EXAMINATION OF SPUTUM BY SMEAR AND CULTURE IN CASE-FINDING

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In case-finding, a diagnosis of tuberculosis can only be firmly established by the finding of acid-fast bacilli in smears, or of *M. tuberculosis* in culture. Radiographic examination may detect cases which are not yet excretors of bacilli, but the diagnosis in such cases remains in doubt. In the developing countries, facilities for bacteriological examinations are often limited, and, when available, are frequently not used as much as is desirable. My purpose today is to try and indicate the most efficient and economical use of bacteriological methods.

RELATIVE VALUE OF DIRECT SMEAR AND CULTURE EXAMINATIONS

The two principal methods for establishing a bacteriological diagnosis are microscopical examination of direct smears and culture. Their relative value depends on:

1. the severity of the disease in the patient,
2. the speed and manner with which the specimen is transported to the laboratory, and
3. the methods of examination in the laboratory.

Table I sets out the results of smear and culture examinations of a single specimen per patient at the Royal Postgraduate Medical School, London and at the Tuberculosis Chemotherapy Centre, Madras (Andrews and Radhakrishna, 1959). In both laboratories, the specimens were collected nearby and were usually examined on the same day. The same bacteriological methods were

TABLE I

*Examination of single collection specimen of sputum from newly diagnosed patients by smear and culture**

Laboratory	Total positive by smear or culture	Culture positive		Smear positive		Positive only on culture	
		No.	%	No.	%	No.	%
London, 1954-62	776	773	99.6	275	35.4	501	64.6
Madras, 1956-58	324	324	100.0	257	82.4	57	17.6

* Conditions for collection of sputum and methods of examination by smear and culture were similar in the two laboratories.

Bull IUAT 1968; 41: 139-47

used; direct smears were examined by fluorescence microscopy, and cultures by a modified Petroff method on Löwenstein-Jensen medium without potato starch (Tuberculosis Chemotherapy Centre, Madras, 1959). The difference between London and Madras lay in the severity of disease in the patients examined. In London the patients came with early disease, often not yet bacteriologically positive. However, in Madras, they usually had severe disease when they first attended for treatment. Among specimens found positive (either on smear or on culture examination), a positive result was obtained only on culture in 64.6% of 776 specimens in London, but in only 17.6% of 324 specimens in Madras.

Rao *et al.* (1966) also found that the value of culture depended on the type of case being examined. The proportion of specimens which were positive only on culture was 52-61% in sputum from people aged 5 years or more with any abnormality in the chest radiography, 28-39% in patients aged 20 years or more with pulmonary symptoms, 18% in patients suspected of having pulmonary tuberculosis, and only 5-13% in patients definitely diagnosed as having tuberculosis but so far untreated.

The value of direct smear examination in chest clinics in developing countries can be improved by examining more than one specimen from each patient. Table II sets out the results of smear and culture examination of specimens obtained from 348 patients who had a positive result by either method on one or more of four sputum specimens examined before the start of chemotherapy at Madras (Andrews and Radhakrishna, 1959). If the results on only one specimen were considered for each patient, 76.7% of the 348 patients had a positive smear. However, the proportion of patients with at least one positive smear result rose to 85.3% when the results on all 4 specimens were considered.

TABLE II

Examination of multiple specimens from 348 patients by smear and culture (Madras laboratory)

Number of specimens per patient	Culture positive		Smear positive	
	No.	%	No.	%
1	324	93.1	267	76.7
2	339	97.4	289	83.0
3	345	99.1	292	83.9
4	346	99.4	297	85.3

In summary, smear examination, especially of several specimens from each patient, is almost as efficient as culture examination in clinics in developing countries where most patients attend with far advanced disease. The additional benefit of culture examination is greater as the case-finding net is spread to include a higher proportion of patients with minimal disease. In particular, culture is advisable in special case-finding programmes when bacteriological examinations are done, for instance, on anyone who can produce sputum. Thus, in the early stages of the development of a Health Service in the developing countries, the provision of extensive facilities for cultural work is usually not

desirable, since it is expensive and requires scarce, trained personnel. However, as the Service is extended, the need for additional facilities for culture will steadily increase.

