Comparison of Stool Collection Techniques in Amebiasis Investigations

KATHLEEN HARPER, B.S., MAURICE D. LITTLE, B.S., and A. L. MARSHALL, JR., M.D.

OUTBREAKS of amebiasis occur unexpectedly and sporadically, and their investigation presents laboratory problems of considerable magnitude. The average diagnostic laboratory has a minimal staff of parasitologists and is unprepared to examine large numbers of stools. Furthermore, routine diagnostic procedures may not be suitable for use in field studies.

Since an investigation of amebiasis should include the detection and identification of both trophozoites and cysts of *Entamoeba histolytica*, freshly passed stools should be submitted to the laboratory only as rapidly as they can be examined. To do this may require setting up a laboratory near the area of investigation and arranging for the services of additional parasitologists.

In Indiana, the State board of health laboratories have been unprepared to provide all the services needed during outbreaks of amebic dysentery. Major difficulties have been insufficient time to make preparations for increased laboratory services, a parasitology staff already fully engaged in normal functions of the laboratory, coordination of the field investigation and laboratory programs so that specimens received at the laboratory while still fresh or adequately preserved would arrive no faster than examiners could handle them, and employment of efficient and feasible parasitological techniques.

In this study, an effort was made to establish a diagnostic procedure which would be readily applicable in epidemiological investigations of amebiasis and which would minimize the difficulties of laboratory participation.

Routinely, stool examinations are made on specimens submitted in polyvinyl (PVA) fixative two-bottle stool collection outfits (*1*, *2*). While these techniques were effective for diagnostic purposes, their performance was too complex for use in extensive investigations of amebic dysentery. The stain preservation technique (*3*) using merthiolate, iodine, and formalin (MIF) and the MIF concentration technique (*4*) showed promise as a survey tool for investigation of sporadic outbreaks of amebic dysentery.

Use of these two techniques in several small surveys for *E. histolytica* gave such favorable results that a comparative study was made of this procedure and of the PVA fixative two-bottle stool collection-examination method. The results of this comparison and the manner of application of the MIF and MIF concentration techniques in an emergency investigation of a possible outbreak of amebic dysentery are presented in this report.

Methods

Specimens were obtained from inmates of a State mental institution. One stool per patient was collected. For each patient, the attendants

Mrs. Harper and Dr. Marshall are respectively chief parasitologist, bureau of laboratories, and director, division of communicable disease control, Indiana State Board of Health, Indianapolis. Mr. Little, formerly with the Indiana State Board of Health, is a graduate student in the division of parasitology, department of tropical medicine and public health, Tulane University, New Orleans, La.

were provided with an empty bottle for an unpreserved portion of the stool, a bottle of prepared PVA fixative solution, and a bottle for the collection of the MIF-preserved stool. The attendants added an appropriate amount of each freshly passed stool to each bottle. Specimens were collected twice a week and promptly transported to the laboratory. Since unpreserved stools were included in this series, specimens were collected only as rapidly as the examiners could handle them. Altogether, 110 specimens of 3 stool portions each were obtained.

Collection Techniques

In the PVA fixative two-bottle collection method, one portion of each freshly passed stool, about the diameter of a quarter, was placed in an empty bottle. In another bottle, an equal quantity of feces was thoroughly mixed with the PVA fixative solution to approximate a mixture of 1 part feces and 2 parts preservative. This method (*2*) was used to prepare the first two portions of the fecal specimens, and an equal amount of each specimen was mixed with MIF solution in a third bottle.

The MIF stain preservative consists of a stable stock merthiolate formalin (MF) solution and Lugol's iodine solution. These solutions were prepared in quantities sufficient to complete the study, dispensed separately, and combined in aliquot proportions by the persons collecting the stools. The stock MF solution (*3*) was prepared by mixing 250 ml. of distilled water, 200 ml. of tincture merthiolate No. 99, 1 : 1,000 (Lilly), 25 ml. of solution formaldehyde U.S.P., and 5 ml. of glycerine, and dispensed at 14.1 ml. per collection bottle. The Lugol's stock 5 percent iodine solution was stored in the refrigerator in a brown glass bottle and dispensed as needed, 0.9 ml. per cork-stoppered 13×75 mm. test tube.

One bottle of MF solution and one test tube of Lugol's iodine solution were provided for each specimen to be submitted in MIF preservative. Immediately after passage of the stool, the attendant added one aliquot of iodine to one bottle of MF solution, then added the appropriate amount of feces, and mixed them thoroughly.

