Macroscopic and Microscopic Methods in the Diagnosis of Intestinal Bilharziasis

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INTRODUCTION

Improvements in the methods of diagnosis of intestinal bilharziasis by the examination of stool samples are urgently needed because those in common use are not sufficiently reliable. The problem is aggravated because laboratory diagnosis tends to be divorced from any consideration of the needs of epidemiological workers in the field. Only in a research organisation which is equally interested in the field and laboratory studies does it become obvious that present methods are not as good as they might be. Sound epidemiological knowledge must be based on sound stool parasitology, and this can only be attained when field workers and the laboratory are at one in their desire to get the most information, and accurate information, from the material collected in the field.

In some parts of the world where *Schistosoma mansoni* infections are very common and intense, the tendency is to rely entirely on a simple direct smear preferably made from a portion of stool containing blood and mucus. This simple method will no doubt detect the majority of cases with a heavy worm load. However, in trying to assess the epidemiological patterns of *S. mansoni* bilharziasis it is necessary to determine the general pattern of infection in all age groups of the population and to attempt to assess the relative intensity of infection in all these groups. It is desirable, also, to show by hatching techniques that the eggs are viable and thus that the infection is active.

Fulleborn (1921) appears to have been the first worker to advocate the hatching of miracidia from eggs to demonstrate their viability. The joint OIHP-WHO Study Group on Bilharziasis in Africa (1950) refers to Fulleborn's methods as a technique for demonstrating viability of infection. The First Report of the Expert Committee on Bilharziasis (1953) took the matter further and recommended the adoption of hatching techniques on both urine and stool specimens. However, the importance of these techniques was later overlooked and the Report of the Scientific Group on the Measurement of the Public Health Importance of Bilharziasis (1967), although it included an annexure on procedures for the examination of excreta, makes no mention of hatching of miracidia in the diagnosis of bilharziasis. Many of the recommended methods and techniques in the report are so complex and idealistic that they are unsuitable for use in the field in the majority of endemic countries. In fact, their very complexity has discouraged their use, particularly by field workers isolated by distance from hospitals and research institutions of the larger cities. This same trend has influenced the evaluation of drug trials.

The excellent oogram techniques (Periera and Barretto, 1949) are extremely valuable in preliminary evaluation of drug activity, but it is not suitable for large-scale trials of drugs, particularly attempts at mass treatment. Despite this, simple direct examination of faeces is often ignored in the "protocols" of drug manufacturers and investigators. If the direct microscopic examination of the excreta is augmented by hatching techniques, valid information on the viability of infection may be obtained. To do this with any degree of accuracy it is necessary to consider the employment of egg concentration methods in order that even mild infections with few eggs may be detected.

This laboratory has been studying refinements in stool preparation and examination for many years, but particularly over the past five years. This has included all aspects of the problem—the collection of the specimen from the patient, the containers used, the handling procedures and the establishment of a diagnosis by both hatching techniques and by microscopic observation.

Earlier Work in Rhodesia on S. mansoni

Eggs in Stool

Bennie (1948; unpublished data) studied the effects of storage and temperature on stool specimens and on the hatching of miracidia from them. Whole stool specimens were exposed to direct sunlight and samples were taken from these each day, both in the morning and afternoon. Each sample was processed by a sedimentation technique; a portion of this was examined microscopically and the remainder diluted with filtered pond water to initiate hatching of miracidia. She found that eggs could be identified five days after such exposure despite the fact that the work was done at the hottest season of the year. Placing of the stool on a bed of damp soil in the jar extended this period. Although on some occa-

sions she demonstrated hatching after exposure lasting as long as six days, in general prolonged exposure tended to decrease the extent of hatching.

Purvis (1964; unpublished data) attempted to determine where, in a formed stool, eggs of *S. mansoni* were to be found. In his experiments stool was passed by the subject directly into a metal dish and frozen by a jet from an ordinary cylinder of compressed gas to facilitate the taking of core samples from a formed stool. Samples were then taken from the leading tip, the tailing tip, the surface and from the core. These samples were allowed to thaw and were processed by a sedimentation technique. The results of this small-scale investigation were not conclusive, but they indicated that most eggs were found in the samples from the two ends and the surface of the stool, with a few eggs seen in the core samples.

