ORIGINAL ARTICLE

Comparative study of ZN Microscopy and LED Fluorescent microscopy along with solid culture for the diagnosis of pulmonary Tuberculosis

S. Kelamane [1] P. Mispah[1,3] S. Sandhya[1,3] S. Rao [1,2]

1. Department of Microbiology, Prathima Institute of Medical Sciences, Karimnagar
2. Malla Reddy Medical College, Hyderabad
3. Department of Microbiology, Kakathiya Medical College, Warangal

Abstract

Background: *The sputum microscopy is most effective method for detection of pulmonary tuberculosis in endemic countries. The Ziehl Nelsen (ZN) staining is highly specific but less sensitive compared to fluorescent microscopy. LED Fluorescent microscopes have now replaced the Mercury vapor lamp fluorescent Microscopes. The objective of the study was to assess the sensitivity and specificity of ZN microscopy and Light Emitting Diode Fluorescent microscopy (LED FM) among the pulmonary tuberculosis suspects.* ***Methods****: A spot and an early morning sample were collected in a sterile container from pulmonary tuberculosis suspects. The specimen was processed under ZN staining, fluorescent staining and inoculated in Lowenstein Jensen Media after decontamination.* ***Results****: Of the 383 pulmonary tuberculosis suspects, 102 (26.6%) were positive for Mycobacterium tuberculosis by culture, 12 (3.1%) contaminated and 269 (70.3%) culture negative after 8 weeks. Sensitivity and specificity of LED FM were 80.17% and 97.4% compared to ZN microscopy was 62.7% & 98.14% respectively.* ***Conclusions****: LED fluorescence microscopy is a reliable alternative to conventional methods i.e. ZN microscopy in rural and high workload settings. Furthermore LED FM is cost effective.*

Keywords*: Pulmonary Tuberculosis, ZN Microscopy, LED Fluorescent Microscopes*

Address for correspondence: Dr. Santosha Kelamane,Villa No-29, Maple Town, Bandlaguda, Suncity, Near Glendale Academy, Hyderabad- 500068 santoshak2020@gmail.com9959269111

Received on :12/10/2016 Revised :22/10/2016 Accepted :25/10/2016

Introduction

Tuberculosis or TB, is an infectious bacterial disease caused by Mycobacterium tuberculosis, which most commonly affects the lungs (Pulmonary TB) but can affect other sites as well (Extra pulmonary TB). The symptoms of active TB of the lung are coughing, sometimes with sputum or blood, chest pains, weakness, weight loss, fever and night sweats. Left untreated, each person with active TB disease will infect on average between 10 and 15 people every year [1] Despite the availability of highly efficacious treatment for decades, TBremains a major global health problem. In 1993, the World HealthOrganization (WHO) declared TB a global public health emergency, at a timewhen an estimated 7–8 million cases and 1.3–1.6 million deaths occurred eachyear. In 2010, there were an estimated 8.5–9.2 million cases and 1.2–1.5million deaths (including Deaths from TB among HIV-positive people). Indiais the highest TB burden country accounting for one fifth (21%) of the globalincidence (Global annual incidence estimate is 9.4 million cases out of which itis estimated that 2 million cases are from India). India is 17th among 22 HighCountries in terms of TB incidence rate. [2, 3] TB is the second leadingcause of death from an infectious disease worldwide (after HIV, which causedan estimated 1.8 million deaths in 2008) [2, 4]The Revised National Tuberculosis Control Program in India to combatTB, one of the main objectives of the program is toachieve and maintain the case detection of at least 70% of estimated NewSmear Positive cases in the community by quality assured sputum smear microscopy facilities which are available

