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A New Method for Counting *Schistosoma mansoni* Eggs in Faeces

With Special Reference to Therapeutic Trials

D. R. BELL [1]

The author describes a new method for estimating the daily egg output from patients with Schistosoma mansoni *infestation. It is suggested that this method—which combines faecal dilution, concentration by filtration and staining of the eggs with ninhydrin—is sufficiently accurate and sensitive to allow the results of treatment to be expressed as the percentage reduction in egg output rather than in the somewhat misleading terms of the "cure rate" usually adopted at present. The author also discusses the theoretical and practical advantages of this approach to the evaluation of treatment.*

An accurate and sensitive method for counting schistosome eggs in the stool has not yet been described, although the importance of egg counting in bilharziasis is well recognized (Second African Conference on Bilharziasis, 1960).

With the high egg output of hookworms, the simple dilution technique (Stoll, 1923; Stoll & Hausheer, 1926) is satisfactory; it has been used to count the eggs of *Schistosoma mansoni* despite its low sensitivity (Scott, 1937), but has never been widely adopted. The present method was devised to be sufficiently sensitive for accurate studies in *S. mansoni* bilharziasis.

METHOD

Principle

The stool is diluted to a known volume and an aliquot is concentrated on to filter-paper by filtration. The eggs are then stained with ninhydrin and counted on the paper.

Technique

Preparing the faecal suspension. Stools are collected for 24 hours. After being weighed, each motion is preserved by mixing to a thin paste with 10% formalin. At the end of 24 hours the entire collection is made up to a volume of 1 litre with tap water, using an electric mixer to ensure thorough comminution. Mixing is continued for 15 minutes, as serial sampling has shown a rise in egg count up to the tenth minute, presumably due to the continued disintegration of egg-containing faecal particles. There is no fall in egg count after this time, suggesting that eggs are not destroyed by the machine.

After mixing, the suspension is shaken vigorously and 50 ml are decanted rapidly into a screw-cap bottle for storage and subsequent counting. The suspension may be stored for at least two months at room temperature without any change in egg content or staining properties becoming apparent.

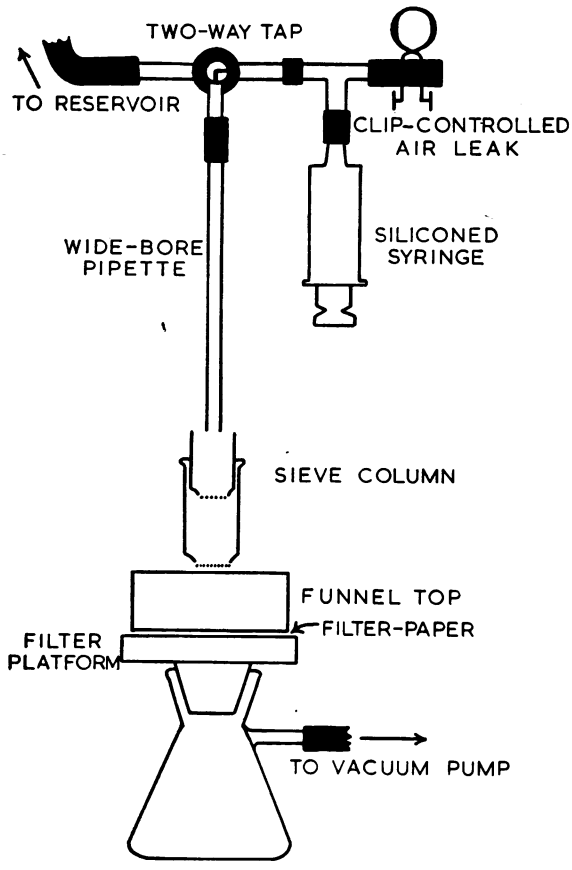
Concentration by filtration. (The apparatus used in this stage of the procedure is illustrated in Fig. 1 and 2.) One millilitre of the faecal suspension is drawn up into a wide-bore pipette, using suction from a siliconed syringe, the suspension having been agitated immediately beforehand. The contents of the pipette are then emptied into the sieve column by releasing the clip controlling the air leak, and the pipette is rinsed thoroughly by means of the two-way tap and water reservoir.

