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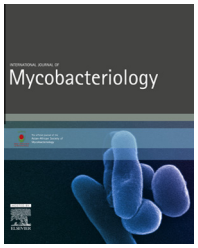

Utility of light-emitting diode microscopy for the diagnosis of pulmonary tuberculosis in HIV infected patients

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ABSTRACT

Objective: To determine the utility of light-emitting diode fluorescent microscopy (LED-FM) for the diagnosis of pulmonary tuberculosis (PTB) in HIV-infected patients.

Material and methods: A cross-sectional study was performed on 400 HIV-infected, clinically or radiologically suspected PTB patients. Two sputum specimens were collected from each patient. Two direct smears were prepared from each sputum specimen. One was stained by ZN method and another by auramine-O method and reported as per the Revised National Tuberculosis Control Programme (RNTCP) guidelines. LED-FM stained smears were reported by two readers. All specimens were cultured on LJ medium after digestion and decontamination. Address and contact details of all the patients were recorded in case record form. They were contacted for follow-up if required.

Results: Of the 800 sputum specimens processed, 130 were positive by LED-FM method and 33 were positive by ZN method; 77 specimens showed growth of MTB on LJ medium. When compared with solid culture as a reference standard, LED-FM has a sensitivity of 67.53%, specificity of 88.71%, PPV of 40% and NPV of 96.08%. Seventy-eight LED-FM positive and culture negative specimens had scanty grading. Of these, 15 were confirmed as having PTB as they responded to anti-TB treatment. The concordance between two readers was 98.75%.

Conclusion: LED-FM can be a good screening test for the diagnosis of PTB in HIV-infected patients. However, all scanty grade positive smears need to be confirmed by WHO approved gold standard.

Introduction

Tuberculosis (TB) is the most common opportunistic infection in Human Immunodeficiency Virus (HIV)-infected patients and is the most common cause of death in Acquired Immunodeficiency Syndrome (AIDS) patients accounting for one in four HIV-related deaths. Globally, in 2011, there were approximately 1.1 million HIV positive new TB cases and 4.3 million deaths due to HIV-associated TB infection [1]. Of the 5.1 million HIV-infected people in India, about half of them are co-infected with TB and approximately 200,000 of these co-infected persons will develop active TB each year [2].

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Under the Revised National Tuberculosis Control Programme (RNTCP), sputum microscopy is the mainstay in the diagnosis of pulmonary tuberculosis (PTB). The staining methods commonly used are Ziehl–Neelsen (ZN) staining and fluorescent staining (Auramine-O/Auramine-rhodamine). The sensitivity of the ZN staining method is 30–70% and still lower in HIV-infected individuals due to the paucibacillary nature of the disease [3]. Hence, smear-negative pulmonary TB is common in HIV-infected patients. Conventional fluorescent microscopy (CFM) is not possible in most health services due to its high cost, short life of the specialized mercury lamp (200 h), higher lamp warm-up time, maintenance and alignment and the need for dark examination rooms [4–6]. Though solid culture is considered the gold standard, it takes weeks for the organisms to grow. Various automated methods are now available for the rapid diagnosis of PTB. They are highly sensitive, but are costly and require trained personnel and infrastructure.

To overcome all these disadvantages, the World Health Organization (WHO) has issued a policy statement in 2010 recommending that CFM be replaced by light-emitting diode fluorescent microscopy (LED-FM) using auramine staining in all settings where CFM is currently used [7]. LED-FM has the combined advantages of light and fluorescent microscopy while minimizing their disadvantages. This low-cost method needs an LED attachment to the conventional light microscope, instead of the conventional fluorescent microscope [8]. LED-FM is 10% more sensitive than ZN microscopy [9,10]. It uses the LED as a light source rather than the mercury vapor lamp or ordinary bulb. Compared with CFM, LED-FM has lower maintenance requirements, requires less power and does not require a dark room [8,11].

Hence, the present study was performed to find out the feasibility of using LED-FM for increasing the sensitivity in the diagnosis of PTB in HIV-infected patients and to compare LED-FM and conventional microscopy using ZN staining.