Methods and Techniques in Current Use

The equipment and routine now employed permit the survey of a group of up to 500 persons, obtaining from each a specimen of urine and of stool for examination. This equipment is standardised so that the staff taking part know their respective roles. Each patient is first identified with a serial number and a tag of paper bearing this number is stapled to his clothing. This number tag is retained on the patient until all sections of the survey have been completed. Each patient's serial number, name, sex, estimated age and any other required information are recorded. It has been found useful in the field to process patients in groups of 20, using a colour code for the identification of each group. These colours are used on tags, bottles, etc., for each group. The group sits together under a flag of the appropriate colour awaiting the next stage in the investigation, and this allows excellent control of the subjects.

Each 6 fl. oz. bottle used for the collection of stool specimens contains about 40 ml. of sodium chloride solution (double strength physiologically normal saline 18 g./l.). Each group of 20 subjects is called up in turn, shown the bottle and a small flat wooden spatula (6.5 x 2.5 cm.) and a demonstration and explanation is given as to how to unscrew the lid, pick up a portion of faeces, put the spatula with its portion of stool into the bottle and re-apply the lid.

It is much easier and more comfortable for the majority of population groups being surveyed if they are able and free to pass their stool on to the ground at a site selected by themselves rather than to have to line up at latrines. When the group has completed this stage of the operation they line up in order and hand in the stool specimen bottle. As the containers are handed in, the security of the lid and the presence of a portion of stool in the bottle are checked and the bottle given a vigorous shaking before being replaced in the carrying case, which holds 100 bottles. The patients are told to put the spatula *and* the stool portion into the liquid in the bottle and the spatula assists in the breaking up of the faeces in the fluid. The hypertonic saline fulfils two roles: it enables the stool specimen to be thoroughly fragmented, releasing schistosome eggs from the solid stool matrix, and the hypertonicity prevents premature hatching of miracidia from these eggs.

The commonly advocated method of sedimentation and centrifugation of the stool specimens is employed. However, all dilutions and washings are carried out with physiological saline (9 g. Na Cl/litre) to prevent premature hatching of miracidia. The steps in the preparation of a stool specimen are:

1. The contents of the stool specimen bottle are thoroughly shaken and poured through a strainer of copper wire gauze (pore size of 0.15 sq. mm.) into a conical urine flask.
2. The stool bottle is thoroughly rinsed with saline and the contents also poured through the strainer.
3. The material held up on the strainer is washed thoroughly with saline from a plastic squeeze bottle, the washings also passing into the conical flask.
4. Sufficient normal saline is added to fill the flask and the suspension is allowed to sediment for at least half an hour.
5. The cloudy supernatant liquid is removed with a water suction pump.
6. The sediment is re-suspended in saline and allowed to sediment for a further 30 minutes. If the supernatant is still turbid, the sedimentation process can be repeated a third time.
7. The supernatant is removed with a water pump to leave 15 ml. in the flask.
8. The conical flask is agitated in a circular manner and the contents poured into a centrifuge tube.
9. The centrifuge tube is centrifuged at 1,000 r.p.m. for 90 seconds.
10. The supernatant is removed with a water suction pump to leave 0.5 ml. in the bottom of the tube.

After thorough agitation of the tube by flicking it with the fingers, a drop of this suspension is put on a microscope slide, covered with a cover slip and the whole preparation examined by a microscope using a low power (X4) scanning lens. A rough assessment of the density of eggs

is made on a one plus to five plus notation as follows:

\+ being less than 5 eggs of *S. mansoni* counted.
2 + up to 20 eggs.
3 + eggs in every traverse of the cover slip preparation.
4 + some eggs in every field.
5 + many eggs in every field.

