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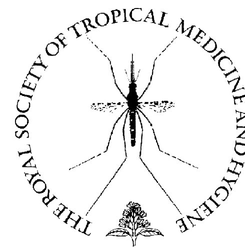

Yield of fluorescence microscopy versus culture for tuberculosis at a middle-income country referral hospital

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Summary The aim of this study was to determine the usefulness of fluorescence microscopy (FM) at a referral centre in a middle-income country. Direct Ziehl—Neelsen (ZN) and direct, as well as concentrated, smear FM were performed on 2179 suspect sputa, with Löwenstein—Jensen (LJ) culture as the gold standard. ZN, direct FM and concentration FM detected 36.0, 38.6 and 37.0%, respectively, of 272 culture-positive specimens. Patient-wise, there were 8.1% (126/1553) positives on any smear compared with 12.0% (187/1553) on any culture. ZN, direct FM and concentrated FM smear were positive in 43.3, 46.5 and 45.5%, respectively, of culture-proven cases. All differences between microscopy and culture were significant ($P < 0.001$), but not those between microscopy techniques. Acid-fast bacilli (AFB) were not rare in 60% of 48 duplicate smears, positive in ZN or FM only. Simple LJ culture, but not FM on direct or concentrated smears, was thus significantly more sensitive than ZN smears. The considerable numbers of AFB found in positive direct smears from discordant microscopy sets suggest that repeating smears can improve microscopy sensitivity more than variations of correctly executed technique, provided that overload is avoided. Thus FM could be particularly useful, as it is time-saving and could protect against the sensitivity loss associated with high workload.
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1. Introduction

Microscopic examination of sputum using the Ziehl—Neelsen (ZN) method is a sensitive tool in identifying transmitters of

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Mycobacterium tuberculosis in a community in industrialized countries (Behr et al., 1999; Grzybowski et al., 1975), and probably even more so in high-burden countries with limited access to diagnostic services. By contrast, the sensitivity of sputum smear microscopy is frequently poor in the diagnosis of an individual with pulmonary tuberculosis (TB). Various approaches to improve the diagnostic sensitivity of microscopy have been proposed. Among others, fluorescence microscopy (FM) has been found to be more sensitive than bright-field microscopy, with a similar specificity (Steingart et al., 2006). While FM has become an uncontested standard in industrialized countries, it does not necessarily follow that this holds in any setting (Tuberculosis Division International Union Against Tuberculosis and Lung Disease, 2005). FM requires a far larger initial investment than bright-field microscopy and requirements for its efficient and continued use are also higher (power supply, replacement lamps and maintenance, a suitable examination room, etc.). Further evaluation of yield and efficiency in various settings is thus indicated (Steingart et al., 2006).

The study presented here was performed at the Cayetano Heredia National Hospital (CHNH) in Northern Lima, Peru. This laboratory has a high workload carried out in a dedicated TB laboratory. The hospital serves as a tertiary care referral level, but also caters directly to a poor segment of the population living in that area. Accessibility is facilitated by the provision of free diagnostic and curative services for TB. Furthermore, the National TB Programme (NTP) has created a high level of awareness over its many years of operation. Numerous health centers in the area are expected to examine monthly quota of suspects by sputum smear microscopy for acid-fast bacilli (AFB) using the ZN technique. Despite an often-heavy workload, FM is not used at this level. The policy requires that patients repeatedly found AFB-negative on series of two sputa should all be referred for culture at referral centers such as CHNH, but only around 10% of NTP-registered cases are detected by culture.

The aim of this study was to compare the efficiency of FM with a basic Löwenstein—Jensen (LJ) culture technique in this population with a presumed high proportion of ZN smear-negative disease.

2. Materials and methods

2.1. Patients and specimens

During the 7 month study period (December 2004—June 2005), all successive adults, 15 years or older, sent to the TB laboratory by the hospital's various departments or by the surrounding health centers (upon finding negative ZN smears), were eligible for the study. Excluded were patients on treatment and salivary and low-volume (less than 2 ml) specimens. Each examinee had one or more specimens included. Free and informed consent was obtained from the study subjects or their legal guardians.