TRANSPORT OF SPUTUM

In a series of prevalence surveys undertaken by the World Health Organization in Africa, sputum specimens were examined by direct microscopy in the local laboratory. Positive specimens, together with a sample of negative specimens, were sent to a European laboratory, usually the State Serum Institute, Copenhagen, for culture. The results of the culture examination were poor; the contamination rate was high and many of the specimens which were positive on direct smear examination were negative on culture. Šula, Sundaresan and Langerova (1960) found a loss of viability of suspensions of tubercle bacilli transported over a 15-day period to Africa and back to Prague. These findings suggested that there might be considerable difficulty in operating a central laboratory for cultural work to which specimens were sent from peripheral clinics.

TABLE III

Effect of transport of sputum specimens on the proportions of smear-positive, culture-negative and contaminated results

Collection of specimens			Total specimens	Positive by smear or culture (%)	Per cent specimens	
Site of patients	Date	Period of transit to laboratory (days)			Smear-positive, culture-negative	Contaminated
Britain	1960–62	1–3	803	74.5	1.4	1.4
Hong Kong	1962	2–7	869	71.1	5.4	0.5
Hong Kong	1965–66	2–7	745	96.9	0.7	1.1
Kenya	1964–65	1–7	869	75.0	0.3	2.6

More recent experience has, however, shown that loss of viability and contamination during transport of specimens need not be so serious a problem (Table III). In a survey of the prevalence of acquired drug resistance in Britain (British Tuberculosis Association, 1963), sputum specimens were sent unrefrigerated by post to central laboratories, and the interval between collection of the sputum from the patient and its receipt in these laboratories was usually 1-3 days. The proportions of specimens which were smear-positive, culture-negative or contaminated were both low (1.4%). During a survey of the prevalence of drug resistance in patients presenting at chest clinics in Hong Kong (Hong Kong Government Tuberculosis Service/British Medical Research Council, 1964), sputum specimens were stored in a refrigerator in Hong Kong, sent unrefrigerated by air to Britain and again stored in a refrigerator before processing in London. The interval between collection in Hong Kong and processing in London was 2-7 days. The contamination rate was only 0.5%. A higher proportion of the specimens was smear-positive, culture-negative (5.4%) than was usually encountered in the laboratory in London, but many of the patients had

already received chemotherapy before the specimens were obtained and such specimens are known to be often smear-positive but culture-negative. Even so, of 618 specimens positive by smear or culture, 79.9% were positive by smear and 92.4% were positive by culture. More recently, sputum specimens from untreated patients, which were flown from Hong Kong and processed in London under similar conditions, yielded a very low proportion of smear-positive, culture-negative results.

In a recent survey of drug-resistance in Kenya, sputum specimens were sent unrefrigerated by various means of transport to the central laboratory in Nairobi. Again, the proportion of smear-positive, culture-negative specimens was only 0.3%, and the contamination rate was also fairly low. In none of these studies was the viability of the organisms in the sputum related to the length of the transit period. Finally, Rao *et al.* (1966) reported the results of a series of surveys in India in which the sputum specimens were transported for 1-7 days to central laboratories for culture. They found that neither the proportion of specimens yielding positive cultures nor the contamination rate were related to the transit period except for a suggestion of a slight loss in viability in specimens which had taken 7 days to arrive in the laboratories. The presence or absence of refrigeration during transport did not affect the results.

On present knowledge, it therefore seems that reasonably satisfactory cultures should be obtained if sputum is sent unrefrigerated and arrives at the laboratory within 5-7 days of collection. Accurate definition of the maximal safe transit time is of great importance because on it depends how large an area can be served by a central laboratory undertaking culture work. More information on this question would be desirable.

DIRECT SMEAR EXAMINATION: FLUORESCENCE MICROSCOPY

Two methods of direct smear examination are well established: (1) the smear may be stained by the Ziehl-Neelsen method and examined by ordinary bright-field microscopy with a high magnification, oil immersion objective; and (2) the smear may be stained with the fluorescent dyes auramine or rhodamine, and examined by fluorescence microscopy, using a low magnification objective. Claims have been made that Ziehl-Neelsen stained smears can be examined more rapidly by using contrasting filters or phase contrast microscopy, but these methods are less well documented.