Experimental Results

Estimation of Egg Numbers in Stored Stool Specimens

An African male, Foster Mavida, aged 23 years, was found to have a double infection of *S. haematobium* and *S. mansoni*, and he volunteered to participate in a trial. He gave a history of two years of intermittent mild pain in the right iliac fossa and he had noted blood in his urine about a year before. He was of medium build and his weight was 126 lb.

The subject was able to produce a stool specimen between 0800 and 0830 hours each morning. This he passed into a plastic container 25 x 18 x 8 cm. in size. When the investigation began early in November, 1967, the specimen was generally unformed; despite this, however, he did not suffer from diarrhoea and very rarely passed more than two stools in 24 hours. In the three-month period November, 1967, to January, 1968, a formed and solid stool was passed by him on only six occasions, invariably on Mondays. The reason for this tendency for the firming up of stool on Monday mornings was not apparent, but could be attributed to increased exercise and the drinking of large quantities of millet beer over the weekend.

In mid-February, 1968, quite suddenly he ceased to pass unformed stools and instead produced soft, but formed, stools. He received treatment with Hycanthone (Etrenol (R) Winthrop) intramuscularly in June, 1968.

From November, 1967, stool specimens have been examined on almost every working day. Portions of each stool were taken and transferred to a series of nine bottles each containing about 35 ml. hypertonic saline (18 gm. Na Cl/litre). An effort was made to take the same amount of stool (about 2 g.) into each bottle. This amount was gauged by eye and no attempt was made in this series of experiments to weigh the quantity of stool. Each bottle was agitated thoroughly to ensure even emulsification of the sample.

One bottle was processed immediately for microscopic examination; four were placed in a cold room (2° to 5° C) and the remaining four left on the laboratory bench, where the temperature range was 20° to 27° C. On each subsequent working day one stool sample from the cold room and one from the bench were processed and examined. A stool passed on Monday morning would therefore have one bottle processed at once and two—one from cold room and one from the laboratory bench—examined on Tuesday, Wednesday, Thursday and Friday. The stool passed on Tuesday would be treated in the same way except that the last two bottles of the series would be processed only on the Monday of the following week. Due to the interruptions of other work and of holiday periods, specimens were on occasion kept over for longer periods—up to 15 days.

An egg count was done on 0.05 ml. of the

Table 1
Average S. mansoni Egg Counts After Storage of Portions of Stool at Room Temperature and in a Cold Room

No. of Days After Passing of Stool	At Room Temperature 20-27° C.		Cold Room (2°-5° C.)	
	No. of Observations	Average Eggs in Sample	No. of Observations	Average Eggs in Sample
Fresh specimens	57	161	—	—
1	42	167	42	198
2	32	169	32	172
3	30	172	30	171
4	28	153	28	190
5	28	139	28	160
6	35	113	35	125
7	13	139	13	162
Total days: 1-7	208	156	208	171
Total days: 8-15	10	172	10	235

0.5 ml. of deposit in the centrifuge tube and to the remaining 0.45 ml. of the deposit, filtered pond water (previously treated to kill infusoria) was added to initiate hatching of miracidia. It will be noted that in this work schistosome egg counts replaced the previously described method of egg estimation used in epidemiological surveys.

Table I sets out the average number of *S. mansoni* eggs estimated in the total sedimented deposit. It will be seen that the average numbers of eggs in samples stored for one, two and three days at room temperature are slightly greater than the average numbers in fresh samples processed immediately on the day the stool was passed. This difference in egg counts between fresh and stored samples is even more marked in those samples kept in a cold room where the average egg count maintains a high level for five days and probably even longer. The number of observations on stool samples stored for more than eight days is insufficient to draw firm conclusions, but even after these long periods of storage—on occasions as long as three weeks—it was demonstrated that viable *S. mansoni* eggs could be recovered. There was even evidence that better egg recovery was possible in specimens stored for one to three days. It is possible that the stool, when stored, disintegrates in the saline and schistosome eggs are freed from the particles of faecal material and are not held up in fragments of faeces on the sieve.

