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EVALUATION OF FLUORESCENT STAINING FOR IMPROVEMENT IN DIAGNOSIS OF PULMONARY TUBERCULOSIS IN SPUTUM SMEAR NEGATIVE CASES

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ABSTRACT

Bright field microscopy of sputum after ZN staining is the backbone of revised national tuberculosis control program (RNTCP) for diagnosis of pulmonary tuberculosis. But reduced sensitivity in overburdened RNTCP centers and HIV-TB co-infection necessitates the use of newer modalities to increase the diagnostic yield. A cross sectional prospective analytical study was done from June 2011 to August 2011, to look for increase in sensitivity in diagnosis of pulmonary tuberculosis by examination of sputum smear negative cases by fluorescent microscopy. Two sets of smears were prepared, of which one set of smear was subjected to ZN stain to confirm sputum smear negativity. The second set of slides was stained collectively by fluorescent stain and observed by Labomed binocular LED Microscope with fluorescent attachment. All the data was maintained in Microsoft Office Excel for analysis Four out of 160 (2.50%) enrolled were found out to be positive on reconfirmation and excluded. Out of 156 samples subjected to fluorescent staining, thirteen samples were reported positive by two independent observers. This rise of 8.33 % over routine RNTCP method which was able to diagnose 5.88% (10/170) cases is highly significant with $P<0.017$ ($\chi 2=5.686$). These 13 additional cases diagnosed constitute 92.85% increase in case detection when compared to RNTCP method which only detected 14 cases.Fluorescence microscopy is much more effective in diagnosis of paucibacillay cases with ease of identification and less eye-strain.

KEY WORDS

Fluorescent Microscopy, Increased sensitivity, Pulmonary Tuberculosis

KEY MESSAGE

Fluorescent microscopy with its ease of staining than ZN staining, increased sensitivity and quick and easy scanning of larger smear surface at lower objective is of tremendous benefit especially in overburdened laboratory system in developing countries and can be implemented at least in RNTCP centers in tertiary care settings.

INTRODUCTION

India accounts for nearly one third of the global burden of tuberculosis with 2 deaths every three minutes in India and 1.6 million deaths world over annually. [1,2]

In revised national tuberculosis control program (RNTCP), microscopic examination of sputum for Acid Fast Bacilli plays an important role in the initial diagnosis of tuberculosis. The microscopic examination requires 10^4 bacilli per millilitre of sputum in order to be detected on smear. Considering the amount of sputum material that is examined in oil immersion field, chances of missing the organism are high thus reducing the sensitivity. Much of the transmission of TB can occur even before the concentration in sputum reaches a critical level when it is diagnosed. [3]

A negative smear does not exclude the diagnosis of tuberculosis, as about 55% of pulmonary tuberculosis cases worldwide harbors low bacillary load.[2] It has also been established that sputum smear microscopy is less sensitive in HIV–TB co infection where sputum HIV smear tends to be negative.[2,3]

Fluorescent microscopes with Light Emitting Diode (LED) is being evaluated by World Health Organisation (WHO) and Foundation for Innovative New Diagnostics (FIND) for increase in sensitivity and specificity, ease of use and for less time being required to scan the slides in low power objectives. It is estimated in a meta-analysis that fluorescent microscopy is having approximately 10 % greater sensitivity in diagnosis of pulmonary tuberculosis.[4]

With this background, we planned our study to look for increase in sensitivity of diagnosis of pulmonary tuberculosis by examination of sputum smear negative cases by fluorescent microscopy.

MATERIALS AND METHODS

This cross sectional prospective analytical study was carried out in Department of Microbiology over a period of three months from June 2011 to August 2011. One hundred and seventy (170) patients of all the ages and either gender coming to RNTCP center having cough for two weeks or more duration or who are contacts of known tuberculosis cases irrespective of duration of cough and patients of Extra pulmonary TB irrespective of duration of cough enrolled in RNTCP register were screened by ZN staining as per RNTCP guidelines

Around 160 samples reported as Negative by the routine RNTCP method employing ZN staining were selected for the study.