The sieve column is designed to remove large particles of debris from the suspension; it consists of two nylon mesh discs, placed one above the other, cemented into Quickfit ground-glass adaptors. This allows the column to be taken apart for easy clean-

[1] Medical Research Officer, East African Institute for Medical Research, Mwanza, Tanganyika.

FIG. 1

DIAGRAM OF THE APPARATUS USED FOR PIPETTING AND FILTERING THE FAECAL SUSPENSION



ing. The upper disc, 2.5 cm in diameter, has a mesh aperture of 500μ; the lower disc is 1.5 cm in diameter, with a mesh aperture of 350μ.

After the pipette has been thoroughly rinsed through into the sieve column, the column itself is washed down with water from a jet bottle to ensure that all the suspension is carried on to the filter.

The filter apparatus consists of a circular perforated Perspex (polymethyl acrylate) platform connected with a source of suction below. The platform supports a 7-cm filter-paper held in place by a removable acrylic cylinder of heavy gauge with a machined base. Whatman No. 541 paper has been found most suitable as it is thin enough to transmit light well, does not clog easily, and has great wet-strength.

When filtration is complete, the sides of the acrylic cylinder are rinsed with water to ensure that all eggs are carried on to the paper.

Staining with ninhydrin. Ninhydrin (triketohydrindene hydrate) reacts with certain amino-acids in proteins to form the dye, Ruhemann's purple. It is ideal for staining eggs in faeces, as the contrast between the umber detritus and the purple eggs is very striking.

The damp filter-paper is removed from the platform with forceps and placed on a drop of freshly made saturated aqueous ninhydrin solution in a flat-bottomed dish. The colour reaction is developed by incubation at 37°C for about 12 hours.

This method gives excellent staining of schistosome eggs without distortion, and hookworm eggs are also stained. Satisfactory staining can be achieved by heating at 60°C for two hours, but slight shrinkage of the miracidium then occurs. Higher temperatures produce distortion of schistosome eggs, and nematode eggs are not shown up, possibly due to a "popcorn" effect.

Examination of the stained paper. The dry paper is bisected with scissors, and each half is mounted on a 3-inch × 2-inch (5-cm × 7.5-cm) microscope slide in a few drops of 0.9% saline without coverglass. All the eggs on both halves of the paper are counted, using a binocular microscope, high-intensity transmitted light and low magnification. The Leitz Ortholux microscope with 3.5 : 1 objective and 10 × eyepieces has been found ideal. A rectangular eyepiece graticule is used to ensure accurate scanning.

When stained with ninhydrin, schistosome eggs have a very characteristic appearance, as the miracidium is stained purple but the eggshell is not.

The purple miracidium surrounded by its refractile shell can hardly be confused with an artefact, but if the identity of a particular egg is in doubt, scrutiny with a higher power almost always reveals the spine.

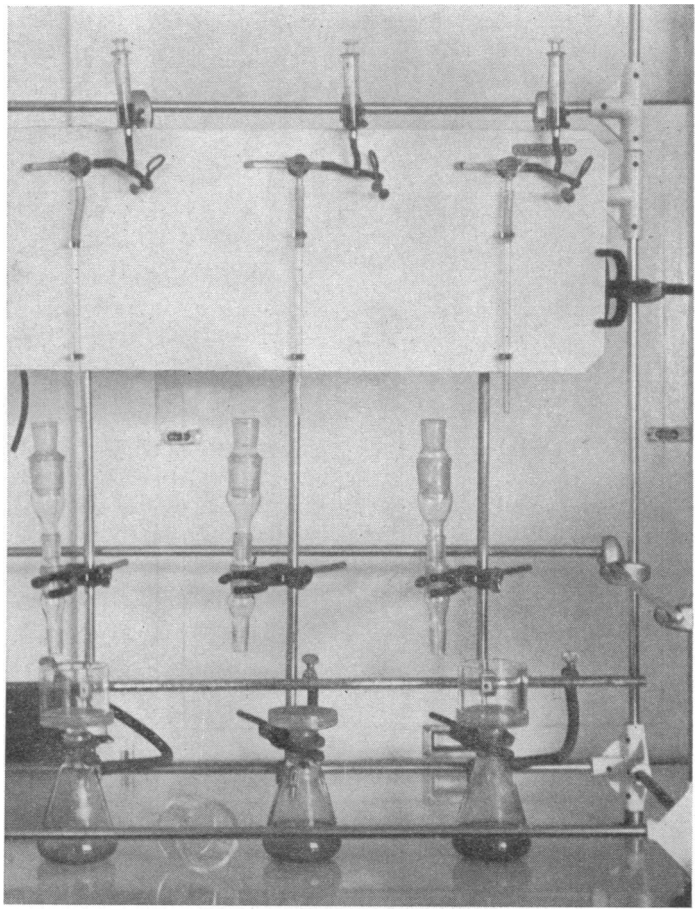
The number of eggs per filter-paper represents the daily egg output in thousands. Three papers are counted as routine.