through more than 13000 sputum microscopy laboratories in the health system across the country.[?] Since its inception, the Program has initiated more than 12.8 million patients on treatment, thus saving nearly 2.3 million additional lives. [5] The diagnosis of TB in any TB Control Program is aimed at early identification and prompt treatment of infectious cases of TB in the community. It is to break the chain of transmission in the community in orderto bring down the problem of tuberculosis. Diagnosis of TB depends on thehistory, physical and radiographic evidence or the presence of Acid Fast Bacilli(AFB) in acid fast smears and cultures. [6]Detection of AFB in direct smearsprepared with sputa, urine and specimens of other body fluids has considerableclinical and epidemiological value and remains the most widely used rapiddiagnostic test for TB in most developing countries. Direct (un-concentrated)smear microscopy using Ziehl-Neelsen staining is still the mainstay of TBdiagnosis in most high burden countries, including India, having remainedessentially unchanged for over 100 years. This method is rapid and inexpensiveand highly specific for Mycobacterium tuberculosisin developing countries. [7]However the main limitation of the direct sputum microscopy using ZN stain is its low sensitivity in programmatic settings, particularly in human immunodeficiency virus (HIV) co-infected patients. Light emitting diode (LED) microscopy is a novel diagnostic tool developed primarily to provide resource-limited settings with access to the benefits of fluorescence microscopy to diagnose tuberculosis. The first use of LED technology was seen, when existing fluorescent microscopes were converted to LED light sources. Considerable research and development have subsequently resulted in inexpensive, robust LED microscopes or LED attachments aimed at routine use in resource-limited settings. LED microscopy is feasible for use in resource-limited settings, having the potential to bring the benefits of fluorescent microscopy (improved sensitivity and efficiency) where needed most. In the present study an attempt has been made about the utilization of LED fluorescent Microscopy in diagnosis of pulmonary tuberculosis. The sensitivity and specificity of LED fluorescent microscopy and ZN microscopy is compared with culture as gold standard.

Materials & Methods

The present study is carried out in the Prathima Institute of Medical Sciences, Karimnagar, Telangana. Pulmonary tuberculosis suspects referred for sputum examination in "Revised National Tuberculosis Control Program-Designated Medical Center of PIMS Hospital, Karimnagar" after informed consent were enrolled in the study.

Inclusion Criteria

Pulmonary tuberculosis suspects undergoing sputum examination for diagnosis in "Revised National Tuberculosis Control Program-Designated Medical Center of PIMS Hospital, Karimnagar", irrespective of their previous history.

Exclusion Criteria

- Cases or suspected extra pulmonary tuberculosis
- Tuberculosis which has relapsed
- Tuberculosis treatment Defaulters

The sample is collected in a sterile screw capped wide neck containers. The sample was transported into PIMS tuberculosis laboratory immediately after the collection without adding any transport medium along with the clinical information form. The sample is processed as early as possible. If any delay is anticipated in processing, they were stored at 4°C. The sputum samples collected from the patients are serially recorded in the tuberculosis laboratory register and processed as follows

Microscopy: Two smears were prepared from the thick mucopurulent portion of sputum on a new unscratched slide on an area of 2 x 3 cm. They were air dried for 30 minutes and fixed theslide by heating it brieflyover the flame 3–5 times, for 3–4 seconds each time and these smears were labeled with their respective laboratory serial numbers.

First Slide: First smear was subjected to ZN staining as recommended by NTI Bangalore. The ZN stained smears were graded as follows using WHO recommendation[8].

Second slide: The smears were stained with Auromine O as recommended by RNTCP Fluorescent Manual [9] and examined under LED

fluorescent microscope Model CH21i Olympus using 10X and 40X objective lens.

***Primary Isolation of Mycobacteria, Culture on L J Medium*:** In house LJ medium prepared as follows.Two loops full of decontaminated deposit was inoculated on the entire surfaceof two LJ slopes which were prepared in our laboratory as per standard 49 in apre-sterilized inoculation hood taking necessary aseptic precautions. Date ofinoculation was noted. The slopes were incubated at 37°C for a maximumperiod of 8 weeks. The slopes were inspected daily for the growth or for thecontamination. In case of growth of Mycobacteria, date of appearance of firstcolony was noted and slopes were further incubated for more growth. In case ofcontamination, the slopes were removed.

