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A simplified technique for counting onchocercal microfilariae in skin snips

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A new method for counting microfilariae in skin snips is described. The method, in which slide specimens are reconstituted after drying, is compared quantitatively with two existing methods that are used when the counting of microfilariae has to be postponed. Counts obtained by the new method were closely correlated with those taken in fresh specimens. Other advantages of the new method are discussed and its use in onchocerciasis field surveys is recommended.

Of the existing methods of diagnosing onchocerciasis, the skin snip method for the detection of microfilariae is the only one suitable for measuring intensity of infection with any degree of accuracy. The number of microfilariae emerging from unteased skin snips is usually counted in fresh preparations that have been allowed to stand for 30–60 min in either normal saline or distilled water. There are two established methods for preserving the specimens when unfavourable field conditions necessitate the postponement of counts: the "fix-and-stain" method (*1*) and the "centrifuge" method (*2*). Both of these methods require great care to ensure minimal loss of microfilariae during processing. To eliminate this totally unpredictable and variable margin of error, I have in the past two years adopted a new technique, the "reconstitution" method, described below.

The aims of this study were to assess both the relative sensitivity and the degree of correlation between the various methods.

MATERIALS AND METHODS

A total of 45 patients with onchocerciasis, attending the endemic diseases clinic of the University College Hospital, Ibadan, were enrolled for the first part of the study. Six skin snips were taken from every patient, three from each buttock, using a corneoscleral punch instrument (Holth) with a 2-mm bite. The snips were taken 1 cm apart to form an equilateral triangle. The three snips from each buttock were then allocated at random to one of the following treatment groups:

The fix-and-stain method. Each snip was placed in two drops of 0.85% saline on a conventional 3 × 1 inch (approx. 75 × 25 mm) microscope slide, left uncovered and kept wet for 1 h after which it was dried, then fixed with methanol and stained with Ehrlich's haematoxylin. The microfilariae were then counted.

The centrifuge method. The snip was placed in a Widal tube containing 0.5 ml of 0.85% saline and incubated for 18–24 h at 37°C. The preparation was then centrifuged at 1000 *g* for 5 min and the deposit placed on to a glass slide. The tube was further washed with saline on to the slide and the microfilariae counted.

The reconstitution method. The snip was placed in two drops of 0.85% saline on a slide, left uncovered and kept wet for 1 h after which it was dried. Then, 12–24 h later, a drop of distilled water was placed on the preparation and spread over it so as to cover the entire area of the original medium, and the microfilariae present were counted. The edge of the area occupied by the original medium is always clearly demarcated after drying.

For the second part of the study, 28 skin snips were taken from 14 patients, one from each buttock. Each snip was placed in saline and microfilariae were counted at 1 h. The preparation was then dried and kept for 12–24 h after which it was reconstituted with a drop of distilled water and recounted. The specimen was once more dried, then fixed and stained, and the microfilariae were counted again. This procedure made it possible to compare the

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Table 1. Two-way analysis of variance to compare the three methods

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Methods (M)	2	2.28	1.139	20.31	$P<0.001$
Patients (P)	44	223.90	5.089		
Interaction M × P	88	4.93	0.056	1.00	
Residual	135	14.34			
Total	269	245			

counts obtained by the fix-and-stain and the reconstitution methods with counts undertaken in the fresh preparation at 1 h, and thus to estimate the loss in material during the staining and reconstitution processes.

In all of the processes the skin snips were left on the slides before, during, and after counting, drying, and reconstitution. Rapid drying was effected by gently warming the slide over the flame of an oil or microscope lamp. The dried smears were then stored in tight fitting slide-box carriers.

STATISTICAL ANALYSIS

The design of the first part of the study was that of a factorial experiment with two factors: one fixed, the other random. The fixed-effect factor, the method of counting the microfilariae, had three levels. These were the different counting methods: the fix-and-stain method, the centrifuge method, and the reconstitution method. The random factor was the group of patients, which can be regarded as a random sample of 45 from the larger overall population of patients. There were two replicates from each patient. The analysis of variance model was the mixed-effects type and the analysis followed standard procedures (*3*). The primary interest of this study was in differences between the three methods. Their effect in the analysis of variance table was tested against the interaction mean square. The interaction mean square also provided estimates of the standard error for the treatment means. Pairwise comparison of the method means was effected by use of the Studentized range. The design and analysis for the second part of the study were as described above. The fixed-effect factor, the counting procedure, was again at three levels: the 1-h count, the reconstitution, and the fix-and-stain procedure. The random-effect factor was, as before, the group of patients. Only 14 patients were included in the second part of the study and, as before, there were two replicates from each. All analyses were done on $\log_{10}$ transforms of the original counts.