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Processing of samples

Two sets of smears were prepared from the muco-purulent portion of the sputum reported as smear negative on new, clean, scratch free glass slides. Smears were fixed using methanol and labelled.

One set of smear was again subjected to ZN staining and observed to confirm sputum smear negativity. The second set of slides was stained collectively by Phenolic Acridine orange with use of known positive AFB slide as staining control.

Preparation of Reagents for fluorescent staining: [5,6]

A) The Acridine orange reagent:

It was prepared by dissolving 5 g of colorless phenol crystals in a solution containing 50 ml of deionized water, 25 ml of glycerol and 25 ml of 95% ethanol .Then 0.1 g of acridineorange was added, and the mixture was stirred briefly. The solution was set overnight to allow the acridine orange to completely dissolve and then was stirred.

***B) Destaining Solution*:**

The acid-alcohol destaining solution was prepared by mixing 74 ml of 95% ethanol, 26 ml of deionize water, 0.5 ml of concentrated hydrochloric acid and 0.2 g of methylene blue .

***Staining procedure*:-**

1) The dried, methanol fixed smear was placed on a staining rack over the sink. Smears of sputum should be thin.
2) The smears were lowered with Phenolic Acridine orange and left to stain at a room temperature for 15 min.
3) The stain was washed off with deionised water.
4) The slides were lowered with excess of acid alcohol with methylene blue and left to decolourize for 5 minutes. [Acid alcohol which gives a debris free background and methylene blue reduces the background fluorescence]
5) Washed with deionised water.
6) All smears were air dried without use of any blotting paper.

Observation

The slides stained were observed by Labomed binocular LED Microscope with fluorescent attachment using Blue excitation filter. Slides prepared were observed by two observers to remove observer's bias. The smears were examined dry by fluorescent microscopy with a 40X objective for scanning of tubercle bacilli which are seen as apple green to orange luminous rods in a dark field. When detected under low power the morphology of bacilli is confirmed with an oil immersion objective [2,5,6]

For fluorescent microscopy a comparative grading system was employed as suggested by Manual for sputum smear fluorescence microscopy by central TB division, Directorate General of Health Services, Ministry of Health and Family Welfare, Govt. of India.[7]

All the data was maintained in Microsoft Office Excel and tests of proportion were applied. Tests of significance like Parchan's Chi square test was applied to know whether the observations are significant or just because of chance. .

RESULT

A total of 170 patients enrolled at RNTCP center during June 2011 to August 2011 were screened for presence of tubercular bacilli in both their spot and morning samples by ZN staining and bright field microscopy. Ten (10) patients (5.88%) were reported as sputum smear positive and were excluded from our study.

A total of 160 morning samples from patients who are reported as sputum smear negative were enrolled in the study. The study population comprised of 108 males (67.5%) and 52 females (32.5%), a ratio of 2.07 to 1 with age variations from 9 yrs to 85 yrs.

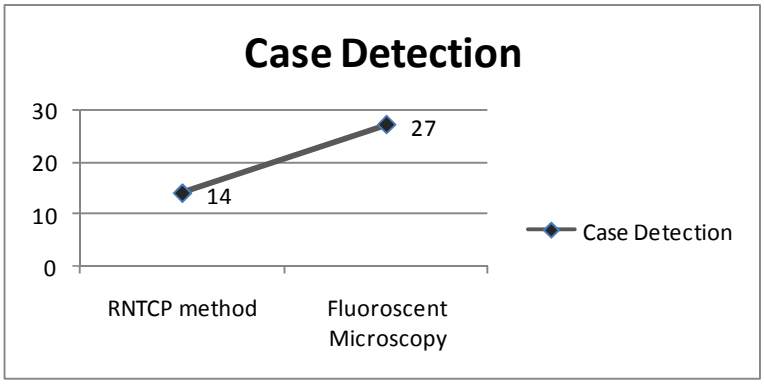
Four (4) out of 160 (2.50%) were found out to be positive when re-examined by modified ZN stain to confirm the sputum smear negativity and were excluded.

