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Original Research Article

Comparison between ziehl-neelsen staining and fluorescent staining of sputum samples to detect acid fast bacilli in suspected case of pulmonary tuberculosis at tertiary care hospital, Amreli, Gujarat

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ABSTRACT

Introduction: In India, Tuberculosis (TB) is one of the major community health problems. Pulmonary tuberculosis (PTB) is a respiratory disease. Causative organism for this is acid fast bacilli known as *Mycobacterium tuberculosis*. It is the most ordinary disease affecting the lower socio-economic class in developing countries. Microbiological diagnosis is the heart for the effective treatment of pulmonary TB (PTB). The look forrapid and efficient method has resulted in several staining techniques. Objective of the study was to compare the results of ZN stain (RNTCP) with fluorescent stain by use of microscopy.
Materials and Methods: The study was carried out in Microbiology Department, SMCGH, Amreli. 350 sputum samples (Spot and early morning sample) collected from 175 suspected case of the pulmonary tuberculosis. All 350 samples were processed by ZN stain and Fluorescent stain to detect acid fast bacilli. By use of microscope, the results of the stained smears were given according to RNTCP guideline.
Results: Out of 350 sputum smears, 52 (14.85%) and 61 (17.4%) were positive by ZN and FM staining respectively. Males are predominantly affected than females. Majority of the patients were in age above 50 years. Early morning samples were more reliable than spot samples for detection of acid fast bacilli for ZN stain, but not for fluorescent stain.
Conclusion: Fluorescent staining with LED microscopy was more efficient than ZN staining for detection of acid fast bacilli from sputum smear.

1. Introduction

Tuberculosis (TB) is one of the major community health problems, in India. Pulmonary tuberculosis (TB) is a lower respiratory tract infection, which is caused by *Mycobacterium tuberculosis*. It is the most common disease affecting the lower socio-economic class in developing countries, approximately one-third of the world's population infected with TB as TB remain a major health problem.[1] Tuberculosis can infect any system or organ of the body, but lung is the most common affected organ.[2] Tuberculosis is one of the leading causes of death, when associated with HIV.[3] There are various methods for diagnosis of tuberculosis eg. Microscopy, Culture, Molecular etc. Microscopy is the simple, quick and practical method for detection of TB bacilli.[4] Specificity of AFB microscopy is high but sensitivity varies depends upon bacillary load.[5] By using of concentration method will increase the sensitivity of the sample.[6] Due to larger field of view and the better contrast, observation of acid fast bacilli by fluorescent

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staining have greater sensitivity than light microscopy.[7] Culture methods are more sensitive than microscopy but takes 4 to 8 weeks as tubercle bacilli are slow growing organism so microscopic examination has the advantage of giving a result at once.[8] So, the most hopeful diagnostic method to overcome this problem is Molecular techniques like PCR.[9]

Microscopic examination with staining methods like Ziehl-Neelson or Fluorescent, allows detection of acid fast bacilli in less than an hour. Ziehl-Neelson is most widely used procedure for the detection of *Mycobacterium tuberculosis* in smear.[10] Fluorescent staining by Auramine stain is another method of staining. It is simpler and can be examined at lower magnification (40x) than ZN (100x), (40x Vs 100x). Fluorescent microscope (FM) may take upto 75% less time than ZN11. This are advantages of fluorescence staining.

2. Objectives

Objective of the study was to compare the results of ZN stain with fluorescent stain by use of microscope.

3. Materials and Methods

The study was carried out in Microbiology Department, SMCGH, Amreli. All age groups of both the gender suspected to be a case of pulmonary tuberculosis as per RNTCP guidelines, both indoor patients and outdoor patients presenting with cough and expectoration for 2 or >2 weeks with or without symptoms like loss of appetite, loss of weight, chest pain and haemoptysis, included in this study. 350 sputum samples (Spot and early morning) collected from 175 suspected case of the pulmonary tuberculosis. Samples other than sputum and Samples macroscopically resembling saliva did not include in this study.

3.1. Sample collection method

Suspected case of pulmonary tuberculosis was asked to take two samples of sputum, one spot sample and another early morning sample into clean, sterile, heat proof, leak proof, wide mouth sputum container. Smear preparation from samples was carried out in a bio safety cabinet by trained RNTCP technician thereby ensuring the quality of smear. Each sample was processed by ZN stain and Fluorescent stain to detect acid fast bacilli. All stained smears were examined microscopically by using an oil-immersion microscope and fluorescent microscope and the results of the stained smears were given according to RNTCP guideline.

3.2. Ziehl-Neelsen stain

Reagent used: 0.1% Carbol fuchsin, 25% Sulfuric acid, 0.1% methylene blue.