Material and methods

A cross-sectional study was performed in a tertiary care hospital in Mumbai between July 2012 and June 2013 after obtaining Institutional Ethics Committee permission (EC/150/2011). 400 HIV-infected patients, clinically or radiologically suspected of PTB visiting this lab for sputum microscopy under RNTCP, were included in the study. Children ⩽5 years of age and patients already on treatment for TB were excluded from the study.

For diagnosis of PTB, each patient submits two sputum specimens (one early morning and one spot). After routine processing, leftover specimens were used for the present study. All processing was carried out in Bio Safety-Cabinet Class II with level 2 biosafety procedures. Two direct smears were prepared from each sputum specimen. One sputum smear was stained by ZN method and another by auramine-O method and reported as per RNTCP guidelines [12]. Reader 1 observed both ZN and fluorescent stained smears and time taken for examination was recorded. The fluorescent stained smears were also examined by a qualified microbiologist trained in reporting these smears under LED-FM (reader 2). Both the readers reported the fluorescent stained smear independently. All smears positive by auramine-O method were re-stained by ZN method for confirmation [13]. All 800 samples were cultured on LJ medium after digestion and decontamination by Modified Petroff's method. Only confirmed culture-positive were considered as true positive. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of both methods was calculated using culture as the gold standard. The statistical difference between the two methods was calculated using the Fischer exact test. *P* value of <0.05 was considered as significant. Address and contact details of all the patients were recorded in case record form. They were contacted for follow-up if required.

Results

Of the 400 patients, the majority of patients were from the age group of 15–40 years (54.5%), and the male to female ratio was 1.7:1. Of the 800 sputum specimens, 130 were positive by LED-FM method. As culture was considered as the reference standard for this study, LED-FM results were compared with culture results; 32 specimens showed contamination on culture, hence excluded from analysis. Of the remaining 768 specimens, 77 showed growth of MTB on culture medium.

Of these 77 culture-positive specimens, LED-FM showed positive results in 52 specimens, while ZN staining showed positive results only in 31 specimens (Table 1). Thus, LED-FM method detected 22 specimens more than ZN staining method. One specimen positive by ZN was negative by LED-FM method.

The remaining 78 LED-FM positive specimens did not show any growth on culture. All these specimens had scanty grading on LED-FM microscopy. Only two specimens were positive by ZN staining showing scanty grading again. All LED-FM positive patients were contacted after 2 months. Only 15 patients were started on anti-TB treatment, and they responded to anti-TB treatment.

All LED-FM stained smears showing 1+, 2+ and 3+ grading were positive by primary as well as re-staining by ZN staining. Of the 111 smears with scanty grading by LED-FM, 13 were positive by primary and 12 by re-staining by ZN staining.

All the smears were reported by two readers. Reader 1 reported 138 smears positive and reader 2 reported 130 of these 138 smears positive by LED-FM technique. All 8 smears reported positive by reader 1 and negative by reader 2 were scanty grading and negative by culture and both primary as well as re-staining by ZN method. Hence, they were

Table 1 – Correlation of results of microscopy with culture (*n* = 768).

	Primary ZN	LED-FM	
		POS (52)	NEG (25)
Culture POS (77)	POS (31)	30	1
	NEG (46)	22	24
Culture NEG (691)		POS (78)	NEG (613)
	POS (2)	2	0
	NEG (689)	76	613

considered as false positive. The concordance between the two readers was 98.75%. By applying Fisher exact test, the difference between the results of reader 1 and reader 2 smears was statistically significant in smear-positive cases ($P < 0.001$).

Discussion

In the present study, the majority of patients were from the age group of 15–40 years (54.5%), which may be because HIV is a sexually transmitted disease and is common in the reproductive age group. The male to female ratio was 1.7:1, which is comparable to that reported by Chaidir et al. (2013) [14]. The predominance of men among HIV patients could be because of the higher male to female ratio in India. Also in India, males are more involved in outdoor and social activities with increased chance of high risk behavior [15]. In comparison, the majority of the women in India are still restricted to household activities.

Increased smear positivity was seen by LED-FM as compared with ZN microscopy and the difference was statistically significant ($P < 0.001$). Similar results have been reported by Xia et al. from China (2013) [16]. This may be because auramine-O staining offers more contrast, with organisms appearing as brilliant yellow against a dark background making it easier for the reader to pick up even scanty bacilli.