Each strain was subjected to following of tests for species identification:

- Rate of growth
- Growth at different temperatures
- Pigmentation
- Niacin test
- Nitrate reduction test
- Catalase test

Quality Control Procedures adopted: all the procedures performed in the study were strictly in accordance to Standard quality control procedures.

Table 1: showing the grading of smears ZN stains

Number of AFB	Result	Grading	Number offields to beexamined
>10 AFB per oil immersionfield	Positive	3+	20
1-10 AFB per oil immersionfield	Positive	2+	50
10-99 AFB per 100 oilimmersion field	Positive	1+	100
1-9 AFB per 100 oilimmersion field	Positive	Scanty	100
No AFB in 100 oil immersionfield	Negative	Negative	100

Table 2: showing the grading of smears with Auromine O for LED Fluorescent Microscope

Number of AFB	Result	Grading	Number offields to beexamined
>100 AFB per field Positive	Positive	3+	20
11 – 100 AFB per field	Positive	2+	50
1-10 AFB per 100 field	Positive	1+	100
1-3 AFB per 100 field	Doubtful Positive/Repeat		100
No AFB in 100 field	**Negative**	Negative	100

DecontaminationModified Petroff Technique was used for decontamination of sputum sample. [10]

Table 3: Reading the culture

Observation	Report
No Growth	Negative
1-100 colonies (1+)	Positive (Actual No. ofcolonies)
>100 discrete colonies Positive (2+)	Positive (2+)
Confluent growth	Positive (3+)
Contaminated	Contaminated
< 20 colonies of only NTM colonies in one orboth slopes	No Growth
> 20 colonies of only NTM colonies in bothslopes	Negative for M. TB

Results

The youngest patient enrolled for this study was 5 years and oldest patient is 80 years. The average age of the patients was 44.13 years. 67 (17.5%) patient were found in 41-50 years age group followed by 63 (16.4%) patients found in 21- 30 and 51-60 years age group. Of the 383

cases enrolled in the study males were 238 (62.1%) compared to females who were 145 (37.9%). The Male: Female ratio is 1.65:1.

18% (68 patients) were positive for AFB in ZN microscopy and 82% (315 patients) reported negative.AFB seen in 89 (23.2%) cases under LED fluorescent microscope and in 294 (76.8%) cases AFB not observed.

Of the 383 cases, culture results were available for all of them. Mycobacterium tuberculosis isolated in 102 (27%) of cases, no growth found in 269 (70%) cases, contamination observed in 12 (3%) patients. No Non Tuberculous Mycobacteria isolated.

The sensitivity and specificity of ZN microscopy when compared to culture was 62.70% and 98.50% respectively. In case of LED fluorescent microscopy, sensitivity and specificity was found to be 80.4% and 98.5% respectively. The results LED fluorescent is encouraging compared to ZN microscopy. The additional yield is approximately 18%.

Table 4: Results of Ziehl Neelsen [ZN] and LED fluorescent Microscopy

ZN Result	No. of cases	Percentage	**LED fluorescent Microscopy**	No. of cases	Percentage
Positive	68	18	**Positive**	89	23.2
Negative	315	82	**Negative**	294	76.8
Total	383	100	**Total**	38	100

Table 5: Solid Culture result

Culture result	**No of cases**	**Percentage**
Positive (M.TB isolated)	102	27
Negative	269	70
Contaminated	12	3
NTM	0	0
Total	383	100

Table 6: Comparative grading of ZN microscopy and LED fluorescent Microscopy

	LED FM						**Total**
		Negative	**Scanty**	**1+**	**2+**	**3+**	
ZN	**Negative**	**293**	**0**	**17**	**4**	**0**	**314**
	Scanty	**1**	**0**	**0**	**0**	**0**	**1**
	1+	**1**	**0**	**14**	**22**	**13**	**50**
	2+	**0**	**0**	**0**	**3**	**2**	**5**
	3+	**0**	**0**	**1**	**0**	**12**	**13**
Total		**295**	**0**	**32**	**29**	**27**	**383**

