Fluorescence Microscopy in the Laboratory Diagnosis and Assessment of Pulmonary Tuberculosis

BY

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The demonstration of acid fast bacilli in smears of sputum remains the most rapid laboratory technique for a presumptive diagnosis of pulmonary tuberculosis. The widely employed and classical staining technique of Ziehl-Neelsen has limitations, however, in Africa, where there are limited laboratory facilities and where in particular there is a shortage of trained laboratory staff. The technique may not always be accurate when employed in the large out-patient clinics encountered in Africa, for technicians are pressed for time and long and tiresome scanning of smears is necessary often before the presence or absence of bacilli can be assumed. There is need, therefore, in those territories which are understaffed and overworked, for the introduction of a simpler method of microscopy that is at once reliable and easy to learn and use.

The method of fluorescence microscopy for the detection of acid fast bacilli was introduced by Hagemann (1937) and has been developed by others over the years. The method consists in staining bacilli by the fluorescent dye auramine, to which they are acid and alcohol fast, and which has the property of converting rays at the lower end of the spectrum to light of longer wavelength. When auramine stained bacilli are subjected to a source of ultra-violet which has been passed through a filter to absorb blue violet and ultra-violet rays, they fluoresce; if a yellow filter is placed in the eyepiece they appear yellow against a dark field. The fluorescence is clearly visible microscopically, and a magnification of 200 to 300 times is sufficient to recognise acid fast bacilli. That the method has not been more widely employed relates firstly to the matter of the equipment for microscopy, which must be used in a darkened room,* and to doubt of the accuracy of the method, and the belief by some that false positive results are produced.

The purpose of this paper is to present evidence for the accuracy of the method of fluorescence microscopy when used by junior technicians in Africa.

Laboratory Methods

Fluorescence Microscopy Equipment

A B.T.H. Mazda mercury vapour electric discharge lamp (box type ME 250 W/50/5) was used as a source of light; a choke was wired in series. Ultra-violet rays were selected by the use of a filter consisting of a bulbous glass vessel containing ammoniacal copper sulphate solution ($CuSO_4$ 7.8 grammes; NH_4OH 30 c.c.; H_2O 270 c.c.); the filter transmits rays of wavelengths from 4,200 Angstrom units to the ultra-violet region. The light source was pro-

* Recently apparatus has been described which can be used in daylight (Holst, Mitchison and Radhakrishna, 1959).

jected through this vessel, which acts as a convex lens, and then to the mirror of the microscope. Since the rays causing fluorescence pass through ordinary glass with minimal loss, quartz lenses and an aluminium surfaced mirror were not required. The light source and the convex lens and filter were integrated in a compact apparatus.† An Ilford minus blue-micro 4 filter was incorporated in the eyepiece of the microscope to absorb all rays passed by the ultra-violet filter. The Ilford filter transmits freely wavelengths from 5,200 Angstrom units upwards.

Staining Techniques

Ziehl-Neelsen Staining.—The method used was the standard technique described by Mackie and McCartney (1956). Methylene blue was employed as a counterstain.

Auramine Staining.—The reagents used were a solution of auramine-phenol, an acid-alcohol decolorisor, and a counterstain of potassium permanganate solution.

Auramine-phenol solution: This consisted of:

Auramine "O" (fluorescent)	0.3 gm.
Phenol	3.0 gm.
Distilled water	97.0 ml.

The phenol was dissolved in water and the solution heated gently until the temperature reached 60° Centigrade. The auramine was added gradually until dissolved, the preparation being shaken vigorously. The final solution was filtered through a Whatman No. 1 paper and stored in a well-stoppered dark bottle. Fresh stock was prepared monthly.

Acid-alcohol decolorisor: this consisted of:

Concentrated hydrochloride acid	0.5 ml.
Sodium chloride	0.5 gm.
Methylated spirit	75.0 ml.
Distilled water	25.0 ml.

Potassium permanganate solution: this consisted of:

Potassium permanganate	0.1 gm.
Distilled water	100.0 ml.

Method of Staining.—The following staining method was adopted:

(1) Smear flooded with auramine-phenol solution (unheated) for ten minutes.

(2) Preparation then washed well in tap water.

(3) Slide decolorised in acid alcohol for five minutes.

(4) Good wash in tap water repeated.

(5) Potassium permanganate solution applied as counterstain for 30 seconds.

