Use of Pooled Sodium Acetate Acetic Acid Formalin-Preserved Fecal Specimens for the Detection of Intestinal Parasites

Maha R. Gaafar*

Parasitology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt

This study aimed at comparing detection of intestinal parasites from single unpreserved stool sample vs. sodium acetate acetic acid formalin (SAF)-preserved pooled samples, and stained with chlorazol black dye in routine practice. Unpreserved samples were collected from 120 patients and represented as Group I. Other three SAF-preserved samples were collected from the same patients over a 6-day period and represented as Groups IIa, IIb, and IIc. The latter groups were equally subdivided into two subgroups. The first subgroup of each of the three samples was examined individually, whereas the second subgroup of each were pooled and examined as a single specimen. All groups were examined

by the routine diagnostic techniques; however, in group II when the diagnosis was uncertain, the chlorazol black dye staining procedure was carried out. Results demonstrated that out of 74 patients who continued the study, 12 cases (16%) were positive in group I, compared with 29 (39%) in the subgroups examined individually, and 27 (36%) in the pooled subgroups. Therefore, pooling of preserved fecal samples is an efficient and economical procedure for the detection of parasites. Furthermore, the chlorazol black dye was simple and effective in detecting the nuclear details of different parasites. *J. Clin. Lab. Anal.* 25:217–222, 2011.

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INTRODUCTION

The purpose of parasite detection is not limited to curing disease in an infected individual, but it is crucial in the prevention and spread of diseases. For these reasons, detection methods must be held at the highest standard of both sensitivity and specificity, so that false negative and false-positive results are avoided (1).

Despite the development of various detection methodologies over the last several years, diagnosis of intestinal parasites in the parasitological laboratories still depends on microscopic examination of fresh, unpreserved stool specimens (2).

Stool specimens that are unformed or semi-formed are initially screened by direct wet smear for the detection of vegetative stages of protozoa, and subsequently examined for protozoan cysts and helminth eggs and larvae by iodine smear, ether sedimentation method, or a flotation concentration technique to detect any scanty parasitic infection. Unfortunately, intestinal parasites are intermittently shed in the stool as their excretion have cyclic basis; thus, examination of multiple stool samples are imperative (3,4). These samples could be examined either individually or as a pooled specimen.

Microscopic examination of multiple samples for each patient is a labor-intensive procedure and of great cost; thus, pooling of specimens can produce significant reduction in processing and cost (5).

For reliable detection of vegetative stages of protozoa, fresh stools should be examined directly after production or stools should be preserved in fixative; otherwise, morphological deterioration of vegetative stages occur. However, these aspects in routine practice are often neglected (2,6).

The most commonly used fixative is 10% formalin. Although being of long shelf life, it is known to rupture the more delicate protozoa and does not adequately preserve organism morphology for a good permanent stained smear. Furthermore, it is suspected to be a carcinogen, and thus a biohazard in itself (7,8).

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*Correspondence to: Maha R. Gaafar, Parasitology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt.
E-mail: drmahagaafar@yahoo.com

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In addition, other fixatives containing mercury, such as the polyvinyl alcohol (PVA), are not favored to be used. Their drawback is the mercury that represents their major component and considered a toxic product, and also the disposal of which is difficult and costly. Moreover, concentration methods (e.g., ether sedimentation) and immunochemical techniques (e.g., copro-antigen tests) cannot be performed on PVA-preserved specimens (3,9).

To overcome these problems, another fixative, such as sodium acetate acetic acid formalin (SAF), could be used. It is a nonmercury-containing fixative having little toxicity and it enables concentration with the ether sedimentation methods (10). In combination with SAF, the iron haematoxylin and the chlorazol black dye stains perform well (10,11). Chlorazol black dye is an acid dye used as a general tissue stain and also used for staining protozoa in fecal smears or in tissues. It is not only a stain, but also a fixative, and the technique of staining in contrast to that of the iron haematoxylin stain is fast and easy to be performed. However, until now, this stain has been used only rarely in routine practice (7,12,13).