Discussion

The objective of the present study was comparison of the two different types of staining and examination viz fluorescent microscopy with the ZN microscopy, which is the standard diagnostic procedure in urban, semi urban and rural settings of India as prescribed by Revised National Tuberculosis Control Program. For the purpose of the study, only pulmonary TB suspects i.e., those patients who were having cough of 2 weeks or more with or without other symptoms were included. The ZN positive cases were 68 (17.6%) out of 383 cases. The sputum smear grading has been done as per WHO guidelines. Of the 68 positive cases 2 scanty cases (0.5%), 49 (12.8%) were 1+, 7 (1.8%) were 2+ and 10(2.6%) were 3+. Among the newly identified TB suspects U Tansuphasiri et al; in 2002 reported positivity rate of 22.6% under ZN microscopy with 14% cases 1+ grading, 4.3% cases 2+ and 4.3% of cases 3+ grading. [11] The study by Paramashivan C.N. and coworkers reported 76.7% patients had 1(+) grade. [12] In our study more than 3 in 5 cases (73%) cases were paucibacillary as per ZN report. By using LED Fluorescent Microscopy, out of 383 patients 89 found to be positive. 41 patients (46.1%), 29 patients (32.6%) and 19 patients (21.3%) were 1+, 2+ and 3+ respectively. Ben J. Marais et al in 2008 and Jessica Minion et al in 2011 reported LED

fluorescent microscopy is very effective in scanty cases and 1+ identified by ZN microscopy. [13, 14] The definitive diagnosis of tuberculosis depends on the isolation and identification of M. tuberculosis. Culture remains the gold standard diagnostic method for tuberculosis. It is a specific and sensitive process that is lengthy because of the slow growth of M. tuberculosis, which requires weeks before a positive culture can be identified. Moreover culture requires at least a moderately well equipped laboratory. Number of sputum samples collected and methodology of collection exerts its influence on rate of isolation. Jena et al. (85.6%) have used three consecutive samples while Narang P. et al; [15] (56.23%)have used 2 samples per patient, one was spot collection and other was overnight collection like other study. In our study, mean duration of isolation on L J medium was 27.2 days. The period of maximum isolation was 4th week followed by 5th week. Our findings differ from those of Bhargava A et al; [16] found average time taken by L J medium for detection of growth to be 5 weeks. This difference may be due to difference in selection criteria. In our study we used Auromine O as primary stain in fluorescent microscopy and Fluorescent microscopy is generally believed to increase the sensitivity of AFB microscopy, particularly for paucibacillary smears. [17] Our study showed that the sensitivity of fluorescent microscopy is more compared to ZN microscopy. The LED fluorescent microscopy detected additional 18% positive cases which would have been missed by ZN microscopy alone. This is similar to findings of a recent systematic review studies in which the LED fluorescent microscope showed additional 2 % positivity (4 additional cases) compared to conventional fluorescent microscope. False positivity of ZN microscopy, and LED fluorescent microscopy is 1% (4/383and 1.3% (5/383) respectively, reported in our study which is not significant. The reasons for false positivity may be specimen from patients on anti-tubercular treatment and processing bloody sputum. [18-20]

Conclusion

ZiehlNeelsen microscopy is less sensitive compared to fluorescent microscopy. By utilizing LED fluorescence microscopy, smear positive pulmonary tuberculosis case detection may be enhanced. The LED fluorescent microscope yielded maximum positivity compared to conventional ZN microscopy with same specificity as of ZN microscopy. **Replacement of the age old ZN technique by fluorescent technique and using LED fluorescent microscope may be considered as alternative for diagnosis pulmonary tuberculosis after conducting large scale feasibility studies in Indian settings.**

Conflict of Interest: *None declared*
Source of Support: *Nil*
Ethical Permission: *Obtained*