To assess the extent to which eggs are trapped when fresh stool is processed, on a number of occasions the material held up on a sieve was washed into a glass specimen jar, using a jet of saline to clean the material into the bottle. The bottle was thoroughly shaken and left at room temperature for 24 hours, and then processed by sedimentation in the usual manner. Here again the material which was retained on the sieve was washed back into another bottle, shaken vigorously and then submitted to sedimentation *without* further sieving. The results showed that eggs could be recovered in numbers from the stored sieved sediment, but the material held up on the second sieving rarely contained eggs.

Hatching of Miracidia from Stored Stool Samples

To the remaining sediment of each sample of stool on which egg counts were made, filtered treated pond water was added. This filtered water was treated by heating to 56° C. in a hot air oven to kill infusoria which might be mistaken for free swimming miracidia. The tubes were then placed in the miracidia hatching racks (Meeser *et al.*, 1948a, 1948b) and examined with a hand lens. They were examined three times daily after the racks and tubes had been placed in sunlight for 15 minutes just before the time of examination. On overcast days the racks were placed in a cupboard into the roof of which had been placed two high wattage electric lamps to provide light and warmth. The tubes were retained for examination for as long as possible, sometimes as long as seven days after the addition of the hatching water. Each morning the tubes were agitated by flicking the tips with the fingers to resuspend the sediment and, when necessary, particularly in hot weather, additional pond water added to keep the meniscus of the fluid just at

Table II

MIRACIDIA HATCHING FROM STOOLS: PROPORTION OF SPECIMENS SHOWING HATCHED MIRACIDIA AT NOON

(Expressed as a percentage)

	Time Elapsed After Excretion (in days)	No. of Observations	1st Day (day of adding hatching water)	2nd Day	3rd Day	4th Day	5th Day and Subsequent Days
Fresh specimen	0	57	54	91	69	34	6
Stored at room temperature	1	42	21	86	67	40	13
	2	32	71	89	27	12	42
	3	30	71	89	70	18	2
	4	28	40	95	59	18	2
	5-8		50	86	52	8	1
Stored in cold room	1	42	50	86	67	37	9
	2	32	62	84	58	36	6
	3	30	33	100	60	50	5
	4	28	40	95	50	44	10
	5-8		24	75	70	18	6

the top of the field of vision in the miracidia hatching rack.

The results of observations on miracidial hatching from fresh and stored stool samples are shown in Table II. The table refers to observations taken at or near noon each day. In practice, specimens were examined at 1000 hours, noon and 1500 hours. The noon figures were generally the highest, except on the day of processing and adding of water (Table III), where it was greatest at 1500 hours. The probable cause of this discrepancy was the short period which was allowed for hatching, since it was seldom possible to add the water before 1000 hours. These results demonstrate that miracidia may be hatched, without difficulty, from stool specimens which have been retained, either at room temperature or stored in a cold room, for several days. The results also show that not all the miracidia hatch within the first few hours after the addition of treated pond water, some only becoming apparent six to seven days after the addition of the water. Since it has been shown by many authors (e.g., Shiff, 1969, in press) that miracidia will not live for this length of time after hatching, it must be assumed that actual hatching from some eggs was delayed. This is contrary to observations on hatching of miracidia from viable eggs of *S. haematobium* which all tend to hatch within two hours after the addition of the treated pond water. In the current observations some miracidia hatched two days after the addition of water to processed samples of stool which had been stored for 13 days, i.e., hatching occurred 15 days after the stool was passed.

It can be seen that hatching was most efficient on the second day after addition of water, whether from fresh stool samples or from samples stored either at room temperature or in a cold room. There were good hatches also on the third day, but after this time there was a marked decrease in the extent of hatching; storage of stools for

Table III

COMPARISON OF EXAMINATIONS OF STOOLS FOR MIRACIDIA AT NOON AND 1500 HOURS ON THE DAY OF PROCESSING

Hatchings expressed as number per cent. of specimens showing hatched miracidia

	Noon	1500 Hrs.
Fresh specimen	54	79
Room temperature—		
Stored for one day	21	75
,, ,, two days	77	77
,, ,, three days	71	78
,, ,, four days	40	78
,, ,, 5-8 days	50	70
Cold Room—		
Stored for one day	50	75
,, ,, two days	62	74
,, ,, three days	33	71
,, ,, four days	40	58
,, ,, 5-8 days	24	40

Observed difference in means three times standard error.