A total of 156 samples were subjected to fluorescent staining. Thirteen samples (8.33%) were reported positive by two independent observers. This increased sensitivity of 8.33 % over routine RNTCP method which was able to diagnose 5.88% cases is highly significant with $P<0.017$ ($\chi 2=5.686$)

With 13 additional cases diagnosed with fluorescent microscopy constituted to 92.85% increase in case detection when compared to RNTCP method which diagnosed only 10 cases and missed out 4 cases constituting to a total of 14 cases.

Both the observers reported 1 to 9 bacilli in 10/13 positive slides (76.92%) and 1+ in 3/13 (23.07%)slides.

Figure1
Shows increase in case detection by Fluorescent Microscopy over routine RNTCP method.



DISCUSSION

Undoubtedly, in low-income countries direct microscopy of sputum is currently the backbone for diagnosing pulmonary tuberculosis in national programs. It is a rapid, inexpensive and highly specific method for the detection of AFB in sputum. But its major disadvantage is the discouragingly low sensitivity when used in overburdened control programs like the RNTCP.

The diagnosis of tuberculosis by fluorescence techniques involves the use of fluorescent stains that tag the bacilli with fluorophores, which are then visualised under UV-light as brightly stained rods, easily identifiable to microscopists. Compared to light-microscopy, these methods reduce eye-strain, use lower magnification and reduce the time needed for slide scanning. Fluorescence microscopy has also been shown to provide an increase in sensitivity of about 10% when examining positive smears, and it provides a cost-effective alternative to the traditional ZN staining.

Out of total 170 patients registered in RNTCP during the study period; 10 patients were sputum smear positive which is lower than that of the expected 10 % in suspected patients according to the RNTCP manual.[2]

Four (4) out of 160 cases (2.5%) turned out to be positive on reexamination, which were reported negative by RNTCP center emphasizing the need of more sensitive and easy method for diagnosis of pulmonary tuberculosis in overburdened RNTCP centers. These findings are similar to the Sarin R. et al who showed that a discordance of about 2% to 6.4 % can occur in sputum smear negative cases between to expert microbiologists.[8]

By fluorescent microscopy 13 samples (8.33%) out of 156 confirmed sputum smear negative cases were reported positive by two independent observers. This increased sensitivity of 8.33 % over routine RNTCP method which was able to diagnose 5.33% cases is highly significant with $P<0.017$ ($\chi 2=5.686$).

Similar findings were reported by K. Prasanthi in 2005 reporting a increase of 16% over RNTCP method.[9] Adithya Cattamanchi et al have also reported a increase of 8 % in diagnosis of pulmonary tuberculosis in HIV prevalent settings.[10] In a similar study done by Laifangbam S. et al; 59.7% (43/72) of the culture positive cases were diagnosed by ZN stained smear light microscopy. The figure increased significantly to 97.2% (70/72) of culture positive cases by AO stained smear fluorescent microscopy.[11]

In our studies with 13 additional cases diagnosed with fluorescent microscopy constituted to 92.85% increase in case detection when compared to RNTCP method which diagnosed only 10 cases and missed out 4 cases constituting to a total of 14 cases.

All these 17 patients i.e. 13 diagnosed by fluorescent microscopy and 4 missed out in routine RNTCP method got the benefit of early detection and prompt treatment which other wise would have gone undetected as open cases spreading infection in the community.
Both the observers reported 1 to 9 bacilli in 10/13 positive slides (76.92%) and 1+ in 3/13 (23.07%) slides and reported as positive according to revised guidelines from WHO for reporting the sputum smear.[12]

Fluorescent microscopy, with its ease of staining than ZN staining, increased sensitivity and quick and easy scanning of larger smear surface at lower objective is of tremendous benefit especially in overburdened laboratory system in developing countries and can be implemented at least in RNTCP centers in tertiary care settings. In our research 13 additional cases diagnosed with fluorescent microscopy constituted to 92.85% increase in case detection when compared to RNTCP method undoubtedly proves it.