Examination Techniques

As the specimens arrived at the laboratory, each set of three stool portions was examined by the following techniques:

Technique 1. At least 1 saline and 1 Lugol's iodine wet mount were examined from different areas of each specimen of unpreserved stool; additional amounts were examined only when necessary to identify species.

Technique 2. Each unpreserved specimen was also examined by the zinc sulfate concentration technique described in an earlier report (*2*).

Technique 3. Routinely, only one fecal film from each PVA fixative preserved stool was permanently stained with iron hematoxylin. When necessary for identification of protozoa, 1 or

Table 1. Number of times protozoa were found in 110 stools and methods of detection

Protozoa	Combined methods	Unpreserved stools		PVA fixative stools	MIF stools	
		Direct wet mount	Zinc sulfate flotation	Hematoxylin stained smear	Direct wet mount	MIFC
Entamoeba histolytica	32	10	10	21	19	27
Entamoeba coli	76	44	58	43	55	70
Iodamoeba bütschlii	1	0	0	1	1	0
Endolimax nana	44	16	14	34	24	27
Giardia lamblia	5	5	2	4	4	4
Chilomastix mesnili	30	12	7	27	18	23
Trichomonas hominis	1	1	0	1	1	1
Unidentified protozoa	19	9	8	5	6	6
Total	208	97	99	136	128	158

2 additional smears were examined. This technique is also described in the earlier report (*2*). A fecal smear was prepared on a 75×25 mm. glass slide, dried overnight, and stained with a modification of the Tompkins-Miller rapid iron hematoxylin phosphotungstic acid method (*5*). If the protozoa could not be identified, additional smears were stained by the Heidenhain long iron hematoxylin method.

Technique 4. At least one direct wet mount from each MIF-preserved stool was examined. A drop of fecal suspension was placed on a glass slide and a coverslip was added. If the suspension was too thick for good visibility, the drop of material was mixed with a drop of saline. With a few exceptions, no additional staining was necessary, although an additional smear was occasionally prepared in a drop of Lugol's iodine or MIF stain formula for the direct smear technique in order to bring out the iodine phase or to enhance specific differential structures.

Technique 5. The merthiolate-iodine-formaldehyde concentration technique (MIFC) developed by Blagg and associates (*4*) for use with MIF-preserved stools was performed on all stools received in MIF. The steps in this procedure follow:

1. Shake the specimen vigorously for about 5 seconds. If mixture appears too viscid to strain easily through gauze, dilute with stock MF solution before shaking.
2. Strain mixture through two layers of wet gauze into a lipless conical paper cup and immediately pour 10 ml. into a 15-ml. graduated centrifuge tube.
3. Add 4 ml. of ether, stopper and shake vigorously. If ether remains on top, add 1 ml. of tap water and reshake.
4. Remove stopper and let stand 2 minutes.
5. Centrifuge for 1 minute at 1,600 r.p.m. Four distinct layers should appear: a top layer of ether, a plug of fecal detritus, an MIF layer, and a small amount of sediment.
6. Loosen the fecal plug by ringing with an applicator stick. Quickly pour out all but the bottom layer of sediment.
7. Mix the sediment and make a coverslip preparation. Slide the coverslip over the drop of sediment, so that coarse particles remain outside the periphery while the fluid and any parasites run underneath the coverslip.

All examinations were made by two parasitologists, with cross-checking for identification purposes.

Results

In the combined examinations of 110 stools, protozoa were found 208 times (table 1). *E. histolytica* was found 32 times and other protozoa 176 times. Ten pinworm infections were found; however, since this report is concerned with protozoa only, these data are not included. Thirteen *E. histolytica* organisms were of the small race type; 19 protozoa were not specifically identified by examination of a single specimen.

The relative efficiency of the techniques for all protozoa ranged from 46.6 percent for direct wet mounts of unpreserved stools to 76.0 percent for the MIF concentration technique; for *E. histolytica* only, from 31.2 to 84.4 percent, respectively. The second most efficient single technique was the PVA fixative hematoxylin stained fecal smear, which yielded 65.4 percent of the total protozoa and 65.6 percent of the *E. histolytica.*

Efficiency of Combinations of Methods

The diagnostic yields of combinations of techniques are shown in table 2. The triple combination of hematoxylin stained PVA fixative smears, direct wet mounts from MIF-preserved stools, and the MIF concentration technique yielded all 32 *E. histolytica* and missed only 3 of the 189 specifically identified protozoa. However, the hematoxylin stained fecal smears plus the MIF concentration technique missed only 1 *E. histolytica* (large race) and 4 other protozoa (1 *Entamoeba coli*, 2 *Endolimax nana*, 1 *Giardia lamblia*). Only 34.4 percent of the *E. histolytica* and 62.4 percent of the other protozoa species could have been reported from examinations of the unpreserved stools alone.

