INT J TUBERC LUNG DIS 3(12):1101–1105

A comparison of fluorescence microscopy with the Ziehl-Neelsen technique in the examination of sputum for acid-fast bacilli

F. Ba,* H. L. Rieder†

*** Laboratoire National de Référence pour la Tuberculose, Programme National de lutte contre la Tuberculose, Dakar, Senegal, † Tuberculosis Division, International Union Against Tuberculosis and Lung Disease, Paris, France**

SUMMARY

SETTING: National Tuberculosis Reference Laboratory in Dakar, Senegal.

OBJECTIVES: Comparison of results with fluorescence and bright-field microscopy for acid-fast bacilli.

METHODOLOGY: Two smears from 2630 consecutive sputum specimens between January 1996 and June 1998 were prepared for blinded examination of one smear each by the Ziehl-Neelsen technique and fluorescence microscopy at 1000× magnification. The time required to declare a slide as negative was determined for both techniques in a sample of 68 slides.

RESULTS: Concordance was 96.9% and 92.3% for diagnostic and follow-up examinations, respectively. The yield was similar with both techniques for specimens with at least 10 bacilli per 100 fields, but higher with fluorescence microscopy in those with fewer than 10 bacilli per 100 fields. The mean time required by fluorescence microscopy before declaring a slide as negative with the same magnification was 3 minutes 34 seconds, compared to 7 minutes 44 seconds with the Ziehl-Neelsen technique.

CONCLUSIONS: The results obtained with one technique are highly reproducible by the other. Fluorescence microscopy appears to be more likely to detect bacilli in paucibacillary cases than bright-field microscopy, and it more than halves the required examination time.

KEY WORDS: acid-fast; auramine O; fluorescence; microscopy; tuberculosis; Ziehl-Neelsen

THE TECHNIQUE for staining acid-fast bacilli (AFB) now attributed to Ziehl and Neelsen has evolved with contributions from many researchers.[1] A standardization of the technique was recommended by the International Union Against Tuberculosis and Lung Disease (IUATLD) in 1978.[2] The utilization of auramine O, a fluorescent dye, instead of carbol fuchsin, was first proposed in the 1930s,[3] but found widespread application in industrialized countries only some 30 years later, after a thorough re-evaluation of the technique, using a combination of auramine O and rhodamine.[4]

A study from Kenya showed superior sensitivity of fluorescence microscopy in comparison with bright-field microscopy for low density smears,[5] and fluorescence microscopy has proved at least as reliable as bright-field microscopy.[6] The most commonly cited advantage of fluorescence microscopy is the possibility to scan a sputum smear at 250× magnification rather than at 1000× magnification, allowing a theoretical reduction of examination time of the same area to one sixteenth as the surface increases by the square of the diameter. Practically, the examination time is reduced about 10-fold with fluorescence compared to bright-field microscopy using a four-fold different magnification (250× vs 1000×).[7] Disadvantages include the higher costs of investment and maintenance, and the lesser robustness of the fluorescence compared to the bright-field microscope.[8] This might explain in part the under-utilization of fluorescence microscopy in many settings.

Despite the postulated equivalence of the operating characteristics of the two methods, it is often recommended that any positive result should be reconfirmed by re-examination of the same smear by the Ziehl-Neelsen technique when the fluorescence method is being introduced.[6,7,9]

In the current study, an approach was chosen to indirectly evaluate the operating characteristics of the two methods, and to compare the time required to examine a smear using the two methods, using the same magnification, before declaring it as negative.

Correspondence to: Dr Fatoumata Ba, Programme National de Lutte contre la Tuberculose, BP 5899, Dakar-Fann, Senegal.
Article submitted 7 January 1999. Final version accepted 7 July 1999.

[A version in French of this article is available from the IUATLD Secretariat in Paris.]