The principal advantage claimed for fluorescence microscopy is increased speed in examining smears. In the fluorescence microscope the bacilli stand out as brightly stained rods against a dark background, and can be seen at a much lower magnification than is possible when Ziehl-Neelsen stained smears are examined by bright-field microscopy. The field seen under a fluorescence microscope is 10-20 times larger in area, and the same area of the smear can thus be examined in a much shorter time.

When fluorescence microscopy was introduced by Hagemann (1937), the equipment available was primitive. These early systems were difficult to set up and had to be used monocular in a dark room, since too much light was lost in a binocular head. Inevitably, some unfavourable reports on the method were published (Holm and Plum, 1943; Darzins, 1958).

Since then, considerable improvements have been made in the design of fluorescence microscopes, spurred on by the need for better equipment for

fluorescent antibody studies in general immunology. Reports on the relative merits of the conventional Ziehl-Neelsen procedure and fluorescence microscopy over the past 10 years have uniformly favoured the fluorescence method (Gray, 1953; McClure, 1953; Von Hubler and Murray, 1954; Needham, 1957; Holst, Mitchison and Radhakrishna, 1959; Kuper and May, 1960; Braunstein and Adriano, 1961; Bell and Brown, 1962; Truant, Brett and Thomas, 1962; Wellmann and Teng, 1962; Gilkerson and Kanner, 1963; De Groat and White, 1964; Koch and Cote, 1965; Yamaguchi and Braunstein, 1965). These authors have almost all used equipment that was efficient by modern standards. We have used fluorescence microscopy at the Royal Postgraduate Medical School for the past 25 years and the method is in use in most laboratories working in association with the British Medical Research Council, for instance, at the Tuberculosis Chemotherapy Centre, Madras, in Nairobi, Kampala and Dar-es-Salaam in Africa, and in Hong Kong. Of particular importance is the study of Bennedson and Larsen (1966) from the State Serum Institute, Copenhagen, who reported that fluorescence microscopy was about five times more rapid than Ziehl-Neelsen microscopy and that the specificity of the results was at least as good.

Although smears can be examined by fluorescence microscopy much more rapidly than they can by Ziehl-Neelsen microscopy, a fluorescence microscope is considerably more expensive than a simple microscope equipped only for bright-field work, and the running costs are also higher. I have therefore attempted to estimate the relative costs of the two methods under varying circumstances. For this purpose, three microscopic systems have been considered:

1. A conventional microscope, at an initial cost of £100.

Many bright field microscopes cost considerably more, but some makes are available at about this price.

2. A cheap form of fluorescence microscope.

For this I have taken the Gillett and Sibert 'Conference' microscope equipped with a 100 watt quartz iodine lamp, a 30/063 exciter filter (corresponding to BG12), an aplanatic fl.4 condenser, Watson ×20 and ×40 (or ×70) paramet objectives for unmounted specimens (these have a greater light-passing capacity than the standard Gillett and Sibert objectives), a 10/285 barrier filter and an inclined monocular head with ×8 eyepiece. The initial cost of this microscope is approximately £250. The performance is not as good as with the best fluorescence equipment and the microscope must be used monocular in a darkened room. The quartz iodine lamps cost much less than mercury vapour lamps but have a shorter length of life than ordinary filament bulbs; they do not deteriorate slowly, as do mercury vapour lamps. If an oil-immersion objective is bought, this microscope can also be used for ordinary bright field work.