Tables 1 and 2 would seem to indicate that when only one technique can be employed, the MIF concentration method is preferable. However, if two techniques can be used, the combination of MIF concentration and hematoxylin stained PVA fixative fecal smears appears to be so efficient that the addition of any

Table 2. Number of times protozoa were found and identified by combinations of techniques

Technique	Total protozoa	*Entamoeba histolytica* race type			Other protozoa
		Large	Small	Both	
Saline and iodine direct wet mount [1]	109	11	0	11	98
Zinc sulfate flotation [1]					
Saline and iodine direct wet mount [1]	168	15	8	23	145
Zinc sulfate flotation [1]					
Hematoxylin stained fecal smear [2]					
Zinc sulfate flotation [1]	158	15	12	27	131
MIF concentration [3]					
Hematoxylin stained fecal smear [2]	184	18	13	31	153
MIF concentration [3]					
Direct wet mount [3]	162	16	12	28	134
MIF concentration [3]					
Hematoxylin stained fecal smear [2]	163	19	9	28	135
Direct wet mount [3]					
Hematoxylin stained fecal smear [2]	186	19	13	32	154
Direct wet mount [3]					
MIF concentration [3]					
All techniques	189	19	13	32	157

[1] Unpreserved stool.
[2] PVA fixative preserved stool.
[3] Merthiolate, iodine, and formalin preserved stool.

of the other techniques would be unnecessary. The MIF direct wet mount technique would appear to be of little additional value in detecting protozoa since the combined MIF direct wet mount and the MIF concentration technique did not significantly increase the number of protozoa found by the MIF concentration method alone.

Trophozoites and Cysts

Trophozoites and cysts when found were recorded for each specimen examined and for each technique employed (table 3). The MIF concentration and hematoxylin stained fecal smear techniques gave comparable results for *E. histolytica* trophozoites. The former appeared less efficient for other trophozoites. However, frequently when trophozoites alone were encountered in the stained fecal films, both cysts and trophozoites were found by the MIF concentration technique. This technique appeared highly efficient for cysts of *E. histolytica* and other protozoa.

In MIF-preserved stools, because of the clearing and staining qualities of the preservative, *E. histolytica* cysts were as easily identified, and, of course, more easily found, in concentrated specimens as in stained PVA fecal films. *E.*

Table 3. Number of times protozoan cysts and trophozoites were found and identified by each examination technique

Technique	All protozoa		*E. histolytica*		Other protozoa	
	Trophozoites	Cysts	Trophozoites	Cysts	Trophozoites	Cysts
Direct wet mount	4	84	0	10	4	74
Zinc sulfate flotation	1	91	0	10	1	81
Hematoxylin stained smear	90	76	14	14	76	62
MIF direct wet mount	51	90	8	14	43	76
MIF concentration	62	126	13	23	49	103
All techniques	101	153	18	27	83	126

histolytica trophozoites were easily identified in MIF-preserved stools, although perhaps not as easily as in the stained PVA fecal films. In preparations from MIF-preserved stools the nuclei and cytoplasm of *E. histolytica* cysts and trophozoites and the chromatoidal bodies of the cysts were clearly visible in the majority of organisms present in any one specimen; the small race *E. histolytica* was exceptionally easy to identify.

Trophozoites of other protozoan species, especially of flagellates, were usually easily identified. Occasionally, there was some hesitancy in specifically identifying the trophozoites of *E. nana* and *E. coli.* Although *Dientamoeba fragilis* was not encountered in this study, observations made with other groups of MIF-preserved stools have shown that *D. fragilis* trophozoites can be identified without too much difficulty after some experience with this species. On the other hand, the protozoan cysts rarely presented any difficulty of identification. Pseudopodia of ameba were frequently present in preparations made from the concentrated MIF specimens and were even more obvious in direct wet mounts made from the unconcentrated specimen. Protozoan cysts are most easily detected by scanning saline wet mounts made from unpreserved stools. However, after experience with wet mount preparations made from MIF-preserved specimens, the cysts are almost as easily found in the latter type of stool.

Application of MIF Technique

Results of the comparative study of methods and the use of the MIF stain preservative method of collecting stools in amebiasis investigations appear to justify planning for this type of stool collection.