(6) Preparation finally washed well in tap water and allowed to dry, but not blotted.

†Supplied by Electrical and Mechanical Installations (Brentford) Ltd.

Preparation of Sputum Samples for Microscopy and Culture

The method employed was that described previously by Brown (1959). In brief, the following procedure was applied.

Preparation of Smears for Microscopy.—Sputum in varying amounts up to 5 ml. was added to 15 per cent. trisodium phosphate (one part sputum to four parts trisodium phosphate approximately) in universal bottles. The outside of each bottle was sterilised in 5 per cent. lysol for one hour. The mixture was then shaken on a rocker oscillating at 200 per minute for one hour. Bottles were incubated at 37° centigrade overnight, and during next morning were spun at 3,000 r.p.m. for 15 minutes. The supernatant was discarded and 25 ml. of sterile distilled water containing 40 units of penicillin per ml. were added to each deposit. Bottles were again spun at 3,000 r.p.m. for 15 minutes, the supernatant discarded, and smears with an area of approximately one sq. cm. made from the deposit.

Cultural Method.—Two freshly prepared Lowenstein-Jensen slopes were employed for each specimen of sputum. Using Pasteur pipettes, 0.5 ml. of concentrate, prepared as above, was inoculated on the surface of each slope. Culture bottles were incubated at 37° centigrade, firstly in the horizontal position for 24 hours and then in the vertical position. Each slope was examined weekly and no culture was labelled negative until the completion of 12 weeks' incubation.

EXPERIMENTAL MATERIAL

Sputum samples were collected from new patients with suspected pulmonary tuberculosis and from patients with tuberculosis already receiving treatment at various clinics throughout Ghana. Each specimen was a single expectoration, and sputum was forwarded from the collection centres at the earliest opportunity to the tuberculosis research unit laboratory in Accra for processing. In all, 1,650 separate samples were collected.

Preliminary Test for Homogenicity of Concentrates

In order to determine whether the method of concentration used adequately homogenised sputum samples and permitted the preparation, therefore, of comparable smears, a preliminary blind test was undertaken.

Concentrates of 102 specimens of sputum were prepared, and two smears of each concentrate were stained Ziehl-Neelsen. All smears were marked, mixed and then examined by one technician. Each was reported "negative" or "positive," and bacilli in positive smears were enumerated according to the Gaffky scale and reported as scanty (Gaffky 1-3), moderate (Gaffky 4-7), or heavy (Gaffky 8-10).

In respect of negativity, positivity and Gaffky counts, identical results were obtained in 93 instances (92 per cent.). Paired slides were negative in 41, scantily positive in nine, moderately positive in 27 and heavily positive in 16 instances. In five instances there were differences in the Gaffky counts (one scanty, one moderate in one pair; one moderate, one heavy in four pairs). Different results in respect of negativity and positivity were obtained in four instances.

Thus differences relating to incomplete homogenisation could be expected in 4 per cent. of paired smears examined and slight differences also in 4 per cent. In 92 per cent. of all comparisons, however, it would be expected that smears had been prepared from adequately homogenised concentrates and that the bacillary content of each member of a pair would be identical.

Comparative Microscopy

Two smears were prepared from concentrates of each sputum sample. The one was stained Ziehl-Neelsen and reported as negative, scanty, moderate or heavy. The other was stained with auramine and reported as negative, scanty (1-4 bacilli per field), moderate (4-10 bacilli per field), heavy (over 10 bacilli per field). All auramine stained smears were restained Ziehl-Neelsen after initial examination and reporting. Ziehl-Neelsen smears were examined with 1/12 oil immersion objective and X6 eyepiece; auramine smears were examined with two-thirds objective and X6 eyepiece.

Slides were marked, stained and mixed before reading, and microscopy was undertaken by one technician. All specimens were cultured. The results were then correlated and are summarised in Table I.

Microscopy Related to Positive Cultures

Of the 379 specimens positive on culture, 370 gave the same result on microscopy (316 positive, 54 negative), and in nine smears the microscopy results were different (six smears being positive only on auramine, three being positive only on Ziehl-Neelsen staining). The results here were closely similar, therefore, but the discrepancy was investigated by restaining auramine smears by Ziehl-Neelsen's method. The results were as follows:

Positive Only on Ziehl-Neelsen Staining—

Ziehl-Neelsen (all positive)	Auramine	Auramine re-stained Ziehl-Neelsen
(1) Scanty	Negative	Negative
(2) Scanty	Negative	Negative
(3) Moderate	Negative	Moderate

In one case (3) restaining by Ziehl-Neelsen revealed bacilli not seen on the initial auramine smear. In the other two cases the initial differences are likely to have been due to faulty homogenisation of the specimens.

