Dx - microscopy

East African Medical Journal Vol 70 No 5 May 1993
A COMPARATIVE STUDY ON THE RELIABILITY OF THE FLUORESCENCE MICROSCOPY AND ZIEHL-NEELSEN METHOD IN THE DIAGNOSIS OF PULMONARY TUBERCULOSIS
W.A. Githui, HDMLT, MSc, Kenya Medical Research Institute, Respiratory Diseases Research Unit, F. Kitui, HDMLT, National Public Health Laboratory Services, Tuberculosis Reference Laboratory, E.S. Juma, HDMLT, D.O. Obwana, CMLT, J. Mwai, DMLT and D. Kwamanga, DPH, DEHSc, MSc (Comm. H), Kenya Medical Research Institute, Respiratory Diseases Research Unit

# A COMPARATIVE STUDY ON THE RELIABILITY OF THE FLUORESCENCE MICROSCOPY AND ZIEHL-NEELSEN METHOD IN THE DIAGNOSIS OF PULMONARY TUBERCULOSIS

W. GITHUI, F. KITUI, E.S. JUMA, D.O. OBWANA, J. MWAI and D. KWAMANGA

## SUMMARY

**Pulmonary tuberculosis (PTB) is the most common presentation of tuberculosis (TB) in Kenya. For the diagnosis of PTB the sputum smear is used because it is technically simple, non-invasive and cheap. The reliability of direct smear examination for the diagnosis of TB has however frequently been questioned. To address this problem, a study comparing the reliability of fluorescence microscopy (FM) and Ziehl-Neelsen (ZN) staining method for examination of direct smear in the diagnosis of PTB was carried out at the Respiratory Disease Research Unit Laboratory, Nairobi, Kenya. A total of 1480 sputum specimens collected from patients with suspected PTB were analyzed. Two direct smears were prepared from each specimen, one stained using FM and the other using the ZN method. Culture results were used as the gold standard for assessment. Specificity was 97% and 96% for FM and ZN methods, respectively. The sensitivity of the FM method was 80% and that of the ZN method 65% ($p < 0.001$). Overall agreement was 86.8%. Positive smears which were missed on the ZN stained smears (15%) contained low density bacilli on both FM stained smears and on culture. The use of FM greatly improves the diagnostic value of the sputum smear especially in patients with a low density of bacilli who are likely to be missed on ZN stained smears. The method is economical in both time and expense and is recommended for laboratories handling large numbers of sputum specimens.**

## INTRODUCTION

The examination of direct sputum smear represents a major tool for diagnosis of pulmonary tuberculosis (PTB) in most developing countries. This is because the method is readily available, non-invasive and cheap. Two standard staining methods, namely, Fluorescence microscopy (FM) and Ziehl-Neelsen (ZN) have been described(1,2). The ZN method has been commonly used, particularly in developing countries, because of its simplicity and low cost. However, the reliability of the direct sputum smear examination as a single test for the diagnosis of TB has earlier been questioned(3). It is estimated that 60% to 70% of all tuberculosis cases is diagnosed through sputum smear examination(4). This low yield by smear may be attributed, partly, to the few numbers of bacilli present in a sputum specimen which greatly increases the chance of obtaining a false negative. Smear positivity has been found to be affected by the number of bacilli in a sputum specimen(5).

Several reports(1,2,6) have indicated a higher sensitivity and a greater saving in time when FM is used for the diagnosis of TB than when the ZN method is used. In developing countries, this facility has largely been confined to research and teaching institutions. One of the reasons proposed as to why FM has been unsuitable for routine use is the high cost of the equipment.

Following the current global increase in tuberculosis cases in part, due to the advent of Human Immunodeficiency Virus infection(7,8), a significant increase in the TB laboratory work-load has been observed. In addition to this, the diagnosis of TB, by smear, is made more difficult than ever before especially among the HIV/TB patients with low concentrations of bacilli in their pretreatment sputum specimens(9) and who may therefore have negative sputum smears. In view of this, the need for quicker and more sensitive methods than those currently used for diagnosis of TB has become imperative. We, therefore, performed a study comparing the reliability of FM and ZN stained sputum smears in the diagnosis of pulmonary TB.