CONCLUSION

Most of the world's tuberculosis cases occur in low-income and middle-income countries, where sputum microscopy with a conventional light microscope is the primary method for diagnosing pulmonary tuberculosis. A major shortcoming of conventional microscopy is its relatively low sensitivity compared with culture, especially in patients co-infected with HIV. In fluorescence microscopy as screening is done under lower power of magnification (400x), it is less time consuming scanning larger smear areas, hence been advocated to be a method of choice where a large number of sputum smears are to be examined. The fluorescing bacilli are easily identifiable and cause less eye-strain. The sensitivity of fluorescence microscopy proved to be much higher than conventional light microscopy in our study and large scale studies involving a larger study populations are required to evaluate its overall cost effectiveness in terms of early diagnosis and treatment of pulmonary tuberculosis.

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REFERENCES

1. Saxena S, Mathur M, Talwar V. Detection of tubercle bacilli in sputum: Application of sodium hypochlorite concentration method. J Comm Dis 2001;33:241-4.
2. RNTCP- An overview. A manual for sensitization of Medical College Faculty. 1st ed. Mumbai: Maharashtra State TB Control Society; Jul 2004)
3. 3.Verma SK, Mahajan V. HIV-Tuberculsis Co-Infection. Int J Pulmonary Med 2008;10.Availablefrom:http://www.ispub.com/journal/the_internet_journal_of_pulmonarymedicine/volume10_number_1_7/article/hiv_tuberculosis_co_infection.html.
4. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 2006 Sep;6(9):570-81
5. Watt B, Rayner A., Harris G. Modern methods in mycobacteriology. In, J Gerald Collee (Ed), Practical Medical Microbiology, 14st edition.New Delhi, Elsevier publication,2006; 4: 97-105.
6. Smithwick RW, Malcolm RB, Ferguson RB, Michael AK and Carolyn KW. Phenolic Acridine Orange Fluorescent Stain for Mycobacteria. J of clin microbiol, 1995;vol 33: 2763–2764
7. Manual for sputum smear fluorescence microscopy, under RNTCP by central TB division, Directorate General of Health Services, Ministry of Health and Family Welfare, Govt. of India.
8. Sarin R.,Singala N.Mukherjee S.and Sharma P. , RNTCP : Quality Control Of

Sputum Microscopy At Sub-District Level. Ind.J Tub, 2002;49: 143-46

9. Prasanthi K., Kumari AR, Efficacy of fluorochrome stain in diagnosis of pulmonary tuberculosis co-infected with HIV, Ind J Med Microbiol,2005;23(3):179-85
10. Cattamanchi A, Lucian J., Worodria W. Boon S., Yoo S., Matovu J. et al, Sensitivity and Specificity of Fluorescence Microscopy for Diagnosing Pulmonary Tuberculosis in a High HIV Prevalence Setting. Int J Tuberc Lung Dis. 2009 ; 13(9): 1130–1136.
11. Laifangbam S, Singh HL, Singh NB, Devi KM, Singh NT. A comparative study of fluorescent microscopy with Ziehl-Neelsen staining and culture for the diagnosis of pulmonary tuberculosis. Kathmandu Univ Med J (KUMJ). 2009 Jul-Sep;7(27):226-30.
12. WHO manual on Improving the diagnosis and treatment of smear-negative pulmonary and extrapulmonary tuberculosis among adults and adolescents Recommendations for HIV-prevalent and resource-constrained settings. WHO /HTM /TB /2007.379