Positive Only on Auramine Staining—

Ziehl-Neelsen	Auramine (all positive)	Auramine re-stained Ziehl-Neelsen
(4) Negative	Scanty	Negative
(5) Negative	Scanty	Negative
(6) Negative	Scanty	Negative
(7) Negative	Scanty	Negative
(8) Negative	Scanty	Negative
(9) Negative	Scanty	Negative

Table I

AURAMINE (FLUORESCENCE) ZIEHL-NEELSEN MICROSCOPY OF SMEARS OF SPUTUM POSITIVE OR NEGATIVE ON CULTURE*

Cultures	Number of Specimens	Identical Results				Different Results			
		Negative Auramine and Ziehl-Neelsen		Positive Auramine and Ziehl-Neelsen		Positive Only Auramine		Positive Only Ziehl-Neelsen	
		No.	%	No.	%	No.	%	No.	%
Positive	379	54	14.2	316	83.4	6	1.6	3	0.8
Negative	1,221	646	52.9	472	38.7	101	8.3	2	0.1

* The discrepancy is accounted for by exclusion from the analysis of 50 specimens contaminated on culture.

Re-staining failed to reveal bacilli noted in the original auramine smear, and the auramine method was superior in all six instances of difference.

Microscopy Related to Negative Cultures

Of the 1,221 specimens negative on culture, 1,118 (91.6 per cent.) gave the same results with Ziehl-Neelsen and auramine staining (472 positive, 646 negative), and in 103 paired smears the microscopy results were different (101 positive only on auramine, two positive only on Ziehl-Neelsen staining). The discrepancy was investigated by counter-staining auramine smears by Ziehl-Neelsen, and the results were as follows:

Positive Only on Ziehl-Neelsen Staining—

Ziehl-Neelsen	Auramine	Auramine smears re-stained Ziehl-Neelsen
(1) Scanty	Negative	Scanty
(2) Moderate	Negative	Negative

In one case (1) re-staining revealed bacilli not noted on the initial auramine smear. It may be that in the other case (2) the discrepancy was related to incomplete homogenisation of the concentrate resulting in an absence of bacilli on the auramine smear.

Positive Only on Auramine Staining

The majority of the smears positive only on auramine had scanty bacillary populations (scanty 91, moderate 10, heavy 0). When the auramine smears were re-stained Ziehl-Neelsen, the following results were obtained:

Lauramine	Auramine smears re-stained Ziehl-Neelsen
91 scanty	75 negative, 16 positive (all scanty)
10 moderate	9 negative, 1 positive (scanty)

Of 101 pairs positive only on auramine staining initially, 84 were negative on re-staining by Ziehl-Neelsen and 17 were scantily positive. Thus 101 specimens could be considered truly positive on auramine staining. All were negative on culture; but of the total, 75 were from patients under treatment. In routine work in our laboratory 10 per cent. of positive sputum concentrate smears from patients under treatment are negative on culture.

The differences between the two methods of microscopy related to culture results are summarised in Tables II and III.

DISCUSSION

In this investigation comparative microscopy was undertaken on paired smears of sputum concentrates, known from preliminary tests to be likely to be comparable in respect of bacillary population in 92 per cent. of examinations. All auramine stained smears were re-stained Ziehl-Neelsen, but details of the results of re-staining are given only in cases of differences in the two methods.

In those specimens positive on culture the results of comparative microscopy were closely similar, and identical results were obtained in 97.6 per cent. of the examinations undertaken. Differences seemed to be related mainly to smears with scanty bacillary content, and the auramine method demonstrated bacilli in such smears in six instances where the Ziehl-Neelsen method failed to do so.

In relation to those specimens negative on culture, the discrepancy was greater. Here identical results were obtained in 91.6 per cent. and results differed in 103 out of a total of 1,221 paired examinations (8.4 per cent.). Almost all differences (101) were related to an apparent superiority of the auramine method (again mainly in smears with a scanty bacillary population), and the question arises as to whether many of the auramine results were not false positives. Of the 101 originally positive only on auramine staining, however, 84 were found to be negative on re-staining Ziehl-Neelsen, and the assumption must be that this number were truly positive on auramine, although negative on Ziehl-Neelsen staining on two separate examinations of different concentrates of the same sputum read blindly.