3.2.1. Preparation and Staining of smear

A portion of the mucopurulent material is taken on a slide and a smear is made and dried. “Fixation of the smear”- fix the smear by gently passing the slide over the flame. Flood the slide with strong carbol fuchsin and heat until steam rises. Allow the preparation to stain for 5 minutes. Heat being applied at intervals until steam rises and stops it and continue in this way for 5 minutes (never heat too much to produce boiling and charring). The stain must not be allowed to evaporate and dry on the slide, if necessary, pour more carbol fuchsin on the slide. Wash in running tap water. Decolourise the smear with 25% sulphuric acid by dipping the slide in the acid solution in a wide mouth jar. Decolourisation should be continued till the film becomes faintly pink. Decolourisation must be done in stages and generally requires about 10 minutes. Wash in running tap water. Counter stain the smear with methylene blue for 1-2 minutes. Wash in running tap water. Blot dry and examine with the oil immersion objective lens of binocular microscope.

3.3. Fluorescent stain

3.3.1. Reagent used

Auramine-Phenol solution, 1% acid alcohol, 0.1% potassium permanganate solution.

Preparation and Staining of smear: A portion of the mucopurulent material is taken on a slide and a smear is made and dried. “Fixation of the smear”- fix the smear by gently passing the slide over the flame. Flood the slide with freshly filtered Auramine – Phenol. Let stand for 7 to 10 mins. Wash well with running tap water. Taking care to control the flow of water so as to prevent washing away the smear. Decolourise by covering completely with acid alcohol for 2 mins, twice. Wash well with running tap water as before to wash away the acid alcohol. Counter stain with 0.1% potassium permanganate for 30 secs. Wash well as before with running tap water and slop the slide to air dry and examine with 40x objective lens of fluorescent microscope.

4. Results

Out of 350 sputum samples collected from 175 patients, 52 and 61 sputum samples were found to be positive for acid fast bacilli by Z-N and Fluorescence staining respectively. The Z-N smear positivity rate and fluorescence staining smear positivity rate in this study was 14.85% and 17.4% respectively.

Out of 175 suspected case of tuberculosis, 113 (64.57%) were males and 62 (35.43%) were females. Males were

Table 1: RNTCP guidelines for grading of slides in AFB microscopy

No. of AFB seen	OIF to be screened	Grading	Result
No AFB in 100 OIF	100	0	Negative
1 to 9/100 OIF	100	Scanty*	Positive
10 to 99/100 OIF	100	1+	Positive
1 to 10/ OIF	50	2+	Positive
>10/ OIF	20	3+	Positive

(* - Record actual number of bacilli seen in 100 fields)

Table 2: Fluorescent microscopy grading system (40x)

No. of AFB seen	OIF to be screened	Grading	Result
No AFB in 40 fields	40	Negative	Negative
1 to 19/ 40 fields	40	Scanty	Positive
20 to 199 /40 fields	40	1+	Positive
5 to 50 / field	20	2+	Positive
>50/ field	8	3+	Positive