The first opportunity to employ this technique in an emergency occurred during the summer of 1956. The diagnosis of three cases of amebiasis among the faculty of a college in northwestern Indiana resulted in a request from college officials for a sanitary survey. The Indiana State Board of Health received this request between the termination of summer school and the beginning of the fall semester. Engineers made a thorough inspection of all plumbing and sanitary installations and sanitarians inspected kitchens and food storage facilities. Although both reports were satisfactory, it seemed advisable to make examinations of stools from the resident faculty, food handlers, and other permanent employees and to take necessary remedial measures before arrival of the students in the fall. It was felt that at least 3 stools from each of the 125 persons involved should be examined.

Basic plans of procedure were devised in consultations among representatives of the college and of the State board of health. With the MIF stool collection technique the field investigators were able to obtain epidemiological data and to collect specimens independently of the laboratory program, thus reducing considerably the time between the request for an investigation and the receipt of specimens in the laboratory.

MIF collection kits were assembled as described under "Methods." To prevent absorption of iodine, the cork stoppers for the test tubes of iodine solution were coated with paraffin. Applicator sticks for adding and mixing the specimens and detailed instruction sheets for the use of investigators were included in the collection kits.

The investigators obtained the history of each person and supplied him with 3 collection kits, with instructions that a stool be collected every 3 or 4 days and that the 3 specimens be delivered together to the college dispensary. The first, second, and third stools were taken to the laboratory in separate cartons. Since approximately half the persons in the study were away on vacation, two collections were made, the second several weeks after the first.

In the laboratory, all specimens were numbered and recorded. Assembly line methods were used in preparing them for examination. During the processing, the first, second, and third stools from each person were kept in separate groups. Individuals from other laboratory units were assigned to the project for brief periods during each step of the processing.

The first specimens were concentrated by the MIF concentration technique, with the aid of one technician. The centrifuge tubes were tightly stoppered and stored in the refrigerator until the sediment could be examined by the parasitologists. Examination of approximately

half of these concentrates indicated that very few would be positive for *E. histolytica* and that all three stools from nearly every person in the study probably would need to be examined. The second and third specimens were then concentrated and stored in the refrigerator.

The assistance of a parasitologist from another institution was necessary to complete on schedule the examination of stools from persons who were on vacation during the first collection. Since all the specimens had been concentrated, schedules of the processing team and the parasitologists did not need to be coordinated.

Since previous studies had indicated that the MIF concentration technique alone was highly efficient, and since three stools were obtained from each individual in the study, no other laboratory technique was employed routinely. However, to check the efficiency of the MIF concentration technique, direct wet mount preparations from a representative number of the MIF-preserved stools were examined; no additional protozoa were found.

Critical reports were obtained on all but six persons. Because rare suspicious *E. histolytica* forms were found in the stools, additional specimens in MIF preservative were requested from three of these individuals. In order to confirm species identification, additional specimens were also obtained in PVA fixative for hematoxylin permanent staining from the other three individuals found to harbor trophozoites of protozoa other than *E. histolytica.*

Altogether, 368 specimens were obtained from 120 of the 125 persons in the study; 5 persons did not submit stools. Twenty-seven persons harbored the following protozoa species: 6 *E. histolytica* (4 small race type), 14 *E. coli*, 15 *E. nana*, 1 *D. fragilis*, 1 *Iodamoeba bütschlii*, 3 *G. lamblia*, 1 *Chilomastix mesnili*, and 1 *Trichomonas hominis.* The incidence of *E. histolytica* was considered to fall within the normal range.

Only 2 of the 6 persons whose specimens were positive for *E. histolytica* were kitchen employees. They were removed from their duties and intensive therapy was begun. In view of satisfactory reports from the engineers concerning the general environmental factors and of the low rate of infection among the staff, the school facilities were not considered to be the source of the infection. The college was advised that it would not be practical to examine the stools of all students. After a conference with the school authorities and the local health officer, the school physician planned to submit stool specimens of any student or member of the faculty who presented himself to the dispensary with symptoms suggestive of amebiasis. This program should insure early treatment of new cases and should alert the school and the health authorities when several cases occur simultaneously.

The investigation of this potential outbreak in a school indicates the need for a relatively simple procedure for collecting and examining stools for amebiasis to insure early attention to any outbreak and to encourage adequate surveillance. If the incidence of infection in this institution had been sufficient to cause concern, the rapid conclusion of the investigation would have permitted the initiation of remedial measures before the situation became alarming.

Discussion and Summary

Results of examining unpreserved stools collected in PVA fixative and in merthiolate, iodine, and formalin (MIF) stain preservative have indicated the value of the MIF preservative technique for collecting stools and of the MIF concentration method of examination for *Entamoeba histolytica* and other protozoa, as well as the high relative efficiency of the combined MIF concentration and hematoxylin stained PVA fixative fecal smear techniques for finding protozoa.