Seventy-eight LED-FM positive specimens were culture-negative. All these 78 smears had scanty grading. All LED-FM positive patients were contacted after 2 months and were asked about their response to drug treatment. Most of the patients responded to routine antibiotics. Only 15 patients were started on anti-TB treatment based on the clinical presentation and radiological findings. These patients responded to anti-TB treatment, hence considered as cases of TB.

Negative culture results of these 15 cases may be due to over-decontamination of specimens. Specimens with low numbers of mycobacteria are particularly prone to being over-decontaminated and can result in false-negative cultures [17]. The choice of the reference standard is a key to any diagnostic evaluation. It is necessary to discuss whether solid culture can function as an adequate comparator, as it is not a perfect reference standard. The underlying assumption that solid culture has a higher specificity than smear microscopy might be debatable. The probability of obtaining a positive culture is related to the number of AFB in the specimen, with only about 50% of cultures of specimens with 1–2 AFB per 100 fields being identified as positive, increasing to 80% and 96.7% for specimens with "scanty" (1–9 AFB per 100 fields) and "1+, 2+ and 3+" AFB grades, respectively [18]. Mycobacterial culture requires a high level of expertise, and even small alterations in protocol can result in decreased yield. Specifically, decontamination is a necessary step in specimen preparation. Contact of decontamination agents for more than 20 min can kill mycobacteria [19,20]. In busy laboratories, there can be implicit motivation to over-decontaminate. While most laboratories aim for an overall contamination rate of 2–5% in mycobacterial cultures, this does not completely eliminate the possibility that some low-burden specimens will yield negative results [21].

This raises the reasonable question of whether another reference standard may be more appropriate when comparing two microscopy methods. Although culture remains the gold standard for the diagnosis of patients with active TB, a microscopy-based reference standard involving rechecking of the same smear or repeat sputum examination may be more relevant for studies designed to optimize smear microscopy [17].

LED-FM was found to be more sensitive than ZN microscopy (67.53% vs. 40.26%), but less specific (88.71% vs. 99.71%) due to many false positive results in scanty grade smears. Similar findings have been reported by Chaidir et al. from the Netherlands (2013) [14], and Albert et al. from Uganda (2013) [22].

It is important to consider that if a trade-off has to be made between sensitivity and specificity, it is preferable to err on the side of increased sensitivity and to treat a small number of patients who do not have TB. The consequences to a false-positive result like patients incorrectly being diagnosed with TB are the cost and inconvenience of taking a six-month course of treatment, and troublesome side effects. But life-threatening treatment-related events (hepatitis and Stevens–Johnson syndrome) are infrequent (5% incidence). However, if results err on the side of specificity and miss out diagnosing TB in HIV-infected patients, it might lead to serious life threatening situations.

Possible explanations for increased sensitivity of LED-FM may be because of a stronger affinity of carbol-auramine than carbol-fuchsin to mycolic acid [23], which would favor increased sensitivity rather than decreased specificity of LED-FM for AFB. Richards et al. (1941) on re-examining the same location on a smear by the ZN microscopy where AFB had been found with FM found that some bacilli were no longer demonstrable [21]. Such a finding would reflect either decreased specificity or increased sensitivity of LED-FM. The low sensitivity of the ZN microscopy may also be due to factors such as quality of smear preparation, staining technique and, as smear is examined under the microscope at 1000× magnification, more lengthy time spent on reading slides and a single bacillus may get missed, resulting in false negativity.

One smear was positive by ZN microscopy with scanty grading, but was negative by LED-FM. The culture of the same specimen was negative for MTB. This was because the specimen was salivary, and it is possible that part of the specimen used for smear preparation for fluorescent staining did not contain any bacilli. Hence, it was considered as false-negative by LED-FM method.

According to RNTCP FM manual, any doubtful smear by fluorescent staining method should be re-stained by ZN staining method to confirm morphology [13]. All smears with grading of 1+, 2+ and 3+ by LED-FM were positive by ZN staining, indicating good correlation with ZN re-staining. The majority of discordant results were obtained by smears with scanty grading.