MATERIALS AND METHODS

The National Reference Laboratory for Tuberculosis in Dakar, Senegal, receives sputum specimens for routine examination for AFB. Examinations are carried out for diagnostic work-up and follow-up of bacteriologic response to anti-tuberculosis chemotherapy in initially sputum smear-positive patients re-examined at 2 months, 5 months, and at the end of an 8- or 12-month treatment regimen. The definition of bacteriologically confirmed pulmonary tuberculosis in Sénégal is based on two consecutive, spontaneously produced sputum specimens positive for AFB,[10] using a standard Ziehl-Neelsen technique.[2]

From January 1996 through June 1998, two slides were prepared from each specimen received in the laboratory, one for examination by the Ziehl-Neelsen technique, the other for examination with fluorescence microscopy.

For the Ziehl-Neelsen technique, fuchsin (basic) for microscopic staining 'Gurr' 'Certistain'® with a minimum dye content of 88% (BDH, Dorset, UK) and methylene blue for microscopic staining 'Gurr' 'Certistain'® with a minimum dye content of 82% (BDH) were used. The IUATLD sulfuric method for decolorization was used.[2] For fluorescence microscopy, Auramine O (Merck, Darmstadt, Germany) in the method proposed by Chadwick with background quenching with 0.5 % potassium permanganate with a minimum content of 99% (Merck) was used. The fluorescence microscope used a mercury vapor burner as a light source.

Clinicians requesting the examination were given only the result obtained by the Ziehl-Neelsen technique in the examination of the specimen. Three technicians took turns in reading slides prepared for either bright-field or fluorescence microscopy, but care was taken that none read the slides from the same specimen with both methods. The technicians were blinded to the result obtained with the other technique. The special register for fluorescence microscopy was appended on a regular basis with the results obtained by the Ziehl-Neelsen technique recorded in the regular microscopy laboratory register. At the end of the study period, all data were computerized and analyzed using Epi Info (US Centers for Disease Control and Prevention, Atlanta, GA, Version 6.04b, 1997).

Examination and recording of the result of each slide was done in exactly the same manner for both techniques. Oil immersion with a 10× eyepiece and a 100× objective was used on both microscopes. Exactly 200 fields were examined before declaring a smear as negative or scanty positive. One hundred fields were examined for specimens found to be 1+ positive, about 50 fields for slides graded 2+ positive and about 20 fields for slides found to be 3+ positive. The grading for the number of observed bacilli was recorded according to the recommendations of the IUATLD.[2,11]

To evaluate the time required for the examinations, an additional study was conducted after the main study where 68 slides found to be negative when examined with the fluorescence method were re-stained and re-read by the Ziehl-Neelsen method. The time needed for the examination was recorded for both methods and both readings were done by the same experienced person.

RESULTS

From the 1687 consecutive patients referred for sputum smear examination during the study period, 1678 had one or more paired reading results. From these, a total of 2630 paired reading results were available (Table 1). Of these pairs, 1491 were diagnostic specimens from 549 patients, and 1139 were follow-up examinations from 1129 patients. For the latter, no attempt was made to identify the point in time during treatment at which the specimen was obtained. The standing recommendation suggests three examinations in the diagnostic process and a single examination for assessment of the bacteriologic progress. Thus, the number of patients with more than one slide at the same point of time during treatment among the latter was very small.

Table 1 Summary of a comparison of results obtained with fluorescence microscopy and microscopy using the Ziehl-Neelsen staining technique

Ziehl-Neelsen	Fluorescence microscopy						
	NEG	1–3	4–9	+	++	+++	Total
	Diagnostic						
NEG	1165	35	3	1	0	0	1204
1–3	4	10	1	2	1	0	18
4–9	1	2	4	6	0	0	13
+	2	3	3	34	6	1	49
++	0	1	1	8	43	7	60
+++	0	0	0	2	23	122	147
Total	1172	51	12	53	73	130	1491
	Follow-up						
NEG	861	48	19	5	0	0	933
1–3	9	9	9	3	0	0	30
4–9	6	4	9	17	0	0	36
+	1	5	6	71	12	0	95
++	0	0	0	9	20	1	30
+++	0	0	0	0	4	11	15
Total	877	66	43	105	36	12	1139
	Total diagnostic and follow-up						
NEG	2026	83	22	6	0	0	2137
1–3	13	19	10	5	1	0	48
4–9	7	6	13	23	0	0	49
+	3	8	9	105	18	1	144
++	0	1	1	17	63	8	90
+++	0	0	0	2	27	133	162
Total	2049	117	55	158	109	142	2630