Experience with the identification of protozoa in saline and iodine wet mount preparations, in hematoxylin stained PVA fecal smears, and in MIF-preserved stools has shown that intestinal protozoa in MIF stain preservative usually are more easily identified than those encountered in wet mount preparations of unpreserved specimens, and in most instances are as readily identified as those found by examining hematoxylin stained PVA fixative stools. The iodine phase of staining disappears in MIF-preserved stools as the specimens age (*3*). This phase may be readily restored by making wet mount preparations with the MIF stain formula for direct wet mounts or with a plain iodine stain. How-

ever, as experience is gained with this technique there is usually less inclination to add additional stain except for an occasional differentiation of a specific structure.

The adaptation of the MIF stool collection method and MIF concentration examination techniques for use in the emergency investigation of a suspected outbreak of amebiasis demonstrates the flexibility and advantages of this procedure as a survey tool. In this and in other surveys, the procedure proved to be efficient in detecting and identifying protozoa, particularly *E. histolytica*. Many problems of collecting and transporting specimens to the laboratory were avoided, and cooperation in the use of this technique from field personnel and others was excellent. The method can be developed in a laboratory far in advance of its actual use and quickly put into operation when needed. Since each group of personnel can work independently of the others, difficulties arising from attempts to coordinate on short notice the activities of the field investigators, the persons to be examined, the laboratory processing crew, and the parasitologists are almost completely eliminated.

An additional advantage of the technique is that, before the specimen is submitted, each individual being examined preserves, fixes, and stains any parasites which he harbors. MIF-preserved specimens may be conveniently concentrated by the MIF concentration technique. The sediments, plus a small amount of MF solution, can be stored in the refrigerator and held for several months without appreciable difference in the ease with which the parasites can be identified. Specimens may be concentrated in one laboratory and the sediments transported to another for examination by parasitologists.

Since parasites usually remain identifiable after long storage in MIF preservative, a laboratory can build up a collection of specimens for training personnel in the identification of MIF-preserved parasites. A few weeks of training is usually adequate, and the personnel are available as examiners when needed.

In this study, almost 100 percent relative efficiency in detecting protozoa was obtained with the combined MIF concentration and hematoxylin stained PVA fixative fecal smear techniques. This combination would appear to be excellent for detecting *E. histolytica*. However, the more complicated PVA fixative hematoxylin staining method may make the combination impractical. If so, the single MIF concentration technique, which requires only one collection preservative, with repeat specimens obtained either in MIF or in PVA fixative, would appear to be far superior to the usual method of collecting unpreserved stools, especially since the MIF concentration technique alone was almost as efficient as the combined techniques for *E. histolytica*, which is the primary concern in amebic investigations.

Helminth eggs are also easily detected and identified by the MIF concentration technique. Blagg and associates (*4*) have reported results indicating that this technique is more efficient in recovering helminth eggs than the concentration methods usually employed.

Results of these investigations and of additional applications of the MIF stool collection technique in a number of smaller surveys indicate that this method is the most effective and practical parasitological survey tool for amebic examinations with which the authors have had experience.

A portion of the data from the comparison of methods has been included in an earlier report on the advantages of the PVA fixative two-bottle stool collection technique (*2*).

REFERENCES

(*1*) Brooke, M. M., and Goldman, M.: Polyvinyl alcohol-fixative as a preservative and adhesive for protozoa in dysenteric stools and other liquid materials. J. Lab. & Clin. Med. 34: 1554, November 1949.

(*2*) Harper, K., Little, M. D., and Damon, S. R.: Advantages of the PVA-fixative two-bottle stool collection technic in the detection and identification of intestinal parasites. Pub. Health Lab. 15: 96, July 1957.

(*3*) Sapero, J. J., and Lawless, D. K.: The "MIF" stain-preservation technic for the identification of intestinal protozoa. Am. J. Trop. Med. & Hyg. 2: 613, July 1953.

(*4*) Blagg, W., Schloegel, E. L., Mansour, N. S., and Khalaf, G. I.: A new concentration technic for the demonstration of protozoa and helminth eggs in feces. Am. J. Trop. Med. & Hyg. 4: 23, January 1955.

(*5*) Tompkins, V. N., and Miller, J. K.: Staining intestinal protozoa with iron-hematoxylin-phosphotungstic acid. Am. J. Clin. Path. 17: 755, September 1947.