This study aimed at comparing recovery of intestinal parasites from single fresh unpreserved stool sample vs. pooled SAF-preserved samples in one specimen and stained with chlorazol black dye in routine practice.

MATERIALS AND METHODS

After obtaining the approval of ethical committee and patient's informed consent, stool samples were collected from 120 patients suffering from gastrointestinal problems attending the outpatient clinic of the Main Alexandria University hospital from January 2010 to December 2010. The samples introduced by the patients upon attending the outpatient clinic were fresh and unpreserved. Then, the patients were instructed to collect other three fecal samples at 2 days interval over a 6-day period, and place each separately into a stool container given to them containing SAF. After 6 days, patients were asked to return the set of the three samples to the laboratory of Parasitology Department, Alexandria University. Patients were informed that, owing to their health and in order to reach the true diagnosis, they have to collect the samples at the precise time and returned them back to the laboratories.

These samples were divided as follows:

Group I: Includes the single unpreserved fresh specimens.

Group II: Includes the three SAF-preserved samples (IIa, IIb, and IIc), which were divided according to the time of collection (the 2nd, the 4th, and the 6th day). These groups were further subdivided into two subgroups (1 and 2). Subgroups IIa1, IIb1, and IIc1 of each

sample were examined individually and the total number of the discovered parasites were calculated and represented as Subgroup IId, whereas Subgroups IIa2, IIb2, and IIc2 were pooled and examined as a single specimen (Subgroup IIE).

This pooled specimen was prepared by pouring 3 ml from each individual SAF-preserved sample into a pooled specimen vial (9 ml total) (5).

Fecal samples of all groups were screened microscopically by the conventional diagnostic method by direct wet saline smear, iodine smear, and ether sedimentation technique, and examined at low magnification ($\times 100$) to locate helminth eggs and larvae and large protozoan cysts; species identification was made at higher magnification ($\times 400$). Then, a portion of each smear was examined for 10 min under oil immersion ($\times 1,000$) for the diagnosis of small protozoans (6,7,12).

When the processed diagnostic methods for samples of Group II were positive and the parasites could be identified directly or were negative, the samples were not further examined. However, when the diagnosis is uncertain or the parasite was not clear, a chlorazol black dye permanent stain was prepared according to the directions of Gleason and Healy (12), Bullock (13), and Van Gool et al. (6). In short, thin smears of SAF-preserved stool were made. After drying, the smears were placed in a container with chlorazol black dye and stained for 2–4 hr. After staining, the slides were dehydrated in 70, 95, and 100% ethanol for 1, 1, and 5 min, respectively, mounted and covered with coverslip, and left to dry in an incubator at 37°C. This study was approved by the Ethics Committee of Alexandria University.

Comparisons were made between Groups I and II as regards the ability of detection of different parasites, the number of positive samples, and the types of the parasites detected.

Statistical Analysis

The number and percentage of positive samples from each group were carried out, and the sensitivity ($\text{true positive}/(\text{true positive} + \text{false negative}) \times 100$) and the specificity ($\text{true negative}/(\text{true negative} + \text{false positive}) \times 100$) of the tested pooled preserved technique for the samples were calculated. The comparison of results obtained by all groups was determined by the percentage of agreement, which is calculated by the number of samples with positive and negative agreement/total number of samples $\times 100$ (14).

RESULTS

From 120 patients, 74 were cooperative and returned back the set of the three preserved stool samples after 6 days. Twelve cases (16%) were positive for one or

TABLE 1. Percentage of Positive Samples and Types of the Detected Parasites Among All Groups