NEG = negative; 1–3 = 1–3 AFB/100 fields; 4–9 = 4–9 AFB/100 fields; + = 10–99 AFB/100 fields; ++ = 1–10 AFB/field; +++ = more than 10 AFB/field.[2,11]

Table 2 Concordance between fluorescence and Ziehl-Neelsen microscopy

Set	Diagnosis	Follow-up	Total
All results	0.969	0.923	0.949
Excluding low scanty*	0.995	0.971	0.985
Excluding all scanty†	0.998	0.994	0.996

* Low scanty = 1–3 bacilli/100 fields.
† Scanty = 1–9 bacilli/100 fields.

An analysis by patient showed that concordance for finding a positive result (at least one AFB in any examination) or a negative result (not a single AFB) was 96.0% for diagnostic examinations and 91.9% for follow-up examinations.

A more detailed study by specimen showed that the overall concordance of the two methods, on a different slide yet from the same specimen, in deciding whether a smear was positive or negative was 96.9% for diagnostic, and 92.3% for follow-up examinations (Table 2). Because concordance decreases with lower bacillary counts, concordance was ascertained for smears showing at least four bacilli per 100 fields in both methods. For diagnostic and follow-up examinations, the respective concordances were 99.5% and 97.1%. Limiting the comparison to smears showing at least 10 bacilli per 100 fields increased concordance to 99.8% and 99.4%, respectively, for the two case categories.

The proportional distribution of positive slides, by quantification of graded smears, case category, and technique, is summarized in Table 3. For diagnostic examinations, each technique identified exactly the same number of cases with at least 10 bacilli per 100 fields. A considerable proportion of positive slides showed scanty results (one to nine bacilli per 100 fields). A large difference was observed between results obtained by the two techniques in those slides with scanty results. The difference was smaller for cases with at least 10 bacilli per 100 fields. The difference between the techniques was particularly pronounced among low scanty results (one to three bacilli per 100 fields). If only specimens with at least four AFB per field are considered, then the differences favoring fluorescence microscopy are found exclusively among follow-up examinations.

Thirty-five of the 48 cases with low scanty results identified with the Ziehl-Neelsen technique were confirmed by fluorescence microscopy on a second slide made from the same specimen. Conversely, only 34 of 117 low scanty results with fluorescence microscopy were confirmed with bright-field microscopy. It has to be considered that the remaining, non-confirmed results could indicate higher sensitivity or lower specificity (yielding more false positive results) of fluorescence microscopy. Overall, fluorescence microscopy yielded virtually the same results as the Ziehl-Neelsen technique, but consistently identified more scanty, particularly low scanty, positive specimens.

The mean time required to examine 200 fields in 68 slides before declaring them as negative by fluorescence microscopy was 3 minutes and 34 seconds, compared to 7 minutes 44 seconds by the Ziehl-Neelsen technique (in one of which one bacillus was found in the second 100 fields).

DISCUSSION

An early study re-examining the same location on a smear by the Ziehl-Neelsen technique where AFB had been found with fluorescence microscopy, indicated that some bacilli were no longer demonstrable.[12] Such a finding would reflect either decreased specificity or increased sensitivity of fluorescence microscopy. One study, done shortly after the first description of fluorescence microscopy, suggested a stronger affinity of carbol-auramine than carbol-fuchsin to mycolic acid,[13] which would favor the notion of increased sensitivity rather than decreased specificity of fluorescence microscopy for AFB.