Time factors

A batch of three unstained papers can be prepared from the faecal suspension in five minutes. Staining is conveniently done in the incubator overnight, and approximately five minutes are needed to count each half-paper.

FIG. 2

PHOTOGRAPH OF THE PIPETTING AND FILTERING APPARATUS, SHOWING THREE COMPLETE UNITS MOUNTED ON ONE CHASSIS TO ALLOW THE SIMULTANEOUS PREPARATION OF THREE FILTER-PAPERS



Accuracy

This method allows every egg in a sizeable sample of faecal suspension to be counted. The use of filtration to achieve concentration enables all surfaces to be adequately rinsed, and ensures that all eggs are washed on to the paper. Eggs are not retained in the nylon meshes of the sieve column, as has been shown both by microscopic scrutiny of the meshes and by reversing the column and collecting the washings on filter-paper.

An egg may sometimes elude detection by being overlain by debris, but the exclusion of large particles by the sieves minimizes this risk.

The consistency of results is illustrated by the table overleaf, which shows the counts recorded from repeated 1-ml aliquots of suspension, using suspensions from three different patients.

Sensitivity

Assuming that the distribution of eggs in the faecal suspension is random, then an output of 1000 eggs per day can be detected with a certainty of 95% if three aliquots each of 1 ml are examined.

Three examinations are made routinely in this laboratory, and the apparatus illustrated in Fig. 2 was designed to allow the preparation of three filter-papers simultaneously.

RESULTS OF REPEATED EGG-COUNTS FROM SUSPENSIONS FROM THREE PATIENTS

Patient	Number of eggs/ml									Mean/ml	Standard deviation	Coefficient of variation (%)
1	471	492	536	531	547	478	473	494	562	509	33	6.5
2	172	230	228	180	177	198	213	219	173	199	23	11.6
3	33	33	40	36	37	47	37	35	25	36	5.5	15.3

The sensitivity of the method may be increased further by using a concentration technique, and examining the entire concentrate on filter-paper. The M.I.F.C. technique (Blagg et al., 1955) gives an extraction rate of 80%-90% when compared with the direct filter-paper count, and it is possible to examine the concentrate produced from 5 ml of faecal suspension on one filter-paper. The use of this method allows an output as low as 200-250 eggs per day to be detected with 95% certainty if three examinations are made.

The sensitivity can be expressed in a more familiar way in terms of eggs per gram of faeces: with a stool of 200 g weight, the direct filter-paper technique will detect 5 eggs/g and the M.I.F.C.-filtration technique, 1 egg/g.

Application to small specimens

Although this method was devised to measure the daily egg output, it can readily be used for counting eggs in small stool samples. A convenient suspension is obtained with 1 g of faeces made up to 5 ml with diluent. It is not feasible to examine more than 1 ml of suspension on each filter-paper, unless concentration is used initially, as the paper tends to clog, and filtration may stop.

Special advantages

Apart from accuracy and sensitivity—already discussed—the method has the advantage that the stained filter-paper preparation is a semi-permanent record, as once the paper has been prepared and stained it can be stored and examined when convenient. Provided it is handled gently and infrequently, the eggs do not appear to detach readily. The paper can be made more permanent by treating it with a fixative such as shellac, and the retention of colour by the eggs is aided by protection from light. The fact that it is possible to carry out a retrospective check on counting which has been delegated constitutes a valuable advantage.