Table IV

S. MANSONI EGGS OBSERVED IN SAMPLES TAKEN OF TIP AND TAIL AND FROM A SCRAPING OF FORMED STOOLS

Stool Specimen No.	Tip		Tail		Scrape of Surface	
	Weight of Sample*	Estimated Eggs/Gram	Weight of Sample*	Estimated Eggs/Gram	Weight of Sample*	Estimated Eggs/Gram
1	1.78	131	1.54	149	0.23	261
2	1.87	208	1.25	344	1.18	322
3	1.35	533	1.64	262	0.96	469
4	1.44	132	1.02	108	0.87	149
5	2.97	118	1.17	273	0.51	451
6	1.24	161	0.79	240	0.60	383
7	2.15	219	1.56	256	1.06	490
8	0.41	292	0.88	148	0.23	261
9	1.13	97	1.25	96	0.90	200
10	0.81	259	1.17	154	0.48	292
11	1.34	261	0.92	456	0.76	487
12	1.52	72	0.97	371	0.23	1,348
13	1.69	296	0.98	235	0.83	265

* Weight of sample in grams.

three days had little effect on hatching, but after this period there was a general decline in the number of miracidia seen.

The Location of S. mansoni *Eggs in a Formed Stool*

Experience over several years, supported by the observations of Purvis (*op. cit.*), has indicated that *S. mansoni* eggs are more concentrated at the leading tip, at the tail and in the surface layer of a formed stool. Further evidence of this was obtained from this series of observations.

As has already been stated, the subject passed formed stools only rarely prior to February, 1968. When formed stools were produced, samples from the leading tip, the tail and a scraping of the surface were taken. It is current practice for a patient to produce a large portion of stool for examination. Since such a portion is liable to have a small proportion of surface layer in relation to bulk, it may be preferable, in fact, to examine only a very small portion of stool which is scraped from the surface or taken from the extreme tip or tail of the formed stool.

In a comparison of eight samples from the extreme ends of the formed stool, containing a high proportion of surface to bulk, with another eight larger samples, also from the tip and tail, but containing a smaller proportion of surface layer to bulk, a greater egg recovery in relation to weight occurred in the smaller samples. These smaller samples ranged in weight from 0.4 to 2.6 g. with a total weight of 14.8 g. The estimated total egg recovery was 2,710 or 183 eggs/g. The larger samples ranged from 2.8 g. to 9.8 g. with a total weight of 38.1 g. The estimated total egg recovery was 1,980 or 52 eggs/g. The difference was accepted as significant since the observed difference in means was twice that of the standard error of difference.

Since taking either the tip or the tail of a formed stool will include part of the sub-surface bulk of the stool, whereas a careful scrape of the surface layer will include only that layer, a comparison was made of such samples from 13 formed stool specimens. The results are shown in Table IV. These confirm a significantly larger number of eggs per gram in the surface scraping than from the tip or tail.

It is not only probable that the surface layer is richer in eggs, but also it is possible that in processing a small sample fewer eggs are retained in the solid particles of stool and thus lost. It is therefore preferable to examine a small portion of stool scraped from the surface layer than a large portion which includes sub-surface bulk. Such a procedure would seldom be practical in epidemiological surveys, but it should be practical for hospital or laboratory workers to ensure the selection of a thin surface scraping from a whole stool specimen passed by a patient.

The Comparison of Microscopic Examination and Hatching Techniques

Experience in epidemiological surveys indicates that, in some cases, miracidia could be detected after hatching even when no eggs were seen by microscopic examination. To assess the relative importance in detection of *S. mansoni* infections, the results of 4,217 stool examinations undertaken in the course of routine epidemiological surveys are presented in Table V.