Establishing the validity of results obtained with fluorescence microscopy by culture of a pathogenic species of the *Mycobacterium tuberculosis* complex as the gold standard is not feasible in many low-income countries. This was also the case in the reference laboratory in Senegal, where the culture tech-

Table 3 Distribution of positive slides by quantification, case category and technique

	Diagnosis				Follow-up			
	Ziehl-Neelsen		Fluorescence		Ziehl-Neelsen		Fluorescence	
Quantified result	*n*	(fraction)	*n*	(fraction)	*n*	(fraction)	*n*	(fraction)
1–3 AFB/100 fields	18	0.063	51	0.160	30	0.146	66	0.252
4–9 AFB/100 fields	13	0.045	12	0.038	36	0.175	43	0.164
All scanty	31	0.108	63	0.197	66	0.320	109	0.416
1+	49	0.171	53	0.166	95	0.461	105	0.401
2+	60	0.209	73	0.229	30	0.146	36	0.137
3+	147	0.512	130	0.408	15	0.073	12	0.046
All 1+ to 3+	256	0.892	256	0.803	140	0.680	153	0.584
All positive	287	1.000	319	1.000	206	1.000	262	1.000

AFB = acid-fast bacilli; 1+ = 10-99 AFB/100 fields; 2+ = 1–10 AFB/field; 3+ = more than 10 AFB/field.[2,11]

nique had not yet been introduced when this study was performed. The results of this study suggest that there is no need for culture as the gold standard, given the high consistency between results obtained with the two techniques on two different slides from the same specimen. In fact, this approach with two different slides indirectly demonstrates the equivalence of the techniques in the national reference laboratory. The high consistency of the two methods resolves a pre-eminent question of quality assurance, using a system of internal quality control without recourse to culture or external proficiency testing. Nevertheless, recourse to culture could be valuable, particularly for microscopically low scanty results, both for confirmation of microscopic findings and for internal quality control.

The finding of AFB during treatment is not necessarily clinically significant, as bacilli found on microscopic examination may be non-viable.[14–18] However, AFB found during treatment of an initially sputum smear-positive patient are highly likely to be tubercle bacilli, either dead or alive, because the prevalence of such a finding is high. Thus, finding AFB in a higher frequency with fluorescence microscopy would indicate a higher sensitivity rather than a lower specificity of the technique in identifying tubercle bacilli. On the other hand, identification of rare non-viable bacilli during treatment can pose a management problem, as it is usually recommended that the finding of AFB late in the course of treatment should prompt change to a second-line treatment regimen,[10] thus subjecting such patients to unnecessary treatment.

The results of this study do not allow a definitive conclusion to be drawn concerning the higher sensitivity of fluorescence microscopy, as although technicians rotated, rotation was not done systematically. It is therefore possible that a technician who was more efficient in identifying scanty positive results worked by chance more frequently with fluorescence than with bright-field microscopy. On the other hand, in the case of cytomorphologic examination of usually particularly paucibacillary specimens from patients with lymphatic tuberculosis obtained with fine needle aspiration, fluorescence microscopy had a superior sensitivity over bright-field microscopy using the Ziehl-Neelsen technique compared to histopathologic and morphologic findings.[19]

Numerous data on the sensitivity and specificity of bright-field and fluorescence microscopy compared to culture were collated and analyzed by Kubica.[6] In this study it was shown that for both methods correspondence with culture decreased with decreasing number of bacilli. Low scanty results in fluorescence microscopy correlated more poorly with culture than low scanty results found with the Ziehl-Neelsen technique. However, the magnifications used by the two techniques were not comparable. When stratified by magnification in fluorescence microscopy, at 450× magnification (the highest studied) the correspondence with culture of 3 to 6 bacilli per 100 fields was actually superior to that of 3 to 6 bacilli per 100 fields at 1000× magnification with the Ziehl-Neelsen technique.[6] It is likely that the utilization of a 1000× magnification in fluorescence microscopy, as used in the current study, would further increase the specificity of the test. No specific recommendations about cut-off points for positivity should be made from the current study, and laboratory technicians should report any AFB they observe,[11] while tuberculosis program managers should decide upon a policy on how to deal with low scanty results with either technique. This will usually entail requesting additional specimens for examination.