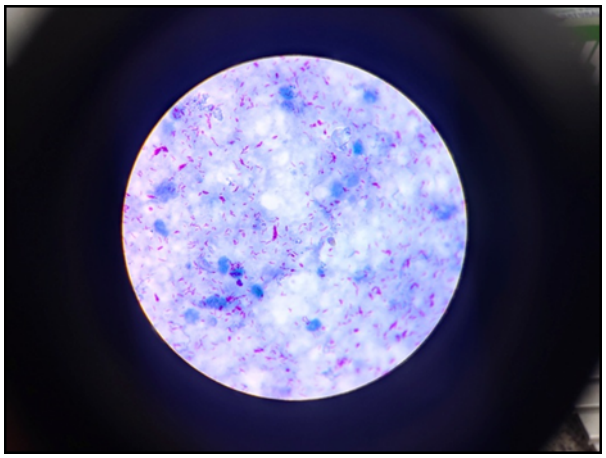


Fig. 1: ZN stain showing acid fast bacilli

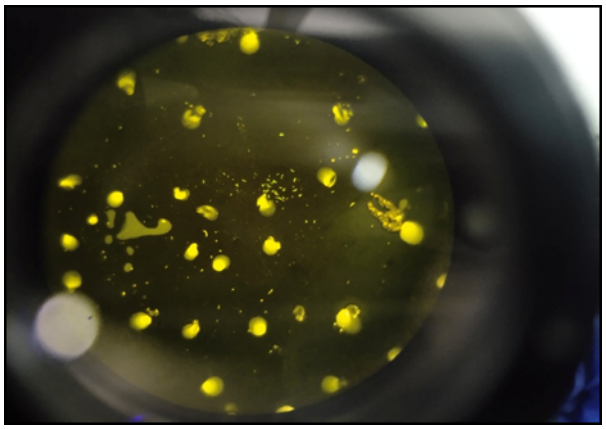
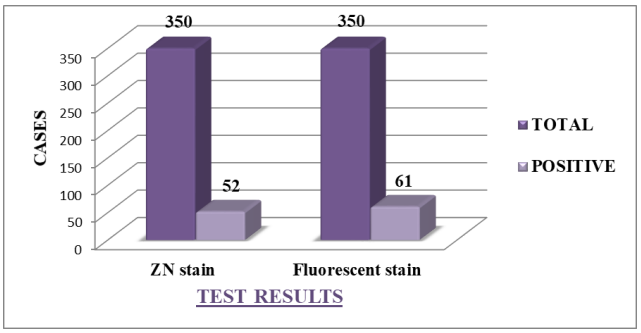
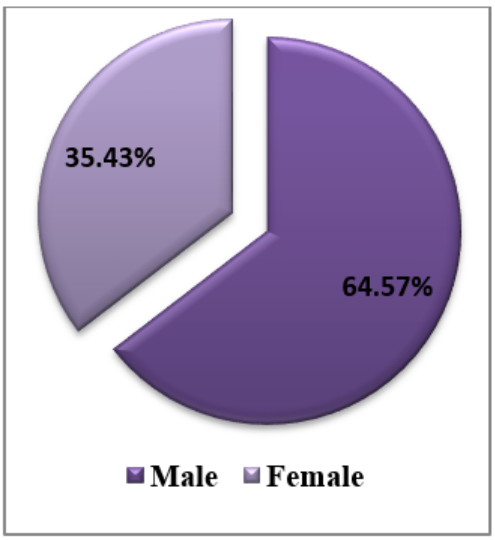


Fig. 2: Fluorescent stain showing tubercle bacilli

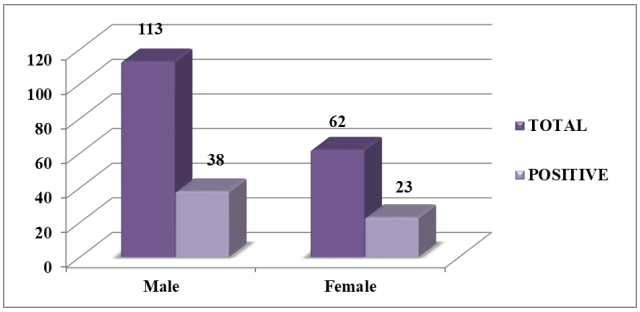


Graph 1: Comparison of ZN and Fluorescent staining report

predominantly affected than females. Out of 61 positive sputum smear by fluorescent staining, 38 (62.29%) smear were of males and 23 (37.7%) smear were of female.

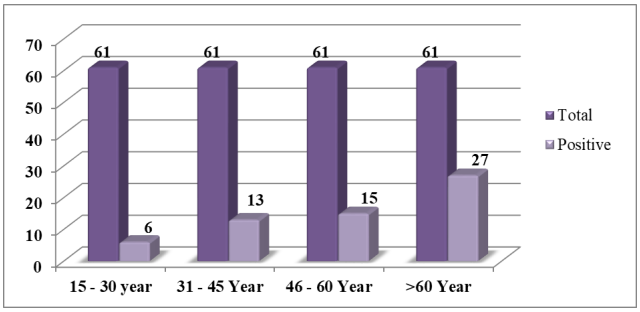


Graph 2: Gender wise distribution in of study participants



Graph 3: Gender wise distribution and positivity

We received sputum samples from different age groups: 15-30 year, 31-45 year, 46-60 year and above 60 year. Out of 61 positive sputum smear 6 (9.84%), 13 (21.31%), 15 (24.59%) and 27 (44.26%) were found positive from different age groups respectively. Majority positive samples were found in the age above 50 years.



Graph 4: Age wise distribution of study participants

5. Discussion

Tuberculosis is the most serious infectious diseases and a major public health problem in India due to its high risk of transmission between person to person, morbidity and mortality. Even though we have many advance methods for early detection of tubercle bacilli and different modalities for treatment and management for tuberculosis still it is a major public health problem in all over the India with poor social and economic consequences. To prevent both morbidity and mortality, early diagnosis of disease followed by adequate treatment for the same is crucial. For the control of tuberculosis we can do early case detection, start treatment as early as possible, and by this prevent the transmission of the disease. Our main focus for its control should be quick and correct identification of the infected individuals by the detection of acid fast bacilli from the given sputum sample of suspected patient.[11] This is considered as the indication of infective stage. The laboratory plays a crucial role in diagnosis of pulmonary tuberculosis by various alternative diagnostic methods like Microscopy, Culture, Molecular methods (PCR) etc. Culture methods are time consuming modality as *Mycobacterium tuberculosis* takes 4 to 6 weeks to grow on culture media. In developing countries, microscopy of the sputum is simplest and rapid method for detection of acid-fast bacilli. Also this is cheapest and more reliable method for diagnosis of pulmonary tuberculosis.