3. A highly efficient, but expensive fluorescence microscope. For this I have taken the Leitz SM microscope with the HBO 200 mercury vapour lamp, a BG12 exciter filter, a ×25 apochromatic objective, a ×40 (or, preferably, ×70) fluorite fluorescence objective for uncovered specimens, an achromatic fl.4 condenser, a K530 barrier filter and a binocular head with ×8 eyepieces. This microscope costs about £450. It can be used binocular in dim daylight with very satisfactory results. The mercury vapour lamp deteriorates slowly and it is important to change the lamp before it becomes too inefficient for satis-

TABLE IV

Running cost of conventional bright field microscopy of Ziehl-Neelsen stained smears (ZN), fluorescence microscopy with cheap equipment (Fl 1) and fluorescence microscopy with expensive equipment (Fl 2)

No. of smears per day	Method	Microscope			Technician's annual salary (£)		Total annual cost (£)	
		Initial cost (£)	Annual depreciation (£)	Annual running cost (£)	Low (£160 p.a.)	High (£800 p.a.)	Low salary	High salary
10	ZN	100	15	2	20	100	37	117
	Fl 1	250	30	9	10	50	49	89
	Fl 2	450	45	30	4	20	79	95
30	ZN	100	15	4	60	300	79	319
	Fl 1	250	30	12	30	150	72	192
	Fl 2	450	45	40	12	60	97	145
50	ZN	200	30	6	100	500	136+	536+
	Fl 1	250	30	15	50	250	95	295
	Fl 2	450	45	50	20	100	115	195
100	ZN	300	45	12	200	1000	257+	1057+
	Fl 1	250	30	30	100	500	160	560
	Fl 2	450	45	65	40	200	150	310
200	ZN	400	60	18	400	2000	478+	2078+
	Fl 1	500	60	60	200	1000	320+	1120+
	Fl 2	450	45	100	80	400	225	545

+ More than one microscope is required and the requirements for bench space are therefore increased.

factory use. At a small additional cost for a transformer unit and oil-immersion objective, the microscope can also be used for ordinary bright field work.

Table IV attempts to estimate the cost of using these different microscope systems according to the average number of smears examined per day. For these calculations it has been assumed that one technician can only spend about 4-5 hours a day looking at slides since a longer period would be very tiring. A total of about 48 Ziehl-Neelsen smears could be examined properly in this time. Using Bennedson and Larson's estimate that efficient fluorescence microscopy can be done 5 times more rapidly, the corresponding maximum by this method would be 240 smears per day. When the average number of smears per day is over 48, two bright field microscopes would be required, though only one fluorescence microscope would be necessary. Similarly, when the average number of smears per day is 100, three bright field microscopes would be needed. These estimates accord well with practice since, in a study undertaken at Madras (Holst, Mitchison and Radhakrishna, 1959) it was found necessary to use three bright field microscopes and one fluorescence microscope when the average number of smears examined per day was about 100. The cheap form of fluorescence microscope is likely to be less efficient than the expensive microscope, mainly because fatigue, due to lesser brightness of the bacilli and

the use of a monocular microscope, would limit the number of specimens that could be examined in a given time; it has been assumed to be capable of examining smears about twice as rapidly as by the Ziehl-Neelsen method.

The total, annual cost (Table IV) is composed of the following items:

1. the annual depreciation of the microscope,
2. the running cost of the microscope, principally replacement of the lamp,
3. the technician's salary while examining smears.

Since the salary of a technician varies greatly from country to country, two salary levels have been assumed: (*a*) a low one of £160 per annum, representative of the amount paid in some developing countries and (*b*) a high one of £800 per annum, representative of salaries paid in economically developed countries.

It is evident that when a low salary is paid to technicians, as in developing countries, Ziehl-Neelsen microscopy is the cheapest method when less than 30 smears are examined per day. When the number of smears increases to 30-50 smears per day, the cheaper form of fluorescence microscope is the most economical method. However, if the number of smears per day is 100 or more, fluorescence microscopy with the more expensive equipment costs least. When high salaries are paid to technicians, fluorescence microscopy is always cheaper than Ziehl-Neelsen microscopy, though the margin of difference is small when only 10 smears are examined a day. When the number of smears examined a day rises to 100 or more, a fluorescence microscope with expensive equipment is the most economical method and costs three to four times less than Ziehl-Neelsen microscopy.

In addition to the costs of the microscope and the technician's time, the space used in the laboratory is a further consideration. When one fluorescence microscope can be used in place of 2, 3 or even 4 bright field microscopes for the Ziehl-Neelsen method, there is an additional saving. This is indicated in Table IV by the '+' sign to show that additional space is taken up by the presence of several microscopes.