This study also demonstrates that fluorescence microscopy, even using oil immersion, is still more than twice as time-saving as examination with bright-field microscopy. This is most likely because the fluorescent contrast caused by stained AFB is seen so much more quickly than the red of AFB against a blue background in bright-field microscopy. It follows that laboratories introducing fluorescence microscopy may well start off with examination of slides under oil immersion until they gain sufficient confidence in the technique to proceed to examination at lower magnification. Once a lower magnification is used routinely, doubtful findings may then be examined by switching to oil immersion rather than re-staining the slide by the Ziehl-Neelsen technique.

Fluorescence microscopy is a useful, rapid, and reliable tool for the examination of specimens for AFB. It should be seriously considered for supplementary use in laboratories that handle large numbers of specimens. Initial capital investment costs, maintenance, and fluctuation in electric current will nevertheless preclude its widespread use at the peripheral level (provincial or regional) in many low income countries, but it should quite clearly be available in every national reference laboratory that handles large numbers of routine specimens.[11]

Acknowledgements

The authors greatly appreciate the technical support of Ms Andrée Mendy and Ms Maguette Diop Ndiaye and their diligence in the preparation of smears, microscopic examination, and meticulous recording of the results. The authors are also grateful to Dr Armand Van Deun for critical comments on the manuscript.

References

1 Bishop J P, Neumann G. The history of the Ziehl-Neelsen stain. Tubercle 1970; 51: 196–206.
2 International Union Against Tuberculosis and Lung Disease. Technical guide for sputum examination for tuberculosis by direct microscopy. Bull Int Union Tuberc Lung Dis 1978; (Suppl No 2): 4–16.
3 Hagemann P K H. Fluoreszenzfärbung von Tuberkelbakterien mit Auramin. Münch Med Wschr 1938; 85: 1066–1068.
4 Truant J P, Brett W A, Thomas W. Fluorescence microscopy of

tubercle bacilli stained with auramine and rhodamine. Henry Ford Hosp Med Bull 1962; 10: 287–296.
5 Githui W, Kitui F, Juma E S, Obwana D O, Mwai J, Kwamanga D. A comparative study on the reliability of the fluorescence microscopy and Ziehl-Neelsen method in the diagnosis of pulmonary tuberculosis. East Afr Med J 1993; 70: 263–266.
6 Kubica G P. Correlation of acid-fast staining methods with culture results for *Mycobacteria*. Bull Int Union Tuberc 1980; 55: 117–124.
7 Smithwick R W. Laboratory manual for acid-fast microscopy. 2nd ed. Atlanta, GA, USA: US Public Health Service, 1976.
8 Toman K. Tuberculosis case-finding and chemotherapy. Questions and answers. 1st ed. Geneva: World Health Organization, 1979.
9 Allen B W. Tuberculosis bacteriology in developing countries. Med Lab Sciences 1984; 41: 400–409.
10 Enarson D A, Rieder H L, Arnadottir T, Trébucq A. Tuberculosis guide for low income countries. 4th ed. Paris: International Union Against Tuberculosis and Lung Disease, 1996.
11 Rieder H L, Chonde T M, Myking H, et al. The public health service national tuberculosis reference laboratory and the national laboratory network. Minimum requirements, role and operation in a low-income country. Paris: International Union Against Tuberculosis and Lung Disease, 1998.
12 Richards O W, Kline E K, Leach R E. Demonstration of tubercle bacilli by fluorescence microscopy. Am Rev Tuberc 1941; 44: 255–266.
13 Richards O W. The staining of acid-fast tubercle bacteria. Science 1941; 93: 190.
14 Frette C, Jacob M P, Kauffmann F, Mitchison D A. Assessment of new sterilizing drugs for treating pulmonary tuberculosis by culture at 2 months. [Correspondence]. Am Rev Respir Dis 1993; 147: 1062–1063.
15 British Thoracic Association. A controlled trial of six months chemotherapy in pulmonary tuberculosis. First report: results during chemotherapy. Br J Dis Chest 1981; 75: 141–153.
16 Singapore Tuberculosis Service, British Medical Research Council. Clinical trial of six-month and four-month regimens of chemotherapy in the treatment of pulmonary tuberculosis. Am Rev Respir Dis 1979; 119: 579–585.
17 Morris C D W. The duration of excretion of viable bacilli in elderly patients in treatment for cavitating pulmonary tuberculosis. S Afr Med J 1996; 86: 958–960.
18 Telzak E E, Fazal B A, Pollard C L, Turett G S, Justman J E, Blum S. Factors influencing time to sputum conversion among patients with smear-positive pulmonary tuberculosis. Clin Infect Dis 1997; 25: 666–670.
19 Kumar N, Tiwari M C, Verma K. AFB staining in cytodiagnosis of tuberculosis without classical features: a comparison of Ziehl-Neelsen and fluorescent methods. Cytopathology 1998; 9: 208–214.