The method is readily adapted to the counting of *S. haematobium* eggs in urine. The entire 24-hour output may be passed through one 7-cm filter-paper if required, but smaller specimens may be examined more conveniently on smaller filter-papers. The absence of debris from urine specimens enables the counting to be done with an extremely low power and therefore much more rapidly than with stool specimens.

SUGGESTED APPLICATION TO THE ASSESSMENT OF TREATMENT

Most treatment trials in bilharziasis express the results of therapy in terms of " cure rate." Although there is no general agreement on the criteria which indicate " cure," the failure to find eggs in the stool, in the case of *S. mansoni*, is the most commonly used. There is, however, still no standardization of the methods employed to detect eggs in the stool, neither is there agreement on the number of examinations which should be made.

No method yet devised can detect all the eggs passing out of the body in a day, and therefore the meaning of " cure " is simply that eggs have not been found at follow-up: it does not necessarily mean that eggs are not being passed.

The standardized direct-smear preparation contains about 2 mg of faeces (Beaver, 1950). With a stool of 200 g weight, 20 direct smears, each of 2 mg, represent altogether only 1/5000th of the total stool; using 20 negative smears as a criterion of cure, the " cured " patient may still be passing in the region of 5000 eggs per day.

The " cure rate," therefore, does not live up to its semantic implications, and its meaning varies with the methods used to establish it.

But there is a further objection to the use of the " cure rate " in assessing treatment which may be even more important: it fails to take into account the weight of the initial infection. It is this factor which may explain discrepancies in the results of treatment even where similar methods are used in assessing cure.

It is well known that in bilharziasis the response to therapy is not an " all-or-none " phenomenon, in that treatment often significantly reduces egg output without eradicating the flukes completely. In patients with a low initial egg output, a comparatively small reduction in egg output induced by treatment may produce apparent cure, because the number of eggs is reduced below the threshold of detection. The contrary holds in patients with a very high initial egg output: a very substantial reduction in the number of eggs may follow treatment, although the number persisting may still be sufficient to allow detection, and the " cure rate " obtained may be nil.

It is obvious, therefore, that unless the initial egg output is known, the results of treatment expressed as " cure rate " must be misleading. Other things being equal, the therapeutic effect will always be overestimated if the pre-treatment egg output is low, and underestimated if it is high. This factor makes it impossible to compare results from different treatment centres where the efficiency of treatment is measured in terms of " cure rate."

The World Health Organization Expert Committee on Bilharziasis (1953) goes some way towards recognizing the importance of the initial egg output, in recommending that subjects used in therapeutic trials for bilharziasis should be " proved to be passing eggs regularly." This presumably implies a fairly high pre-treatment egg output, although the recommendation is far from precise and is interpreted in different ways by different workers.

In the case of hookworm infestation, the limitations of the " cure rate " have long been recognized, and it is now common practice to express results in a therapeutic trial as " percentage reduction in egg count " (Goodwin et al., 1958), using the Stoll technique. It is suggested that the new method described here makes the same quantitative approach feasible in the case of *S. mansoni* bilharziasis, with all its attendant advantages, and that the use of the " cure rate " in therapeutic trials should be abandoned.

Before such a quantitative approach to bilharziasis can be made, it must be shown that the natural variation in egg count is sufficiently low to make results meaningful without the use of a vast number of patients. This has been done by Scott (1938), who showed no greater variation in the egg counts obtained for *S. mansoni* than for hookworm. He also compared the variability of egg output in terms of eggs/g and eggs/day, and found the former more constant. Preliminary investigations using the new technique suggest little difference in constancy between the two parameters, and the daily egg output is the measure provisionally adopted because of its fundamental nature, and because it would seem more likely, on first principles, to bear a close relation to parasite load.

If the quantitative approach to the problem of treatment in bilharziasis is adopted, it should enable valid comparisons to be made between different centres. It should also show up smaller differences in drug efficiency than can be detected by the crude " cure rate " and it should enable definitive trials to be conducted without the use of very large numbers of patients. It might also help to stem the flow of contradictory papers on the efficacy of particular drugs.

If, notwithstanding the use of this quantitative method, significantly different results are still reported from different centres, then a search for differences in host or parasite would be rationally justified.