RESULTS

Table 1 shows the two-way analysis of variance to compare the three methods used in the first part of the study. As shown by the F-test, there was a significant difference between the three methods ($P<0.001$). Table 2 shows the mean log count for each method while Table 3 shows a pairwise comparison of the three methods. Both the centrifuge and the reconstitution methods yielded significantly higher mean counts than the fix-and-stain method

Table 2. Mean log values for the three methods

Method	Mean	S.E.[a]
Fix-and-stain	0.94	0.025
Centrifuge	1.16	0.025
Reconstitution	1.09	0.025

[a] Estimated from the mean square value for interaction in the analysis of variance given in Table 1.

Table 3. Pairwise comparison of the three methods[a]

Contrast	Difference between mean values	Probability
Fix-and-stain against Centrifuge	0.22	$P<0.01$
Fix-and-stain against Reconstitution	0.15	$P<0.01$
Centrifuge against Reconstitution	0.07	$P>0.05$

[a] Comparison is by use of the Studentized range to estimate the least difference, D, required for significance: $D = 0.084$ at the 5% level and $D = 0.106$ at the 1% level.

Table 4. Two-way analysis of variance to compare the three procedures

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Procedures (R)	2	1.66	0.830	32.90	$P<0.001$
Patients (P)	13	30.64	2.357		
Interaction R × P	26	0.66	0.025		
Residual	42	9.46			
Total	83	42.42			

($P<0.01$ in each case) but there was no significant difference ($P>0.05$) between the centrifuge and the reconstitution methods.

Table 4 uses the analysis of variance to compare the three procedures of the second part of the study. The F-test here also indicates significant differences ($P<0.001$) between the mean counts of the procedures. Tables 5 and 6 show, respectively, the mean log count of each procedure and a pairwise comparison of the three mean values. The fix-and-stain procedure had a significantly lower mean count than both the 1-h count and the reconstitution procedure ($P<0.01$ in each case) but the difference between the mean counts of these latter two methods was not significant at the 5% level.

Table 5. Table of mean log counts for the three procedures

Procedure	Mean log count	S.E. [a]
1-h count	1.22	0.030
Reconstitution	1.24	0.030
Fix-and-stain	0.93	0.030

[a] Estimated from the interaction mean square in the analysis of variance given in Table 4.

Table 6. Pairwise comparison of the three procedures [a]

Contrast	Difference between mean values	Probability
1. 1-h count against Reconstitution	0.02	$P>0.05$
2. 1-h count against Fix-and-stain	0.29	$P<0.01$
3. Reconstitution against Fix-and-stain	0.31	$P<0.01$

[a] Comparison is by use of the Studentized range to estimate the least difference, D, required for significance: $D = 0.105$ at the 5% level and $D = 0.135$ at the 1% level.

DISCUSSION

To enable comparisons to be made between different onchocerciasis surveys, it is essential that estimates of infection rates based on microfilarial counts should be well correlated, irrespective of the methods used. This study and a previous one (*4*) have shown that counts obtained by the centrifuge method, where the snips remain immersed in the medium for 24 h, were higher than those obtained with the different slide methods, where counts were taken after 1 hour. The higher yield of the centrifuge method was almost certainly due to the longer interval allowed for microfilariae to emerge. However, whereas the counts by the centrifuge and the fix-and-stain methods were significantly different ($P<0.01$), those by the centrifuge and reconstitution methods were not ($P>0.05$). Equally revealing is the fact that the reconstitution method was more sensitive than the fix-and-stain method, the mean microfilarial count being significantly higher in the former ($P<0.01$). Thus there is a closer correlation between the results of the centrifuge and the reconstitution methods than there is between either of these two methods and the fix-and-stain method. This suggests that a significant quantity of microfilariae is lost during the staining process of the last named procedure.

This impression is further strengthened by the results of the second part of the study which was designed to determine the degree of correlation between counts undertaken on slide specimens examined in the fresh state and those obtained with the reconstitution and fix-and-stain methods. With the fix-and-stain method and the reconstitution method, the time allowed for microfilariae to emerge can be regulated with a degree of precision that matches that for fresh specimens. An emergence interval of 1 h was allowed in all three slide procedures in this study since it has been shown that 90%

of all microfilariae would have emerged from skin snips after 1 h in saline or distilled water (*5*, *6*).