## MATERIALS AND METHODS

*Sputum specimens:* A total of 1480 sputum specimens were received at the Tuberculosis Reference Laboratory in Nairobi to examine for acid fast bacilli (AFB). The specimens were collected from patients attending chest clinics both in Nairobi and in the peripheral hospitals. Prior to preparation

KW: lab, dx, microscopy, ZN, fluorescence, specificity, sensitivity

2083

of smears all specimens were homogenized using a mechanical shaker. Two direct smears were prepared from each specimen.

*Smear preparation:* Using a standard procedure(5), two direct smears were prepared from each specimen.

*Staining: Fluorescence microscopy:* A standard staining procedure previously described by Lempert(1) was used. One set of heat fixed smears was stained with auramine phenol, rinsed, decolourized and counterstained in bulk lots of 48 using an automatic (Shandon-Elliot) staining machine(10).

*Ziehl-Neelsen (ZN) method:* Another set of heat fixed smears were stained with carbol fuchsin(1,2) heated to steamrise after which rinsing, decolourization and counterstaining was carried out using a bulk staining method(11).

Smears for both FM and ZN staining methods were examined using a Leitz SM binocular fluorescence microscope and a bright field binocular microscope respectively. The smears were quantified for the presence of bacilli by eye as scanty (+) moderate (++) and heavy (+++)(12). Smears were considered negative when less than 3 bacilli were found in the entire smear.

*Culture:* Sputum specimens were processed for culture using Aber's modified sodium hydroxide technique(13). For each specimen 2 slopes of Lowenstein-Jensen (LJ) medium were inoculated and incubated for a total of 8 weeks before they were declared negative. Growth was graded, according to the number of viable colonies: for 1-20, the actual number was recorded, 21-100 as moderate growth, more than 100 distinguishable colonies as innumerable colonies (IC) and for more than 100 indistinguishable colonies as confluent growth (CG).

*Data analysis:* Culture results were used as the gold standard in the assessment of sensitivity and specificity. Sensitivity was defined as the proportion of PTB cases correctly identified by microscopy to the total number of cases and specificity as the proportion of AFB negative cases correctly identified by microscopy to the total number of AFB cases correctly identified by culture. Using the EpiInfo package for statistics, the Chi-square test was used for comparison of differences between the two techniques.

## RESULTS

Table 1 indicates smear and culture results of 1480 sputum specimens. Of the total specimens, 952 (64.3%) were culture positive and 778 (52.5%) were smear positive while 600 (40.5%) were positive by both FM and ZN staining methods and 160 (10.8%) by FM and 1.8 (1.2%) by ZN methods only. There were 174 (11.8%) culture positive specimens which were negative on both FM and ZN stained smears. The sensitivity of the FM was 80% and that of the ZN method 65% ($p < 0.001$). Of the 528 (35.7%) specimens which were negative on culture, 500 (33.8%) were also negative on smear. Of the 28 (1.9%) smear positive but culture negative, 10 (0.7%) were positive by both FM and ZN methods, while 12 (0.8%) were positive by FM only and 6 (0.4%) by ZN method only. The specificity of FM and ZN methods was 96% ad 97%, respectively. Overall agreement was 86.8%.

**Table 1**

*Smear and culture results of 1480 sputum specimens*

| Culture | Smear | | Specimens | |
|---|---|---|---|---|
| | Fluorescence | Ziehl-Neelsen | Number | % |
| Positive | Positive | Positive | 600 | 40.5 |
| | Positive | Negative | 160 | 10.8 |
| | Negative | Positive | 18 | 1.2 |
| | Negative | Negative | 174 | 11.8 |
| Sub-total | | | 952 | 64.3 |
| Negative | Positive | Positive | 10 | 0.7 |
| | Positive | Negative | 12 | 0.8 |
| | Negative | Positive | 6 | 0.4 |
| | Negative | Negative | 500 | 33.8 |
| Sub-total | | | 528 | 35.7 |
| Total | | | 1480 | 100 |

**Table 2**

*Relationship of staining method to quantified sputum smear result*

| Smear Quantify | Fluorescence Number | % | Ziehl-Neelsen Number | % |
|---|---|---|---|---|
| *Scanty (+) | 180 | (23.7) | 50 | (8.1) |
| Moderate(++) | 250 | (32.9) | 243 | (39.3) |
| Heavy (+++) | 330 | (43.4) | 325 | (52.6) |
| Total | 760 | (100) | 618 | (100) |