RÉSUMÉ

Les méthodes de numération des œufs de schistosomes, appliquées jusqu'ici pour établir le degré d'infestation par la bilharziose n'étaient ni assez précises, ni assez sensibles. L'auteur en décrit une, convenant aux recherches sur la bilharziose à *Schistosoma mansoni*: les selles sont diluées à un volume donné et une partie aliquote concentrée par filtration sur papier. Les œufs sont colorés par la ninhydrine et comptés sur le papier même.

Après avoir exposé le détail des opérations, l'auteur discute la sensibilité de la méthode: l'examen de trois fractions de 1 ml de la dilution des selles permet de déceler, avec une approximation de 5%, une émission de 1000 œufs par jour. La précision de la méthode peut

être augmentée par l'examen de tout le concentré filtré sur papier. En outre, la méthode a l'avantage de permettre la conservation des papiers filtres, après traitement par un fixatif. Elle peut être adaptée à la numération des œufs de *S. haematobium* dans l'urine, l'examen étant encore plus facile par suite de l'absence de matières étrangères.

Quant au « taux de guérison », établi d'après le nombre d'œufs subsistant après traitement, c'est un concept imprécis, car aucune standardisation des méthodes de numération n'a été proposée ni un nombre fixe d'examens requis. Ainsi, le fait de ne pas trouver d'œufs au microscope, dans les selles, n'implique pas nécessairement qu'il n'y en a pas. Au contraire, les normes actuellement en vigueur, selon lesquelles 1/5000 seulement des selles journalières est examiné, permettent de considérer comme « nulle » une émission effective de 5000 œufs par jour. Actuellement, l'estimation du taux de guérison ne tient pas compte du degré initial d'infection. On sait que, dans la bilharziose, la réponse au traitement n'est pas « tout ou rien », et que souvent, le traitement provoque une réduction significative de la quantité d'œufs évacués, sans supprimer complètement l'infestation. Dans les cas d'infestation légère, une réduction moyenne ne laissant subsister qu'un faible nombre d'œufs, peut faire croire à une guérison. Au contraire, en cas d'infestation massive, le traitement peut provoquer une réduction importante, qui laissera cependant subsister un nombre d'œufs assez élevé pour que l'on considère le taux de guérison comme nul. Il s'ensuit que le taux de guérison est un critère sans grande valeur, si le nombre initial d'œufs n'est pas indiqué. Le taux de guérison sera surestimé si le nombre initial d'œufs est faible et sous-estimé si ce nombre est élevé.

La méthode recommandée par l'auteur pour la recherche des œufs de *S. mansoni* a l'avantage de permettre une évaluation quantitative — comme c'est le cas pour la méthode de Stoll dans l'ankylostomiase. La notion du « taux de guérison » devrait être abandonnée. Si l'évaluation quantitative des résultats du traitement de la bilharziose était adoptée (en nombre d'œufs par jour), elle permettrait de comparer les résultats obtenus dans différents centres, avec différents médicaments, sans faire appel à un trop grand nombre de malades. Si, malgré l'emploi de la méthode quantitative, des différences significatives entre les centres sont observées, il sera temps de chercher l'origine de ces différences chez l'hôte ou le parasite.

REFERENCES

Beaver, P. C. (1950) *J. Parasit.*, **36**, 451

Blagg, W., Schloegel, E. L., Mansour, N. S. & Khalaf, G. I. (1955) *Amer. J. trop. Med. Hyg.*, **4**, 23

Goodwin, L. G., Jayewardene, L. G. & Standen, O. D. (1958) *Brit. med. J.*, **2**, 1572

Scott, J. A. (1937) *Amer. J. Hyg.*, **25**, 546

Scott, J. A. (1938) *Amer. J. Hyg.*, **27**, 155

Second African Conference on Bilharziasis (WHO/CCTA) (1960) *Wld Hlth Org. techn. Rep. Ser.*, **204**, 11

Stoll, N. R. (1923) *Amer. J. Hyg.*, **3**, 59

Stoll, N. R. & Hausheer, W. C. (1926) *Amer. J. Hyg.*, **6**, Suppl. 1, p. 80

World Health Organization, Expert Committee on Bilharziasis (1953) *Wld Hlth Org. techn. Rep. Ser.*, **65**, 40