While comparing both the staining methods, the auramine-O staining was found to be simple as there was no heating required. However, it required 20 min for staining which was almost double the time required for ZN staining. In the present study, auramine-O staining was done in a batch

of 20 slides. Though fluorescent staining takes more time to stain as compared with ZN staining, the overall time required until reporting is reduced, and it is less strenuous for the microscopist to examine the smear using LED-FM.

In the present study, the average smear reading time was 1 min 40 s for LED-FM compared with 3 min 30 s for ZN microscopy. The examination time for reporting of LED-FM was half as compared with ZN microscopy. Similar findings have been reported in different studies [8,11,14,24,25]. The time-saving achieved with LED-FM did not result from a reduction in the number of fields screened (100 fields were screened with both modalities). However, it can be ascribed to quicker scanning of each field because of increased visibility of the mycobacteria and the use of decreased magnification [26].

For comparing the cost of LED-FM and ZN microscopy, the cost of reagents and additional equipment required was considered. In the present study, consumable cost (reagents for staining) per slide for fluorescent staining was ₹ 4.14 and for ZN staining was ₹ 1.94. The light microscope used for reporting ZN microscopy was modified by using the LED attachment. The additional onetime cost for LED attachment for LED-FM used in this study was ₹ 2.5 lakhs. It was then used for reporting both types of staining methods and did not require any maintenance. The high initial cost of the equipment and expertise required for reporting the smear were its limitations.

However, these drawbacks should be weighed against the benefits, such as increased sensitivity, shorter reading time, the simplicity of the staining method and less strain on human resources before implementing LED-FM in a new set-up. Also, the development and validation of adequate and sustainable external quality assessment systems for LED-FM still remain a pre-condition for the scale-up of LED-FM.

Due to significant inter-reader variability, there is a need for proper training and very close monitoring of readers in the early stages of implementation of LED-FM.

The limitation of this study may be the use of solid culture as a reference standard. WHO-approved liquid culture or molecular method might have given a better comparison.

Conclusion

LED-FM can be a good screening test for the diagnosis of PTB in HIV-infected patients. However, all scanty grade positive smears need to be confirmed by WHO approved gold standard.

Conflict of interest

We have no conflict of interest to declare.

REFERENCES

[1] World Health Organization, HIV-Associated TB facts, 2013, <http://www.who.int/tb/challenges/hiv/>.

[2] G.R. Khatri, T.R. Frieden, Controlling tuberculosis in India, N. Engl. J. Med. 347 (2002) 1420–1425.

[3] Central TB Division, Ministry of Health and Family Welfare, Revised National Tuberculosis Control Programme, Module for Laboratory Technicians, March 2006.

[4] T. Hanscheid, The future looks bright: low-cost fluorescent microscopes for detection of *Mycobacterium tuberculosis* and *Coccidiae*, Trans. R. Soc. Trop. Med. Hyg. 102 (2008) 520–521.

[5] A. Somoskövi, J.E. Hotaling, M. Fitzgerald, D. O'Donnell, L.M. Parsons, M. Salfinger, Lessons from a proficiency testing event for acid-fast microscopy, Chest 120 (1) (2001 July) 250–257.

[6] G. Torrea, J. Chakaya, M. Mayabi, A. Van Deun, Evaluation of the FluoreslenS™ and fluorescence microscopy blinded rechecking trial, Nairobi, Kenya, Int. J. Tuberc. Lung Dis. 12 (2008) 658–663.

[7] WHO, Fluorescent light emitting diode (LED) microscopy for diagnosis of tuberculosis policy statement, 2010, <http://www.who.int/tb/dots/laboratory/who_policy_led_microscopy_july10.pdf>.

[8] A. Whitelaw, J. Peter, H. Sohn, D. Viljeon, G. Theron, M. Badri, et al, Comparative cost and performance of LED-microscopy in HIV-TB co-infected patients, Eur. Respir. J2011 (38) (2011) 1393–1397, http://dx.doi.org/10.1183/09031936.00023211.

[9] M. Bonnet, L. Gagnidze, W. Githui, P.J. Gué rin, L. Bonte, Performance of LED-based fluorescence microscopy to diagnose tuberculosis in a peripheral health centre in Nairobi, PLoS One 6 (2) (2011) e17214, http://dx.doi.org/10.1371/journal.pone.0017214.