2.2. Microscopic examinations

From each specimen, a total of three smears were made: two direct smears, and the third smear made from the decontaminated and concentrated sediment that had been prepared for culture inoculation. One randomly chosen direct smear was used for ZN staining and examination with bright-field microscopy; the other two were stained with auramine and examined using FM. ZN staining followed the standard WHO technique used in the NTP (5 min staining with 0.3% carbolfuchsin, heated three times; 3% acid alcohol as decolorizer) (WHO, 1998). Auramine staining was performed according to standard technique (0.1% auramine O for 15 min), except for a 1% acid alcohol decolorizer and 0.1% potassium permanganate (Rieder et al., 1998; WHO, 1998). Reading of ZN smears was done at 1000× magnification. For FM, a 50 Watt mercury vapor epifluorescence system was used, with screening at 200× and confirmation of doubtful findings at 400× magnification. In both systems, one length of the smear had to be checked before declaring negative (corresponding to 100 high-power fields in ZN, or about 30 low-power fields in FM). The WHO/Union scale for quantification was applied to both ZN and FM smears. To account for the larger field with the lower magnification when using FM, quantification was calibrated by dividing the number of bacilli seen at 200× by 10 (WHO, 1998).

To make the additional study microscopy possible, the regular staff was reinforced by one technician plus the study coordinator, but care was taken to let the various technicians rotate in reading ZN- and FM-stained smears. Duplicate slides from the same specimen were read in a blinded way by different technicians. A 6 month pilot phase preceding the study intake assured that all readers were sufficiently proficient in the newly introduced FM technique.

The study coordinator compiled the results, and identified microscopic series with discordant results (defined as partly negative, partly positive for AFB). By way of internal quality assurance and for continuous motivation, the FM slides from such series were then reviewed by the most experienced reader, but the original results served for the study analysis.

2.3. Culture and decontamination

Decontamination used a modified Petroff method, with double volume 4% NaOH for 20 min, followed by topping up of the 50 ml conical Falcon tubes with distilled water and centrifugation at 3000 *g* for 25 min. The sediment was re-suspended in distilled water, and two LJ slants were inoculated. Incubation was done at 37 °C for a maximum of 8 weeks. *Mycobacterium tuberculosis* complex was presumptively identified based on growth rate and morphological criteria and characteristic cord formation in a ZN stain, in accordance with national guidelines.

2.4. Statistical analysis

The required sample size estimate was based on the following assumptions: 15% culture-positive prevalence among suspects, of whom 60% also positive on FM; ZN sensitivity reaches 85% of FM, or 51% compared to culture. Because of limited resources, 60% statistical power at 95% confidence level was chosen, thus arriving at 321 culture-positives or total 2140 samples to be processed by each method.

Table 1 Comparison of results on individual specimens in Ziehl—Neelsen (ZN) and fluorescence microscopy (FM), by culture status (specimens with contaminated culture excluded)

Direct FM smear	Concentration FM smear								
	ZN-positive			ZN-negative			Any ZN		
	Positive (*n*)	Negative (*n*)	Total (*n*)	Positive (*n*)	Negative (*n*)	Total (*n*)	Positive (*n*)	Negative (*n*)	Total (*n*)
Culture-positive									
Positive	69	14	83	12	10	22	81	24	105
Negative	13	2	15	9	143	152	22	145	167
Total	82	16	98	21	153	174	103	169	272
Culture-negative									
Positive	4	2	6	1	5	6	5	7	12
Negative	1	4	5	5	1834	1839	6	1838	1844
Total	5	6	11	6	1839	1845	11	1845	1856
Any culture result									
Positive	73	16	89	13	15	28	86	31	117
Negative	14	6	20	14	1977	1991	28	1983	2011
Total	87	22	109	27	1992	2019	114	2014	2128

McNemar's χ^2 test was used to compare the yield of the various microscopic examinations, with culture as the gold standard.