The cost of preparing smears, staining and recording the results is likely to be fairly similar for the different methods and has therefore not been estimated.

CULTURE EXAMINATION

A developing country can only afford a small number of laboratories that are properly equipped for culture of tubercle bacilli. Since the capital cost of equipment and its satisfactory maintenance are much larger items of expenditure than the salary of technicians, it is usually not economical to use highly simplified cultural procedures which are less efficient than slightly more complicated procedures. For instance, methods involving the use of a centrifuge have been shown to be more efficient than simple decontamination and culture of sputum directly onto medium (Public Health Laboratory Service, 1958). The additional cost of a centrifuge and of the time taken in processing the specimen is very small compared to the total cost of the running of the laboratory. It is far more expensive to use inefficient methods and therefore to send out misleading reports. It is not appropriate to discuss the great variety of technical methods of culture here, but the principle of using a reasonably efficient method, even in developing countries, should be stressed.

IMPLICATIONS ON THE ORGANIZATION OF LABORATORY SERVICES IN DEVELOPING COUNTRIES

I. In the earliest phases of development of a bacteriological service for tuberculosis in a developing country, the most economical and efficient arrangement appears to be:

i. The performance of Ziehl-Neelsen microscopy in small laboratories at peripheral clinics and hospitals where the average number of smears per day does not exceed 30. These peripheral laboratories would also use the microscope for other purposes. Cultures would not be undertaken.

ii. A central laboratory (or, in a large country, more than one such laboratories) which would do fluorescence microscopy with expensive equipment, culture for tubercle bacilli and sensitivity tests. Sputum specimens would be sent from the peripheral clinics and hospitals as long as the time taken in transit was not likely to exceed 5 days.

iii. Special case-finding procedures, such as sputum surveys or bacteriology done in association with mass radiography, should be undertaken at the central laboratory. For this purpose, sputum specimens should be collected in the field and smears made at the central laboratory, since it is unreasonable to expect that proper conditions for the preparation of satisfactory smears will be available under field conditions. All specimens should be examined by fluorescence microscopy and if possible by culture.

II. As the bacteriological services extend in a developing country and especially as the clinician begins to utilise bacteriological methods in preference to radiography, there would be an increasing demand for smear and culture examinations. In the busier peripheral centres it would then become economical to purchase the cheaper type of fluorescence equipment, which could also be used for non-tuberculous examinations. The number of larger laboratories capable of doing culture for tubercle bacilli would also need to be increased since this would mean shorter delay in the transport of sputum specimens and in the reporting of the results. However, sensitivity tests should still be confined to one reference laboratory as it is usually quite easy to transport cultures of tubercle bacilli within the country and personnel with the necessary skill and interest would be hard to find.

REFERENCES

ANDREWS, R. H. and RADHAKRISHNA, S. (1959): A comparison of two methods of sputum collection in the diagnosis of pulmonary tuberculosis. *Tubercle (Lond.), 40*, 155.

BELL, W. J. and BROWN, P. P. (1962): Fluorescence microscopy in the laboratory diagnosis and assessment of pulmonary tuberculosis. *Centr. Afr. J. Med., 8*, 4.

BENNEDSON, J. and LARSEN, S. O. (1966): Examination for tubercle bacilli by fluorescence microscopy. *Scand. J. resp. Dis., 47*, 114.

BRAUNSTEIN, H. and ADRIANO, S. M. (1961): Fluorescent stain for tubercle bacilli in histologic sections. *Amer. J. clin. Path., 36*, 37.

BRITISH TUBERCULOSIS ASSOCIATION (1963): Acquired drug-resistance in patients with pulmonary tuberculosis in Great Britain — A national survey, 1960-61. *Tubercle (Lond.), 44*, 1.

DARZINS, E. (1958): In: *The Bacteriology of Tuberculosis*, p. 191. University of Minnesota Press, Minneapolis, Minn.

DE GROAT, A. and WHITE, M. (1964): Observations on fluorescent staining for the detection of acid-fast organisms in sputa and tissues. *J. Mich. med. Soc., 63*, 34.