RÉSUMÉ

CADRE : Laboratoire National de Référence pour la Tuberculose à Dakar au Sénégal.

OBJECTIFS : Comparaison des résultats obtenus par microscopie à fluorescence ou à champ clair pour la recherche des bacilles acido-résistants.

METHODOLOGIE : Entre janvier 1996 et juin 1998, deux frottis provenant de 2630 échantillons consécutifs d'expectoration ont été préparés pour un examen à l'aveugle d'un frottis soit par la technique de Ziehl-Neelsen, soit par microscopie de fluorescence avec un agrandissement de 1000 fois. La durée nécessaire pour déclarer une lame négative a été déterminée pour les deux techniques sur un échantillon de 68 lames.

RÉSULTATS : La concordance fut respectivement de 96,9% et de 92,3% pour les examens de diagnostic ou de suivi. Le rendement fut similaire pour les deux techniques pour les échantillons comportant au moins 10 bacilles pour 100 champs, mais meilleur pour la microscopie à fluorescence chez ceux ayant moins de 10 bacilles pour 100 champs. La durée moyenne exigée par la microscopie à fluorescence avant de déclarer une lame négative avec le même agrandissement est de 3 minutes 34 secondes par comparaison à 7 minutes 44 secondes avec la technique de Ziehl-Neelsen.

CONCLUSIONS : Les résultats obtenus avec une technique sont hautement reproductibles avec l'autre. La microscopie à fluorescence semble plus apte que la microscopie à champ clair à détecter les bacilles dans les cas paucibacillaires, et elle réduit de plus de moitié le temps nécessaire à l'examen.

RESUMEN

MARCO DE REFERENCIA : Laboratorio Nacional de Referencia de Tuberculosis en Dakar, Senegal.

OBJETIVOS : Comparación de los resultados con microscopia de fluorescencia y por transiluminación para los bacilos ácido-alcohol resistentes.

METODOLOGIA : Se prepararon dos extendidos de 2630 muestras consecutivas de esputos entre enero de 1996 y junio de 1998 para examen ciego, uno de ellos por la técnica de Ziehl-Neelsen y el otro por microscopia fluorescente a un aumento de 1000×. El tiempo requerido para diagnosticar un preparado como negativo se determinó con ambas técnicas en una muestra de 68 extendidos.

RESULTADOS : La concordancia fue de 96,9% y de 92,3% para el diagnóstico y seguimiento, respectivamente. El rendimiento fue similar con ambas técnicas para las muestras que tenían como mínimo 10 bacilos cada 100 campos, pero fue mayor con la microscopia fluorescente para las muestras con menos de 10 bacilos por 100 campos. El tiempo medio requerido para diagnosticar un preparado como negativo con microscopia fluorescente fue de 3 minutos 34 segundos, comparado con los 7 minutos 44 segundos con la técnica de Ziehl-Neelsen.

CONCLUSIONES : Los resultados obtenidos con una técnica son muy reproducibles con la otra. La microscopia fluorescente parece detectar con más probabilidad los bacilos en los casos paucibacilares y requiere la mitad del tiempo que la microscopia por transiluminación.