Parasites	Group I	Group II				Subgroup IIe
		IIa1	IIa2	IIa3	Total (Sgp IIId)	
<i>Protozoa</i>						
<i>Giardia</i>						
Troph.	0	1	0	0	1	1
Cyst	3	1	2	3	6	6
Troph and cyst	0	0	0	1	1	1
<i>E. coli</i>						
Troph.	0	0	1	0	1	1
Cyst	1	1	0	1	2	2
Troph and cyst	0	0	0	0	0	0
<i>Blastocystis hominis</i>						
Vacuolated form (only)	0	0	1	0	1	1
<i>Blastocystis hominis</i> and <i>Giardia</i> cyst	2	0	2	3	5	3
<i>Blastocystis hominis</i> and <i>E. coli</i>	0	1	0	0	1	1
<i>Iod. butsch</i>						
Troph.	0	0	1	0	1	1
Cyst	2	1	0	1	2	2
Troph and cyst	0	0	0	0	0	0
<i>Iod. butsch</i> cyst and <i>Blastocystis hominis</i>	1	1	1	0	2	2
<i>Iod. butsch</i> and <i>Blastocystis hominis</i> and <i>Giardia</i> cyst	0	1	0	1	2	2
<i>D. fragilis</i>						
Troph.	0	0	0	1	1	1
<i>Helminth</i>						
<i>Fasciola</i> egg	1	0	1	0	1	1
<i>Ascaris</i> egg	1	0	1	0	1	1
<i>Schistosoma</i> egg	1	1	0	0	1	1
Total number of positive samples	12				29	27
Percentage of positive samples	16%				39%	36%

more intestinal parasites in Group I (single fresh unpreserved stool samples), compared with 29 cases (39%) positivity in Subgroup IIId (total number of recovered parasites in multiple SAF-preserved stool samples examined individually of Group II) and 27 cases (36%) positivity in Subgroup IIe (pooled SAF-preserved samples of Group II) (Table 1).

Giardia lamblia, *Entamoeba coli*, *Blastocystis hominis*, *Iodamoeba butschlii*, *Dientamoeba fragilis* were observed more frequently in Group II than in Group I. On the other hand, eggs of *Fasciola*, *Ascaris lumbricoides*, and *Schistosoma* were recovered at similar frequencies in both groups. Thus, helminth infection could be diagnosed easily by the conventional diagnostic method in all groups.

The vegetative forms of protozoa were only demonstrated in Group II. The most commonly discovered parasite was *B. hominis*, and it was detected either alone or associated with other parasitic infections and its detected number was significantly higher by the improved technique.

Intermittent shedding of parasites was clearly demonstrated in the individually examined stool samples of Group II (IIa1, IIb1, and IIc1), as the parasites were detected in only one or two of the three samples.

The pooled samples (Subgroup IIe) were negative in only two cases in which the total of the individual specimens (Subgroup IIId) was positive. The detected organisms were *G. lamblia* and *B. hominis*. The sensitivity and the specificity of the pooled preserved technique in the examination of the samples were 97 and 100%, respectively.

The percentage of agreement was determined between Group I and Subgroup IIId, between Group I and Subgroup IIe, and between Subgroup IIId and Subgroup IIe, and was calculated to be 74, 77, and 97%, respectively (Table 2).

Figures 1–4 show some of the detected parasites stained by chlorazol black dye stain. This stain resulted in clear visibility of the different intestinal parasites and excellent detection of their nuclear details.

DISCUSSION

The widely used microscopy is a very specific but less sensitive method for the laboratory detection of several parasites in feces. The routine conventional diagnostic methods usually result in false-negative findings, especially in protozoal infections (15).

TABLE 2. Agreement Between the Results of the Different Groups in Examination of Fecal Samples

Relation between the results of Group I and Subgroup II d	Number of samples	Relation between the results of Group I and Subgroup II e	Number of samples	Relation between the results of Subgroup II d and Subgroup II e	Number of samples
Agreement		Agreement		Agreement	
Positive/positive	12	Positive/positive	12	Positive/positive	27
Negative/negative	43	Negative/negative	45	Negative/negative	45
Disagreement		Disagreement		Disagreement	
Positive/negative	0	Positive/negative	0	Positive/negative	2
Negative/positive	19	Negative/positive	17	Negative/positive	0
Total number of samples	74	Total number of samples	74	Total number of samples	74
Agreement %	74%	Agreement %	77%	Agreement %	97%

Fig. 1. Stool sample showing oval cysts of *Giardia lamblia* with axostyle and nuclei (Chlorazol black dye $\times 1,000$). **Fig. 2.** Stool sample showing pear-shaped trophozoites of *Giardia lamblia* and *Giardia* cyst. The trophozoite has two large nuclei and axostyle (Chlorazol black dye $\times 1,000$).