The results show that the reconstitution method gave counts that are correlated more closely with those in the fresh specimen than did the fix-and-stain method. This can be attributed to the better preservation and prevention of loss of material with the reconstitution method.

Six major disadvantages of existing techniques for estimating microfilarial densities in onchocerciasis have been listed by Scheiber et al. (*7*). To overcome these disadvantages, these workers adapted for use in onchocerciasis the membrane filtration technique which was first developed for the quantification of *Wuchereria bancrofti* microfilaraemia (*8*).

The reconstitution method does not suffer from any of the disadvantages of the existing techniques; rather, it has the following advantages: (*a*) it is a very simple and easy technique that can be performed in the field by relatively unskilled technicians whose number need not be more than two for any series; (*b*) operational costs are kept to the barest minimum since expensive and cumbersome equipment for filtration, centrifugation, etc., is not needed, and there is conservation of valuable fixative and staining reagents which, with this new method, are used only when specifically necessary for the identification of microfilarial species; (*c*) since at the time of reconstitution of the specimen the microfilariae are nonmotile, counting is much easier and more accurate than in the fresh specimen in which rapid movement and clumping of microfilariae occur; (*d*) the technique permits counts to be repeated if necessary, and dried smears have remained in good condition for reconstitution and counting for up to six weeks. Although skin snips were placed in saline in this study, further experiments have shown that microfilariae retain their morphological characteristics better and longer when the snip is immersed in distilled water rather than saline.

No appreciable difficulties were experienced with hygroscopic salt crystals, and the use of tight-fitting slide boxes has been effective in preventing dust, ants, cockroaches, moulds, etc., from destroying the dried specimens. Since the reconstitution method has most of the advantages and few of the disadvantages of other methods, its use may be preferred in quantitative onchocerciasis surveys.

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RÉSUMÉ

TECHNIQUE SIMPLIFIÉE DE NUMÉRATION DES MICROFILAIRES ONCHOCERQUIENNES DANS DES BIOPSIES CUTANÉES (" SKIN SNIPS ")

Une nouvelle méthode de comptage des microfilaires dans des biopsies cutanées est décrite et les résultats obtenus sont comparés à ceux d'examens pratiqués avec la méthode dite de coloration après fixation et la méthode par centrifugation. Selon la nouvelle méthode, on place le fragment de peau prélevé dans quelques gouttes de solution saline où il demeure à découvert pendant une heure avant sa dessication à chaleur modérée au-dessus d'une lampe. La préparation peut alors être conservée dans une boîte à couvercle à glissière bien ajusté et reconstituée ultérieurement au moyen d'une goutte d'eau distillée pour le comptage des microfilaires.

Les densités microfilariennes relevées avec cette méthode de reconstitution se sont révélées en corrélation plus étroite avec les densités observées sur des prélèvements frais examinés au bout d'une heure que ne l'étaient les résultats obtenus avec la méthode de coloration après fixation. Les densités obtenues au moyen des méthodes de reconstitution et de centrifugation n'étaient pas statistiquement différentes.

On dispose donc, avec la méthode de reconstitution, d'un procédé simple dont l'application peut être confiée à deux assistants de terrain sans qualification particulière. On peut attendre jusqu'à six semaines pour reconstituer les fragments de peau desséchés.

REFERENCES

1. DUKE, B. O. L. *Bulletin of the World Health Organization*, **27**: 629-632 (1962).
2. GRATAMA, S. *Acta Liedensia*, **35**: 47 (1966).
3. BOWKER, A. H. & LIEBERMAN, G. J. *Engineering statistics*, 2nd ed., New Jersey, Prentice Hall Inc., 1972, Chapter 10.
4. KALE, O. O. ET AL. *Bulletin of the World Health Organization*, **51**: 547-549 (1974).
5. BRINKMAN, U. K. *Zeitschrift für Tropenmedizin und Parasitologie*, **24**: 397-403 (1973).
6. PICQ, J. J. ET AL. *Bulletin of the World Health Organization*, **45**: 517-520 (1971).
7. SCHEIBER, P. ET AL. *Bulletin of the World Health Organization*, **53**: 130-133 (1976).
8. BELL, D. *Annals of tropical medicine and parasitology*, **61**: 220-223 (1967).