[10] J. Minion, H. Sohn, M. Pai, et al, Light-emitting diode technologies for TB diagnosis: what is on the market?, Expert Rev Med. Devices 6 (4) (2009) 341–345.

[11] H. Albert, Y. Manabe, G. Lukyamuzi, P. Ademun, S. Mukkada, et al, Performance of three led-based fluorescence microscopy systems for detection of tuberculosis in Uganda, PLoS One 5 (12) (2010) e15206, http://dx.doi.org/10.1371/journal.pone.0015206.

[12] Central TB Division, Ministry of Health and Family Welfare, TB India 2013 Revised National Tuberculosis Control Programme Annual Status Report.

[13] Central TB Division, Ministry of Health and Family Welfare, Revised National Tuberculosis Control Programme, Manual for Sputum Smear Fluorescent Microscopy.

[14] L. Chaidir, I. Parwati, J. Annisa, S. Muhsinin, I. Meilana, et al, Implementation of LED fluorescence microscopy for diagnosis of pulmonary and HIV associated tuberculosis in a hospital setting in Indonesia, PLoS One 8 (4) (2013) e61727, http://dx.doi.org/10.1371/journal.pone.0061727.

[15] S.K. Sharma, A. Mohan, T. Kadhiravan, HIV-TB co-infection: epidemiology, diagnosis & management, Indian J. Med. Res. 121 (April 2005) 550–567.

[16] H. Xia, Y.Y. Song, B. Zhao, K.M. Kam, R.J. O'Brien, Z.Y. Zhang, et al, Multicentre evaluation of Ziehl-Neelsen and light-emitting diode fluorescence microscopy in China, Int. J. Tuberc. Lung Dis. 17 (1) (2013) 107–112, http://dx.doi.org/10.5588/ijtld.12.0184.

[17] S. Shenai, J. Minion, V. Vadwai, T. Tipnis, S. Shetty, et al, Evaluation of light emitting diode-based fluorescence microscopy for the detection of mycobacteria in a tuberculosis-endemic region, Int. J. Tuberc. Lung Dis. 15 (2011) 483–488.

[18] L.E. Cuevas, N. Al-Sonboli, L. Lawson, M.A. Yassin, I. Arbide, et al, LED fluorescence microscopy for the diagnosis of pulmonary tuberculosis: a multicountry cross-sectional evaluation, PLoS Med. 8 (2011) e1001057.

[19] T.V. Burdz, J. Wolfe, A. Kabani, Evaluation of sputum decontamination methods for *Mycobacterium tuberculosis*

using viable colony counts and flow cytometry, Diagn. Microbiol. Infect. Dis. 47 (2003) 503–509.
[20] P.T. Kent, G.P. Kubica, Public Health Mycobacteriology: A Guide for the Level III Laboratory, Atlanta, GA, USA, US Department of Health and Human Services, Public Health Service, Centers for Disease Control, 1985.
[21] O.W. Richards, E.K. Kline, R.E. Leach, Demonstration of tuberculosis bacilli by fluorescence microscopy, Am. Rev. Tuberc. 44 (1941) 255–266.
[22] H. Albert, L. Nakiyingi, J. Sempa, O. Mbabazi, S. Mukkada, et al, Operational implementation of LED fluorescence microscopy in screening tuberculosis suspects in an urban hiv clinic in Uganda, PLoS One 8 (9) (2013) e72556, http://dx.doi.org/10.1371/journal.pone.0072556.
[23] O.W. Richards, The staining of acid-fast tubercle bacteria, Science 93 (1941) 190.
[24] B.J. Marais, W. Brittle, K. Paincyzk, A.C. Hesseling, N. Beyers, et al, Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum, Clin. Infect. Dis. 47 (2008) 203–207.
[25] M. Bonnet, L. Gagnidze, W. Githui, P.J. Gué rin, L. Bonte, Performance of LED-based fluorescence microscopy to diagnose tuberculosis in a peripheral health centre in Nairobi, PLoS One 6 (2) (2011) e17214, http://dx.doi.org/10.1371/journal.pone.0017214.
[26] J. Minion, S. Shenai, V. Vadwai, et al, Fading of auramine-stained mycobacterial smears and implications for external quality assurance, J. Clin. Microbiol. 49 (2011) 2024–2026.