* Scanty $p < 0.001$

**Table 3**

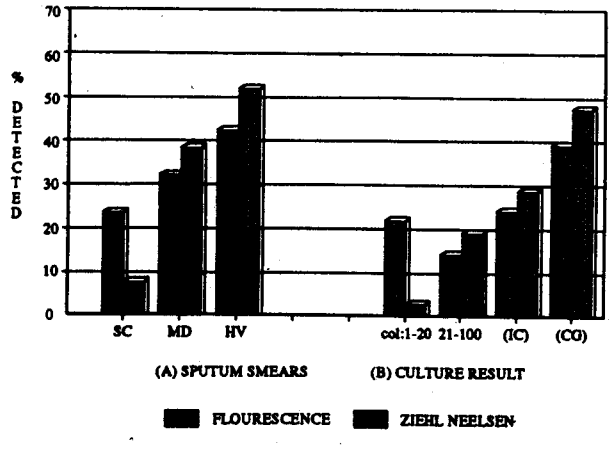
*Relationship of staining method to quantified culture results*

| Number Colonies | Fluorescence Number | % | Ziehl-Neelsen Number | % |
|---|---|---|---|---|
| * 1-20 | 168 | (22.1) | 20 | (3.2) |
| 21-100 | 110 | (14.5) | 123 | (19.9) |
| >100 (IC) | 182 | (23.9) | 181 | (29.3) |
| >100 (CG) | 300 | (39.9) | 294 | (47.6) |
| Total | 760 | (100) | 618 | (100) |

* 1-20 colonies $p < 0.001$
IC = Innumerable colonies
CG = Confluent Growth

**Figure 1**

*Relationship of staining method to quantified (A) sputum smear results and (B) culture results*




SC = Scanty, >100 = Innumerable colonies (IC)
MD = Moderate, >100 = Confluent Growth (CG)
HV = Heavy, col = Colonies

Fluorescence microscopy yielded more positive both on smear (23.7%) and culture (22.1%) than the ZN method which showed 8.1% and 3.2%, ($p < 0.001$ and $p < 0.001$, respectively, Tables 2, 3 and Figure 1). All specimens which were positive on smear by FM only also had low density of bacilli (scanty on smear and 1-20 colonies on culture). However, there were neither difference between FM and ZN methods in the overall grading of smears with moderate and heavy bacilli, nor in the numbers of isolated colonies greater than 21.

## DISCUSSION

This study has shown that fluorescence is more sensitive than the Ziehl-Neelsen method in particular where small numbers of bacilli are present in sputum specimens. This confirms the observations of previous studies(1,2,6).

The observation that the majority of specimens which were positive by FM only were also positive on culture, supports earlier findings(2) that FM does not yield more false positive than the ZN method. However, in the case of the specimens which were positive on smear but negative on culture (Table 1) the possibility of dealing with a population of dead bacilli, from patients who could have already been on treatment, cannot be excluded. This information was not obtained. On the other hand, the possibility of false positive smears(14) cannot also be excluded. When dealing with FM, skill is essential to distinguish genuine acid fast bacilli from fluorescent artifacts, the failure of which could lead to a high false positivity rate. It is therefore advisable to have all doubtful smears counterchecked by a more experienced senior technician and/or re-stain the smear over the fluorescence stain by the Ziehl-Neelsen method(2). This is an additional advantage of using FM method as the same technique cannot be applicable to the ZN stained smear.

Lempert(1) described two reasons for the superiority of FM over the ZN method with respect to weakly positive sputum specimens: (i) an increased area of smear per field and, (ii) an increased contrast between the stained bacilli and the background. In addition to these observations, it was found in our study that the use of bulkstaining method with an automatic staining machine(10) was an added advantage in terms of saving time. Furthermore, heating is not required during staining and immersion oil is also not required for smear examination.

Although this study did not address the influence of HIV infection on smear positivity, FM may be more appropriate in the diagnosis of HIV-associated tuberculosis where sputum smears are repeatedly reported as negative due to the low bacillary content. The FM method may also be useful in detecting a significant number of early PTB cases who may be excreting small numbers of bacilli with negative smears by ZN method

but who will later cavitate and become infectious.

During the study, an average of 45 sputum specimens were processed daily. Apart from time used for the preparation of smears, staining and examination by one technician using the FM method required an average time of two and a half hours. It is envisaged that three technicians using three light bright field microscopes would be required to process the same number of specimens within the same period of time when the ZN method is used. In this study however, for the purpose of comparison, only one technician was involved for each of the staining methods.