From the point of view of clinic practice, the method of fluorescence microscopy is neither

Table II

COMPARISON OF SMEARS STAINED AURAMINE OR ZIEHL-NEELSEN RELATED TO CULTURE RESULT

Auramine and Ziehl-Neelsen Comparison	Positive Cultures		Negative Cultures	
	No.	%	No.	%
Same result	370	97.6	1,118	91.6
Auramine superior	6	1.6	84	6.9
Ziehl-Neelsen superior	1	0.3	1	0.1
Differences accounted for by inadequate homogenisation of sputum	2	0.5	18	1.4
	379	100.0	1,221	100.0

Table III

RESULTS OF COMPARISON OF AURAMINE AND ZIEHL-NEELSEN STAINED SMEARS RELATED TO CULTURE RESULTS

Culture Result	Smear Result		Specimens		Same Result	
	Auramine	Ziehl-Neelsen	No.	%	No.	%
Positive	Positive	Positive	316	19.8	370	*23.9
	Negative	Negative	54	3.3		
	Positive	Negative	6	0.4		
	Negative	Positive	3	0.2		
Negative	Positive	Positive	472	29.5	1,118	*69.9
	Negative	Negative	646	40.4		
	Positive	Negative	101	6.3		
	Negative	Positive	2	0.1		
			1,600	100.0	1,488	*93.8

* Per cent. of 1,600 specimens.

superior nor inferior to formal microscopy using the Ziehl-Neelsen method when dealing with sputa heavily populated with bacilli; it is superior, however, when sputa with scanty bacillary populations are being investigated. Heavily populated sputa are more likely to be found in cases of symptomatic disease reporting for diagnosis, and scantily populated sputa are more likely in those undergoing treatment and on whom microscopy is being undertaken as a measure of assessing progress and response to the therapy—the group of patients constituting the great majority on whom microscopy is undertaken in day-to-day clinic practice.

It might be argued that in the culture negative group (mainly a group receiving treatment), since bacilli were not demonstrated by the Ziehl-Neelsen method, there is no evidence that acid fast bodies demonstrated by the fluorescence microscope were bacilli. It might indeed be that the fluorescing bodies were merely particles of bacilli disintegrating during the course of treatment, but still retaining their acid-alcohol fast properties, particularly in cases with negative culture results; but this does not apply to untreated cases or to cases with positive cultures unless we postulate here the demonstration of developing bacillary forms.

The apparent ability of fluorescence microscopy to demonstrate acid fast bacilli more readily in scantily populated smears underlines one of the main advantages of the method, for it is the examination of the smear containing few bacilli that is so time-consuming in the routine laboratory when the ordinary method of microscopy is used. The area of the slide included in one field when fluorescence microscopy is used is about 50 times greater than when Ziehl-Neelsen microscopy is employed. Thus, if bacilli can be demonstrated more easily in scanty populated smears by a quick scanning of slides, the method employed has a decided advantage in saving of time, in saving of fatigue and in more accurately demonstrating the presence of bacilli.

The method of fluorescence microscopy is easy to learn and is reliable in the hands of junior technicians when they have acquired some practice and have been taught the need for careful adjustment of the optical equipment. When smears have been stained with auramine and there is doubt about the result, they can quickly be re-stained by the classical Ziehl-Neelsen method and smears re-examined.

SUMMARY

An investigation has been undertaken to determine the efficacy of fluorescence microscopy (using auramine stain) relative to ordinary microscopy (using Ziehl-Neelsen stain) by

comparing paired smears of sputum concentrates read blindly.

Preliminary to comparative microscopy, the method of homogenisation of sputum was critically examined to determine whether paired smears were likely to have comparable bacillary populations.

Fluorescence microscopy was shown to be superior to the Ziehl-Neelsen method in smears with scanty bacillary populations, but there was little difference in the two methods in smears with high bacillary content.

The method of fluorescence microscopy is recommended for use in busy understaffed laboratories where large amounts of tuberculous materials are being handled. It is sensitive, quick to learn, easy to use and confers a great saving of time and fatigue on technicians.

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