3. Results

A total of 2179 specimens had complete microscopy results, but 51 (2.3%) were excluded from analysis because of a contaminated culture. Of the excluded specimens, one was positive on all smears, another one was positive only on the concentrated FM smear and the other 49 were negative on all microscopic examinations. Of the remaining 2128 specimens, 272 (12.8%) were positive on culture and 151 (7.1%) were positive on at least one microscopic examination.

Table 1 compares the yield of the various smears (ZN or FM, the latter direct or from the decontaminated sediment). Overall, ZN was positive in 109 (5.1%), direct FM in 117 (5.5%) and FM of the concentration specimen in 114 (5.4%) of all specimens. Considering only the 272 culture-positive specimens, 98 (36.0%), 105 (38.6%) and 103 (37.9%), respectively, were positive on direct ZN, direct FM and concentration FM. Among the 151 specimens with any positive microscopy result, 72.2, 77.5 and 75.5% were detected by ZN, direct FM and concentration FM, respectively. All differences between yield of smears versus cultures were highly significant ($P < 0.001$), but differences between smear techniques were non-significant (NS).

Twenty-two specimens were microscopy-positive but culture-negative, with four positive only on ZN, five only on direct FM, and five only on concentration FM. The eight others were positive on at least two microscopic examinations.

To analyze the data per examinee rather than specimen, a hierarchy decided on the classification. With an allowable maximum delay of 2 months, the results of each examinee were classified as positive or negative. Any positive examination resulted in classification as 'positive examinee'; any negative culture result was defined as 'negative', even if the other cultures were contaminated. Five hundred and seventeen (32.7%) patients had two results, and 80 (5.0%) had three results, but for 985 (62.3%) patients only one specimen was examined (data not shown). In 29 patients all cultures were contaminated. These were excluded from analysis (one was positive on all smears, the other 28 were smear-negative on all examinations). Of the remaining 1553 patients, 126 (8.1%) were positive on any smear, compared with 187 (12.0%) on any culture.

Table 2 compares the patient-based yield of the various microscopy methods by culture result. Eleven and 12 patients were culture-negative but positive on ZN or on FM (both direct and concentrated smear). Among the 187 culture-positives, 81 (43.3%, ZN) to 87 (46.5%, direct FM) were positive by one of the smear techniques. Overall, ZN yielded 92 (5.9%), concentrated FM 97 (6.2%) and direct FM 99 (6.4%) smear-positive cases. The increase in yield examining more than one sample was very similar for all techniques (5.6—6.6% positive cases in ZN, 5.9—7.0% in concentrated FM and 6.0—7.2% in direct FM; data not shown). The differences between culture- and smear-positives (individual techniques, or any positive smear) were highly significant ($P < 0.001$), but not those between yields of the individual microscopy techniques.

Table 3 compares quantifications of AFB smears reported for the different techniques. Strongly positive (2+ and 3+) smears made up only 30% of ZN, but 41% of direct FM and 56% of concentrated FM smears. Smears with scanty AFB were rare with ZN (5%).

Considering all microscopy results (excluding those from specimens with contaminated culture), six specimens were exclusively positive on ZN, compared with 15 on direct FM and 14 on concentration FM. Table 4 shows the quantified results of the discordant series, considering direct ZN and FM smears independent of the non-comparable concentrated FM result. Sixty percent of the 48 isolated direct smear positives contained at least 10 AFB/100 fields (1+ to 3+ smears).

Table 2 Comparison of patient-wise results in Ziehl—Neelsen (ZN) and fluorescence microscopy (FM), by culture status (patients with all cultures contaminated excluded)

Microscopy result		Culture result		
		Positive (n = 187)	Negative (n = 1366)	Total (n = 1553)
ZN	Positive (%)	81 (43.3)	11 (0.8)	92 (5.9)
	Negative	106	1355	1461
Direct FM	Positive (%)	87 (46.5)	12 (0.9)	99 (6.4)
	Negative	100	1354	1454
Concentration FM	Positive (%)	85 (45.5)	12 (0.9)	97 (6.2)
	Negative	102	1354	1456