References

1. Revised National Tuberculosis Control Program, India Medical Officer Training Module 1-4 Central TB Division, Directorate General of Health Services, Ministry of Health and Family Welfare, Nirman Bhavan, New Delhi; Module 1-Course Introduction; 1-14
2. International statistical classification of diseases and related health problems, 10th revision (ICD-10), 2nd ed. Geneva, World Health Organization, 2007; WHO TB 2011; 15-30
3. International statistical classification of diseases and related health problems, 10th revision (ICD-10), 2nd ed. Geneva, World Health Organization, 2007; http://apps.who.int/ghodata. These HIV deaths include 0.4 million deaths from TB.WHO TB 2011; 35-36 [Accessed on 20-10-2016]
4. Powell DA, Hunt WG. Tuberculosis in children: an update. AdvPediatr. 2006;53: 279-322
5. RNTCP TB India Report 2011. Chapter 1-Tuberculosis Burden; RNTCP TB India Report 2011; 5-10
6. Truffot-Pernot C, Véziris N, Sougakoff W. Modern diagnosis of tuberculosis. Presse Med 2006;35 (11 Pt 2):1739-46.
7. Goel MM, Ranjan V, Dhole TN, Srivastava AN, Mehrotra A, Kushwaha MR, et al. Polymerase chain reaction vs. conventional diagnosis in fine needle aspirates of tuberculous lymph nodes. ActaCytol 2001;45:333-40.

8. Revised National Tuberculosis Control Program, EQA Module**,** Central TB Division, Directorate General of Health Services, Ministry of Health and Family Welfare, NirmanBhavan, New Delhi; 5-30.
9. Revised National Tuberculosis Control Program; Manual for Sputum Smear Fluorescence Microscopy Central TB Division Directorate General of Health Services Ministry of Health and Family Welfare, NirmanBhavan, New Delhi; 4-16.
10. Allen BW, Baker FJ. Modified Petroff Technique Treatment of specimens, chapter 3 in Mycobacteria Isolation, Identification and Sensitivity Testing. Allen BW, Baker fj butter worth & Co. London 1968; 9-16.
11. UnchaleeTansuphasiri and BooncherdKladphuang, evaluation of sputum staining by modified cold method and comparison with ziehlneelsen and fluorochrome methods for the primary diagnosis of tuberculosis southeast Asian j trop Med public health 2002; 33(1):128-135.
12. Paramasivan CN, Bhaskaran K, Venkatraman P, Chandrasekaran V, Narayanan PR. Surveillance of drug resistance in tuberculosis in the state of tamil Nadu. Ind J Tuber 2000 ; 47: 27-33.
13. Ben J. Marais, Wendy Brittle, KatrienPainczyk, Anneke C. Hesseling, NuldaBeyers, Elizabeth Wasserman, Dick van Soolingen, and Rob M. Warren; Use of Light-Emitting Diode Fluorescence Microscopy to Detect Acid-Fast Bacilli in Sputum; LED Fluorescence Microscopy to Detect AFB • CID 2008:47 (15 July) • 203 – 207
14. Jessica Minion, MadhukarPai, Andrew Ramsay4, Dick Menzies, Christina Greenaway; Comparison of LED and Conventional Fluorescence Microscopy for detection of Acid Fast Bacilli in a Low-Incidence Setting. PLOS one 2011; 6(7): 1-6.
15. Narang P, Mendiratta DK, JyotiKannathe. Characterization of M. tuberculosis variants in Wardha district. Ind J Tub 1997 ; 44 : 21-26.
16. Bhargava A, Jaiin A, comparison of liquid and solid culture media with radiometric system for detection of mycobacteria in clinical specimens Ind J Tub 2001;48:9-12.
17. Steingart K R, Henry M, Ng V, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis 2006; 6: 570–581.
18. K. Toman Microscopic examination of sputum smears during and at the end of treatment TOMAN'S Tuberculosis Case Detection, Treatment, and Monitoring, 2nd Edition, World Health Organization; Page No. 8-9.
19. Somoskovi A, Hataling JE, Fitzgerald M, O'Donnell D, Parsons LM, Salfinger M, Lessons from proficiency testing event for acid fast microscopy. Chest 2001; 120;250-57.
20. Toman K. What are the advantages and disadvantages of fluorescence microscopy? In Frieden T, ed. Toman's tuberculosis case detection, treatment, and monitoring—questions and answers (2ndEdn). Geneva. WHO, 2004: 31–34.