The cost of a bright light microscope is estimated at US$4,000. The cost of a fluorescence microscope and its accessories is approximately US$10,000. The salary of a qualified technician paid at a minimum rate is about US$1,200 per year. From the above estimate, a total of US$15,600 would be required for the purchase of three bright light field microscopes and a yearly pay for three technicians if the ZN method is to be used for the same amount of workload to be performed when using the FM which would require a total amount of US$11,200. When all aspects of cost including equipment and manpower as well as ease of use are taken into account, the FM method is superior to the ZN. In fact the saving could be used to buy an automatic staining machine which would lighten the workload even more. For operational convenience as well as quality control it would be ideal to have the FM facilities based at the central TB reference laboratory where skilled technical personnel are based. This will facilitate earlier diagnosis of a number of cases which would be negative on ZN method and later become positive on culture.

## CONCLUSION

In conclusion, this study shows that the use of FM greatly improves the diagnostic yield in pulmonary tuberculosis, particularly in patients with a low density of bacilli who are likely to be missed on ZN stained smears. In addition, we have observed that the FM method is easier to use, quicker and cheaper especially where large numbers of sputum specimens are processed The method is recommended for routine use in a national TB control programme.

## ACKNOWLEDGEMENTS

To Dr. P.G. Waiyaki, Director of Microbiology Research, KEMRI, Dr. H. Hawken of the British Medical Research Council/KEMRI project for their critical comments during the preparation of the manuscript. This paper is published with the permission of the Director, Kenya Medical Research Institute. The results of this paper were presented at the 5th International Congress for Infectious Disease (ICID), Nairobi, June, 1992.

## REFERENCES

1. Lempert, H. Fluorescence microscopy in the detection of tubercle bacilli. *Lancet.* **2**:818, 1944.
2. Holst, E., Mitchson, D.A. and Radhakrishna, S. Examination of smears for tubercle bacilli by fluorescence microscopy. *Ind. J. med. Res.* **47**:495, 1959.
3. Boyd, J.C. and Marr, J.J. Decreasing reliability of acid fast smear techniques of detection of tuberculosis. *Ann. intern. Med.* **82**:487, 1975.
4. Nagpaul, D.R. District Tuberculosis Control Programme in concept and outline. *Ind. J. Tubercle.* **14**:186, 1967.
5. Technical guide for sputum examination for tuberculosis by direct microscopy. *Bulletin of IUAT*, 3rd Edn, (Suppl. No. 2), 1978.
6. Allen, B.W. Isolation of mycobacterium tuberculosis from faeces. *Med. Lab. Sci.* **46**:101, 1989.
7. Slutkin, G., Leowski, J. and Mann, J.M. Tuberculosis and AIDS. The effects of the AIDS epidemic on the tuberculosis problem and tuberculosis programmes. *Bull. Int. Union Tuberc. Lung Dis.* **63**:21, 1988.
8. Harries, A.D. Tuberculosis and human immunodeficiency virus infection in the developing countries. *Lancet.* **335**:387, 1990.
9. Brindle, R.J., Nunn, P.P., Githui, W.A. Allen, B.W., Gathua, S. and Waiyaki, P. Quantitative bacillary response to treatment in HIV associated pulmonary tuberculosis. *Amer. Rev. Res. Dis.* **147**: 958, 1993.
10. Clancey, J.K., Allen, B.W., Rogers, D.T., Smith, L.S., Aber, V. and Mitchson, D.A. Comparison of machine and manual staining of direct smears for acid-fast bacilli by fluorescence microscopy. *J. Clin. Path.* **29**:931, 1976.
11. Fodor, T. Bulk staining of sputum by Ziehl-Neelsen method. *Tubercle.* **65**:123, 1984.
12. Mitchson, D.A. Standard smears for grading the content of acid-fast bacilli in sputum. *Tubercle.* **47**:289, 1966.
13. Aber, V.R., Allen, B.W., Mitchson, D.A. *et al.* Quality control in tuberculosis bacteriology. Laboratory studies in isolated positive cultures and the efficiency of direct smear examination. *Tubercle.* **61**:123, 1980.
14. Collins, C.H., Yates, M.D. and Down, G.F. False positive direct films in tuberculosis bacteriology. *Med. Lab. Sci.* **38**: 129, 1981.