This study demonstrated that the use of SAF-preserved stool samples examined individually or pooled followed by permanent staining in a routine clinical setting strongly improved the detection of intestinal parasites, when compared with the conventional method of examination of unpreserved stool specimens after ether sedimentation. Also, the vegetative form of

Fig. 3. Stool sample showing cyst of *Entamoeba coli* having five nuclei with eccentric karyosome (Chlorazol black dye $\times 1,000$).

protozoa is discovered only in the improved technique. This was approved by Van Gool et al. (6) and Shetty and Prabhu (16), who suggested that SAF fecal preservation of multiple stool samples and subsequent staining was superior to wet mount examination for the diagnosis of intestinal parasites and for the detection of the trophozoite stages of protozoa, and it avoided the need for fresh specimens.

Intermittent shedding of parasites was clearly demonstrated in the various stool samples of Subgroups IIa1, IIb1, and IIc1, and the number of the detected protozoa was significantly differed between Group I and Group II. This was adopted with the work of Van Gool et al. (6), who stressed on the need for multiple samplings to ensure accurate diagnosis of protozoal infection, because their shedding is intermittent in stool samples. In addition, Sawitz and Faust (17) showed that the rate of recovery of *Entamoeba histolytica* was under 50% if only one stool specimen was examined. However, if six specimens were examined, the rate was more than 90%. Moreover, Heymans et al. (18) supposed that parasitological examination of multiple stool specimens on

Fig. 4. Stool sample showing *Dientamoeba fragilis* with two fragmented nuclei (Chlorazol black dye $\times 1,000$).

nonconsecutive days showed a higher sensitivity with very low false-negative rate.

Examination of preserved specimens increases the rate of diagnosis of parasitic infections (19). In this study, the use of SAF fixative as a preservative for the specimen proved its effectiveness, in addition to its safety depending on its contents of nonmercury compounds. Mank et al. (10) supposed that examination of SAF-preserved stool specimens increases the chance of recovering intestinal protozoa as compared with the conventional procedure. Furthermore, Giarcia and Shimizu (20) stated that as a result of disposal problems related to the use of mercury compounds, many laboratories have switched to the nonmercury-based preservatives.

In this work, we compared recovery of intestinal parasites from pooled specimen with recovery from three individual samples. The pooled samples were negative in two cases infected with both *G. lamblia* and *B. hominis*. This could be a false-negative result for the pooled samples. However, there was a statistically significant agreement (97%) between the results of the examination of the samples by both techniques. Furthermore, the sensitivity and the specificity of pooling technique were determined to be 97 and 100%, respectively. This coincided with Aldeen et al.'s work (5). Therefore, pooling was proved to be effective in detecting parasites for which intermittent shedding is

common and not associated with labor-intensive procedure, as only one concentration is done for the three specimens and the parasitologist evaluates this single concentration only. This was approved by Aldeen et al. (5) and Wahlquist et al. (21) who stated that pooling of individual specimens is an efficient and cost-effective procedure for screening of ova and parasites, and it is useful in laboratories when multiple specimens are received at one time.

Several stains can be used in combination with SAF fixative, such as iron haematoxylin stain, chlorazol black dye, and modified Ziehl-Neelsen stain (12,19). Iron hematoxylin staining is quite stable and can sharply stain fine structures, and thus used in the identification of intestinal protozoa. However, it is often thought to be too complex and time consuming to be used in routine fecal examinations (22). On the other hand, chlorazol black dye staining procedure used in this study was simple, rapid, stable, and effective in detecting the fine details of the nuclei of different parasites. These were supported by Van Gool et al. and Bullock (6,13). Baker (23) advised the use of chlorazol black dye as a stain for general purposes in ordinary microscopic technique, owing to its possessing three azo linkages instead of the two linkages present in most of the dyes. Thus, it is more useful than other dyes. Also, Gleason et al. (24) supposed that the chlorazol black staining technique for fecal films was found to be a simple, efficient