GILKERSON, S. W. and KANNER, O. (1963): Improved technique for the detection of acid-fast bacilli by fluorescence. *J. Bact., 86*, 890.

GRAY, D. F. (1953): Detection of small numbers of mycobacteria in sections by fluorescence microscopy. *Amer. Rev. Tuberc., 68*, 82.

HAGEMANN, P. (1937): Fluoreszenz mikroskopische Untersuchungen über Virus und andere Mikroben. *Zbl. Bakt., I. Abt. Orig., 140*, 184.

HAGEMANN, P. (1938): Fluoreszenzsäbrung von Tuberkelbakterien mit Auramin. *Münch. med. Wschr., 85*, 1066.

HOLM, J. and PLUM, N. (1943): On the demonstration of tubercle bacilli in the sputum in man. *Acta tuberc. scand., 17*, 13.

HONG KONG GOVERNMENT TUBERCULOSIS SERVICE/BRITISH MEDICAL RESEARCH COUNCIL COOPERATIVE INVESTIGATION (1964): Drug-resistance in patients with pulmonary tuberculosis presenting at chest clinics in Hong Kong. *Tubercle (Lond.), 45*, 77.

HOLST, E., MITCHISON, D. A. and RADHAKRISHNA, S. (1959): Examination of smears for tubercle bacilli by fluorescence microscopy. *Ind. J. med. Res., 47*, 495.

KOCH, M. L. and COTE, R. A. (1965): Comparison of fluorescence microscopy with Ziehl-Neelsen stain for demonstration of acid-fast bacilli in smear preparations and tissue sections. *Amer. Rev. resp. Dis., 91*, 283.

KUPER, S. W. A. and MAY, J. R. (1960): Detection of acid-fast organisms in tissue sections by fluorescence microscopy. *J. Path. Bact., 79*, 59.

MCCLURE, D. M. (1953): Development of fluorescence microscopy for tubercle bacilli and its use as an adjunct to histological routine. *J. clin. Path., 6*, 273.

NEEDHAM, G. M. (1957): Comparative efficacy of direct microscopy (two methods) and cultures in the diagnosis of tuberculosis. *Mayo Clin. Proc., 32*, 1.

PUBLIC HEALTH LABORATORY SERVICE (1958): The effect of centrifugation on the results of culture of tubercle bacilli from sputum treated by the sodium hydroxide method. *Mth. Bull. Minist. Hlth Lab. Serv. (Lond.), 17*, 103.

RAO, K. P., NAIR, S. S., COBBOLD, N. and NAGANATHAN, N. (1966): Some operational factors influencing the utility of culture examination in the diagnosis of pulmonary tuberculosis. *Bull. Wld Hlth Org., 34*, 589.

ŠULA, S., SUNDARESAN, T. K. and LANGEROVA, M. (1960): Effects of storage and transport on the cultivability of mycobacteria. *Bull. Wld Hlth Org., 23*, 635.

TRUANT, J. P., BRETT, W. A. and THOMAS, Jr., W. (1962): Fluorescence microscopy of tubercle bacilli stained with auramine and rhodamine. *Henry Ford Hosp. med. Bull., 10*, 287.

TUBERCULOSIS CHEMOTHERAPY CENTRE, MADRAS (1959): A concurrent comparison of home and sanatorium treatment of pulmonary tuberculosis in S. India. *Bull. Wld Hlth Org., 21*, 51.

VON HAEBLER, J. and MURRAY, J. F. (1954): Fluorescence microscopy as a routine method for the detection of *M. tuberculosis* and *M. leprae*. *S. Afr. med., J., 28*, 45.

WELLMAN, K. F. and TENG, K. P. (1962): Demonstration of acid-fast bacilli in tissue sections by fluorescence microscopy. *Canad. med. Ass J., 87*, 837.

YAMAGUCHI, B. T. and BRAUNSTEIN, H. (1965): Fluorescence microscopy of tubercle bacilli stained with auramine and rhodamine. *Amer. J. clin. Path., 43*, 184.