Table 3 Distribution of quantified smear results (positive or scanty only), by technique; specimens with contaminated culture have been excluded

Quantification grade	Percentages by technique		
	ZN (n = 109)	Direct FM (n = 117)	Concentration FM (n = 114)
Scanty, %[a]	5	22	14
1+, %[a]	65	37	30
2+, %[a]	17	20	13
3+, %[a]	13	21	43

FM, fluorescence microscopy; ZN, Ziehl—Neelsen.
[a] Scanty, 1+ to 3+: refers to grading according to the IUATLD/WHO quantification scale for ZN microscopy, converted for lower magnification used with FM.

Table 4 Quantified results of discrepant direct smears (series with contaminated culture excluded)

Quantified positive result	ZN-negative, direct FM-positive (n = 28)	ZN-positive, direct FM-negative (n = 20)	Total (%) (n = 48)
Scanty[a]	15	4	19 (40)
1+[a]	10	15	25 (52)
2+[a]	0	0	0
3+[a]	3	1	4 (8)

FM, fluorescence microscopy; ZN, Ziehl—Neelsen.
[a] Scanty, 1+ to 3+: refers to grading according to the IUATLD/WHO quantification scale for ZN microscopy, converted for lower magnification used with FM.

One quarter of the 48 yielded a negative culture (about equally divided among scanty and 1+; data not shown).

4. Discussion

More than 30 years ago, Mitchison recommended FM for settings with a high workload, showing that FM was cheaper than ZN if at least 30 smears were examined daily, and where salary costs were low (Mitchison, 1974). The surmise that the reported higher sensitivity came at the cost of specificity has accompanied FM virtually since it was introduced and has survived for a long time, even after Kubica's publication showed high similarity in specificity between ZN and FM (Kubica, 1980). A recent review confirmed that there is sufficient evidence of increased sensitivity compared with AFB conventional microscopy, without apparent loss of specificity (Steingart et al., 2006). On average, sensitivity in studies using culture as gold standard increased by 10%, while in those without the incremental yield over ZN was 9%. In a few studies this translated into a more than double detection of AFB-positive cases (Habeenzu et al., 1998; Kivihya-Ndugga et al., 2003), but more commonly the proportional detection gain remained in the 5—20% range. The relative gain might be particularly high if the yield of ZN is low because of poor technique.

The technician's quality has long been recognized as the decisive factor determining the yield from AFB microscopy (Slosarek et al., 1977). An excessive workload reduces examination time, which in turn is known to substantially diminish the yield of ZN (Cambanis et al., 2007). This may be a major reason for the large variation in gained sensitivity by FM compared to ZN in different settings, as the lower magnification and superb contrast require less time for quality reading. Compared to its perceived advantages, FM remains under-utilized in low- and middle-income countries.

Equipment costs are prohibitive but do not fully explain why this technique is used so rarely. Field visits show that many fluorescence microscopes remain unused, even in high-volume settings where they would seem to be needed desperately. Maintenance poses problems (the usual mercury-vapor lamps are short-lived, expensive and not available locally), and technicians are unfamiliar with proper adjustment of the instrument. Other factors play a role as well, including unfamiliarity with the technique, unpleasant working conditions (in isolation in a hot and dark room) and unfounded fears of ultraviolet light. There is good hope that the light emitting diode (LED) fluorescence systems under development will overcome most of these problems, allowing more decentralized use of FM for TB detection.

We evaluated ZN and FM versus culture on LJ, using a population attending a large referral center of a middle-income country to determine the usefulness of FM as an intermediary step between ZN and culture. Because of the urban setting and strong emphasis on TB detection by the NTP (i.e. awareness-raising campaigns; quota of smears required), case detection may have also been rather early. This was further suggested by the distribution of quantified ZN results, over 70% being 1+ or scanty, despite the low prevalence of HIV.