Table V

STOOL EXAMINATIONS FOR S. MANSONI EGGS: COMPARISON OF RESULTS OBTAINED BY MICROSCOPIC EXAMINATION AND BY MIRACIDIAL HATCHING TECHNIQUES

Total number examined	4,217
Total number positive	1,983
Number positive by microscopic examination	348 (17.5%)
Number positive by miracidial hatching	471 (23.8%)
Number positive by both methods	1,164 (58.7%)

It is seen that, for accurate diagnosis, it is essential to use microscopic examination and miracidial hatching as complementary observations on each sample. Nearly one-quarter of the *S. mansoni* infections would have been missed if no hatching had been attempted, and in fact the results indicate that miracidial hatching was the more reliable single technique. This is not surprising as miracidial hatching employs almost the entire sediment, whereas a single microscopic preparation employs only one-tenth of the sediment. It is, however, acknowledged that miracidial hatching may detect the occasional *S. haematobium* infection in the bowel.

It can be expected that miracidial hatching will be even more valuable where only light infections exist and in the evaluation of drug therapy. It is obviously a most reliable measure of viability of infection.

DISCUSSION

The examination of stool specimens for *S. mansoni* eggs has not reached the level of reliability and accuracy which is both possible and required. The development of sophisticated techniques such as ninhydrin staining of filtered eggs (Bell, 1963), although giving quantitative results, is not suitable for detecting light infections. They are thus of limited value, yet workers throughout

the endemic world have rejected the traditional sedimentation techniques in favour of these newer methods. The very nature of the material on which the work must be done has discouraged the study of technique. The collection of stools in dry plastic containers or discarded bottles has made them unpleasant to handle, and as a result, even when more reliable techniques are developed, these are seldom applied by the workers undertaking routine specimen examination.

This investigation has indicated methods and techniques whereby accurate and reliable examination of stool material is possible. With attention to detail, the unpleasantness of these investigations is reduced. Great importance is placed on the use of wide-mouthed glass bottles with screw-on lids for the collection of the stool specimens. If specimens are placed directly into the saline in the bottles they are rendered less obnoxious, and in this state they may also be kept for several days before processing.

In this investigation it was found that greater reliability of examination was possible if stool specimens were stored for 24 to 48 hours, and that even from such stored specimens hatching of miracidia from eggs is greatest 24 to 48 hours after processing of the stool and addition of hatching water.

It would appear that eggs are released from the solid particles of stool if they are retained in saline for a period of time. It is possible that these eggs adhere to the stool particles and that they are released by the saline, thus allowing them to wash through the sieve and also making it easier for the microscopist to see the eggs in the microscopic preparation.

No reason can be suggested for the increased hatching if the sediment is kept for 24 to 48 hours after the addition of the hatching water. However, this improvement is invariably apparent in practice.

There appears to be no advantage in selecting the more complex sedimentation techniques, especially those involving the use of such chemicals as ether, which would prevent hatching.

The apparent distribution of eggs in the formed stool makes it important that, wherever practicable, surface scrapings are selected for examination. Nursing staff and other personnel who would have the opportunity to select the material should be taught to take surface scrapings to obtain the most reliable diagnosis.

Summary

Studies have been made to improve the methods of collection and processing of specimens of stool for examination for *S. mansoni* infections.

Employing sedimentation techniques, it has been shown that a stool placed by the subject into a hypertonic saline solution may be retained for several days and eggs will still be found in it and hatch from it. It is shown that there is a definite advantage in not processing the material immediately it is collected. It is suggested that stored in hypertonic saline the faecal matrix disintegrates, releasing the schistosome eggs and preventing them from being trapped on the sieve during processing.

Stool specimens subjected to hatching should be retained and observed at intervals for at least 48 hours after addition of hatching water before being discarded as negative.

The type of stool sample taken has an important bearing on the harvest of eggs. It is shown that it is preferable to take a small sample by scraping from the surface of the body of a formed stool.

Ideally, both microscopic examination and miracidial hatching techniques should be used, but hatching methods used alone will generally diagnose more cases than microscopic methods, and they have the added advantage of proving the viability of the eggs being passed by the patient. This is of very great significance in the follow-up examination of patients in trials of chemotherapeutic agents.

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