially carcinogenic (29), requiring processing in a fume hood and special handling for disposal. An added disadvantage is the need for a fluorescence microscope, an additional expense of about $9,100.00 for those laboratories not equipped with one.

Three of the positive specimens were found by the SSF technique only. Filtering through gauze may have allowed mucous strands with adherent *Cryptosporidium* oocysts to pass through more easily than with FEC (1, 6, 32, 40). Although the materials were inexpensive, it was technically exacting to prepare the SSF solution to the correct specific gravity. The procedure did not adapt well to the work flow in the laboratory and specimens could not be easily batched, which required reading of results within 15 min of preparation because the oocysts tended to collapse and disappear (1). The solution was difficult to work with because of its high viscosity. The preparations were more difficult to read because they had more debris than the FEC-prepared specimens. The oocysts did not appear as pink or refractile as in the MCSF technique. The cost comparison of this technique did not include FEC, but because FEC would normally be done to detect other parasites, the cost of FEC would still exist in most laboratories.

The IIF technique was the most expensive and lengthy of all the procedures, and special equipment was required to perform the test; this equipment included microdilutors, reciprocal shakers, and a fluorescence microscope. When scanning at a magnification of ×100, the apple green fluorescence of the oocysts was not easily differentiated from the yellow fluorescence of the background debris. Because the monoclonal antibody bound to the oocyst cell wall, internal detail could occasionally not be seen, and confirmation would be required only when these ghost cells were seen. Batches of specimens could easily be used for the procedure, with 13 specimens with controls being processed at a time. The FEC method may have been less efficient than other concentration methods (23) for the IIF technique. An advantage of the IIF technique is its high specificity (3).

The ZN staining technique, although lengthy, had fewer steps than some of the other staining methods and could easily be integrated into the work flow of a parasitology laboratory. It was moderately inexpensive, specimens could be used in batches, and the stained slide could be kept as a permanent record. Use of a cold acid-fast technique is preferred in our laboratory because of the use of highly volatile, extremely flammable liquids such as diethyl ether. Rare oocysts may have been more difficult to detect by the ZN staining technique than by the AR staining procedure, but the morphology was better with the ZN stain.

TABLE 3. Cost to perform each procedure

Procedure	Cost/test for materials ($)[a]	Cost of technologist time ($ [min])[b]	FEC cost ($)[a]	Total cost ($)[a]
IIF	2.57	1.56 (6)	3.83	7.96
GS	0.28	2.60 (10)	3.83	6.71
AR	0.89	1.30 (5)	3.83	6.02
ZN	0.75	1.30 (5)	3.83	5.88
SSF	0.84	3.90 (15)	0	4.74
MCSF	0.10	0.52 (2)	3.83	4.45

[a] Costs are in 1988 Canadian dollars.
[b] Values in parentheses indicate time to perform (in minutes).

TABLE 4. Overall ranking of diagnostic procedures

Procedure	Ranking for the following attribute:			
	Yield	Cost	Ease of handling	Ability to process large numbers of specimens
AR staining	6	3	3	4
SSF	5	5	2	1
IIF	4	1	1	3
ZN staining	3	4	5	5
MCSF	2	6	6	2
GS	1	2	4	6

TABLE 5. Comparison of combinations of two procedures for detection of *Cryptosporidium* spp.

Procedure combination	No. of specimens		Yield (%)
	Total positive	Total negative	
AR staining-SSF	54	51	100
AR staining-GS	52	53	96
AR staining-IIF	51	54	94
AR staining-ZN staining	51	54	94
AR staining-MCSF	51	54	94
SSF-IIF	52	53	96
SSF-ZN staining	49	56	91
SSF-GS	48	57	89
SSF-MCSF	47	58	87
IIF-GS	49	56	91
IIF-ZN staining	47	58	87
IIF-MCSF	47	58	87
ZN staining-GS	43	62	80
ZN staining-MCSF	41	64	76
MCSF-GS	39	66	72

The MCSF procedure provided a rapid method for detecting oocysts. It was very inexpensive and versatile, being prepared with formalin-ether concentrates from fresh or SAF-preserved stool specimens. The pink oocysts stood out very clearly, allowing the preparation to be read in 3 min or less. The 18 positive specimens missed by this technique contained rare oocysts only. No more than five specimens could be prepared at a time because the hypertonic solution burst the oocysts if it was left standing for longer than 15 min. The MCSF solution was considerably easier to use than the SSF solution.

GS was difficult to read and assess because of poor color contrast, as has been noted by other investigators (4, 39). The microscopic search for oocysts was tedious and required more technological judgment than the acid-fast procedures or sugar flotation methods (1, 2). Although detection was facilitated by use of a magnification of ×1,250, this required longer examination times. The entire procedure was expensive and time-consuming, but the stained slide did serve as a permanent record.

Because no single procedure was able to detect all 54 positive specimens, a combination of techniques would increase the diagnostic yield. The results obtained by using a combination of techniques (Table 5) permitted comparison of techniques to maximize diagnostic yield.

Each procedure was compared by using a linear ranking scale (1 being the least desirable characteristic, 6 being the most desirable characteristic) (Table 4). This approach assumes that each attribute is of equal importance and that within each attribute the ranking scores are proportionally related on a linear scale. These assumptions may not be true for each laboratory, and certainly could not be generalized between diagnostic laboratories. The multiattribute utility theory and the analytical hierarchy process can be applied to this type of decision-making in the laboratory. The application of multiattribute utility theory or the analytical hierarchy process to the linearly ranked attributes permits the individual laboratory to apply its own value, or utility, to these results.

Hence, the ranked results of this laboratory study for the diagnosis of cryptosporidiosis can be analyzed by using these techniques to determine the appropriate resource allocation for testing for cryptosporidiosis in each health care setting. With our results, a utility can be generated for each of the attributes evaluated. Specifically, how important are the attributes of diagnostic yield, cost of testing, ability to use batches of specimens, and ease of processing with respect to each other? By determining a weight for each attribute, the relative desirability of each diagnostic procedure can be assessed, and ultimately, the utility of that diagnostic procedure for the diagnosis of cryptosporidiosis can be determined.

For example, if each attribute were equally desirable the ranking of the tests would be as follows (from best to worse): ZN staining, AR staining and MCSF, SSF and GS, and IIF. If an assessment of the utility of each attribute found that the diagnostic yield was desirable (utility, 0.7) but less desirable than low cost (utility, 0.95) and that a value for ease of handling was assigned at 0.9, being more desirable than the ability to process large numbers of specimens (utility, 0.5), a new ranking of tests would be as follows (from best to worse): MCSF, ZN staining, AR staining, SSF, GS, and IIF.

The concept of evaluation in laboratory diagnosis has usually been limited to diagnostic yield and has occasionally been extended to cost-effectiveness (34, 47). Multiattribute assessment is a logical extension of this process of evaluation. This report has demonstrated how this approach to multiple-criteria decision-making can be applied to the parasitology laboratory diagnostic techniques available for *Cryptosporidium* spp.

As newer techniques become available for the laboratory diagnosis of cryptosporidiosis, including the currently available second-generation immunofluorescence tests and enzyme-linked immunoassays, comparative evaluation on multiple attributes of the tests will assist in the rational choice of laboratory procedures for the detection of *Cryptosporidium* oocysts.

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