The quality of culture at CHNH might be a limiting factor in our study. Only homemade egg-media with Petroff decontamination were used, to stay closer to prevailing routine conditions in resource-limited settings (which might equally or more apply to some middle-income countries without generous external support). The decontamination technique used, i.e. double volume NaOH diluted with water during centrifugation, is a rather harsh procedure, reflected in the fairly low frequency of contamination and 15% of false-negative cultures. However, this variant is commonly encountered in such settings, as the quite common delay in specimen arrival would result in excessive contamination rates if more gentle techniques were used. Our culture results are thus likely to be a good average of what can be realistically achieved under these conditions. It is nevertheless clear that more sparing but expensive decontamination techniques, combined with liquid cultures, could have resulted in a higher yield of positive cultures. However, fast processing of sputum would be a prerequisite. Even so, significantly more cases were detected by culture, with around 45% of culture positives detected by any microscopy technique. However, microscopy also identified a number of culture-negative cases, reducing the difference between microscopy and culture to 8.1% patients detected by any smear compared with 12.0% by any culture.

Although patient-wise they fall within the 5—13% incremental yield confidence interval of the Steingart review, our results suggest that FM does not allow detection of an appreciable proportion of ZN-negative but culture-positive cases in this type of setting. Sensitivity compared to culture was only 2.2% (concentrated smear) to 3.2% (direct smear) higher than that of ZN, for an incremental yield of five to seven cases (or percent), a non-significant difference. This might be correlated with the bacillary load of sputa examined (and the patient population): although the proportion of highly positive (2+ and 3+) smears increased in FM and particularly after concentration, most of those could still be easily detected by ZN as 1+ smears by the diligent technicians, executing both techniques correctly. The lack of FM increment from NaOH/high-power centrifugation concentration is surprising, but has already been reported by others. Slosarek emphasized that only concentration after mechanical but not after NaOH homogenization of sputum resulted in better sensitivity (Slosarek et al., 1977). Murray reported the highest yield of ZN as well as FM smears from dithiotreitol-digested, centrifuged sputum, but a reduced sensitivity after NaOH digestion, particularly if not followed by centrifugation concentration. He questioned the standard procedure of making such smears in culture laboratories (Murray et al., 2003), without further elucidating the causes. It is known that NaOH-treated sputum sticks very poorly to the slides, so that many AFB might be washed off during staining, but a negative effect on AFB staining properties cannot be excluded.

Sixty percent of duplicate pairs, negative on one of the direct smears, contained sufficient numbers of AFB in the other smear to be readily recognized as positive, in both ZN and FM. Such relatively frequent discrepancies between duplicate smears are probably due to inhomogeneous distribution of the AFB in fresh sputa and have been found in other studies (Van Deun et al., 2005). This suggests that examination of a reasonable number of fields [i.e. one length (Van Deun et al., 2002)] from different smears/specimens may be more rewarding than an extended search of one smear or variations of technique, when aiming at improved sensitivity of AFB smear microscopy. However, because of the extreme negative impact of workload on smear microscopy quality, examination of more smears might better be applied only to selected suspects with persistent signs and symptoms, or abnormal chest radiography findings. At the same time careful consideration of efficiency should be given to the definition of a suspect and the number/type of sputa required for their first screening. Besides a more rational strategy, FM might then also be very useful to avoid the deleterious effect of excessive workload on AFB-microscopy sensitivity, particularly if the new LED systems would allow its expansion to the intermediate level. Culture sensitivity was clearly superior to smears in this referral setting, with early detection but low levels of HIV co-infection. However, it was not possible to compare registrations for treatment based on late results of culture versus repeated series of smears over the same period, which would be the overriding determinant of utility for the control program.

Authors' note

The opinions expressed herein are those of the author(s) and do not necessarily reflect the views of the U.S. Agency for International Development.

Authors' contributions: AVD, RC, JA, GT, KV and EG designed the study protocol; JA and RC carried out the experimental work and data collection; AVD, RC, JA and GT were responsible for data cleaning, analysis and interpretation; AVD, RC, JA, GT, KV and EG drafted the manuscript. All authors read and approved the final manuscript. AVD and RC are guarantors of the paper.

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Conflicts of interest: None declared.

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