In this study shows, males are predominantly affected than female which is also seen in other studies.[12,13] The reason behind it is increased outdoor activity of men and therefore they are more prone to come in contact with active case of tuberculosis.

In this study, out of 350 sputum samples, 52 (14.85%) and 61 (17.4%) sputum samples were found to be positive for acid fast bacilli by Z-N and Fluorescence staining respectively. Rate of smear positivity depends upon the type (spot and early morning sample) and quality of sputum sample also presence of tubercle bacilli in the sputum sample. 10^4 bacilli/ml is required to give positive result.[14] Fluorescent staining is a superior method of microscopy for demonstration of acid fast bacilli than ZN staining method. Many other studies have also revealed the increased efficacy of fluorescent staining.

In this study we conclude early morning samples are more reliable than spot samples due to accumulation of the secretion get concentrate with bacilii over night.[19]

In this study it was observed that total number of 9 sputum smears which were negative by ZN staining and positive by Fluorescent staining method and So, we conclude that fluorescent staining with LED microscopy was more efficient over ZN staining.

Though Fluorescence staining has been added in Revised National Tuberculosis Control Programme (RNTCP) for elimination of TB because of its sensitivity and fast results.

6. Conclusion

Sputum smear examination for the acid fast bacilli is usually carried out for patients which are clinically and radio logically suspected for pulmonary tuberculosis. Though, the usual method of sputum examination, eg, ZN staining is not sensitive enough and same suspected cases are not confirmed. Also they remain undiagnosed and fail to get treatment. Therefore our study concludes that Fluorescent staining with LED is more efficient over ZN stain in detecting acid fast bacilli in sputum smear, and also Fluorescent Microscope LED (40x) has been found to be less time consuming as compared to Light Microscope (1000x) in the diagnosis of pulmonary tuberculosis. Nowadays, Molecular methods like PCR are even more sensitive and specific over microscopic methods. The only disadvantage with molecular method is high cost and time consuming and may not be affordable for developing countries.

Table 3: Comparison between spot sample and morning sample by ZN staining and Fluorescent staining

	By ZN staining		
	Positive	**Negative**	**Total**
Spot sample	23	152	175
Morning sample	29	146	175
Total	52	298	350
	By Fluorescent staining		
	Positive	**Negative**	**Total**
Spot sample	30	145	175
Morning sample	31	144	175
Total	61	289	350

Table 4: Comparisons of results of various studies with same objectives

Study	**Slide +ve rate by ZN stain**	**Slide +ve rate by Fluorescent stain**	**Sample size**
Z. Khatun et al [15]	10.4%	17.8%	300
Suria et al [16]	12.4%	19.1%	225
Golia S et al	10.41%	16.56%	634
Ulukanligil et al [17]	9.89%	12.47%	465
Rahul achaeya et al [18]	12%	19.8%	500
Our study	14.85%	17.4%	350

7. Source of Funding

None.

8. Conflict of Interest

The authors declare no conflict of interest.

References

1. Caulfield AJ, Wengenack NL. Diagnosis of active tuberculosis disease:From microscopy to molecular techniques. *J Clin Tuberc Other Mycobact Dis*. 2016;4:33–43. doi:10.1016/j.jctube.2016.05.005.
2. Hajia M, Rahbar M, Amini R. Is PCR assay reliable for diagnosis of extrapulmonary tuberculosis? *Afr J Microbiol Res*. 2009;3(2):877–81.
3. Scherer LC, Sperhacke RD, Jarczewski C, Cafrune PI, Michelon CT, Rupenthal R, et al. Comparison of two laboratory developed PCR methods for the diagnosis of pulmonary tuberculosis in Brazillian patients with and without HIV infection. *BMC Pulmonary Med*. 2011;11(15). doi:10.1186/1471-2466-11-15.
4. Daniel TM. The history of tuberculosis. *Respir Med*. 2006;100(11):1862–70.
5. Oguz VA, Sezak N, Oztop A. A comparison of two different fluorochrome stains for the detection of acid fast bacilli in sputum specimens. *Turk J Med Sci*. 2011;41(3):411–7.
6. Chakravorty S, Dudeja M, Hanif M, Tyagi JS. Utility of Universal Sample Processing Methadology, Combining Smear Microscopy, Culture and PCR for Diagnosis of Pulmonary Tuberculosis. *J Clin Microbiol*. 2005;43(6):2703–8.
7. Torrea G, Chakaya J, Mayabi M, Deun AV. Evaluation of the Fluoreslen S TM and fluorescence Microscopy blinded rechecking trial. *Int J Tuberc Lung Dis*. 2008;12:658–63.
8. Nandagopal B, Sankar S, Linesan K, Appu KC, Sridharan G, Gopinathan AK. Evaluation of a nested PCR targeting IS6110 of Mycobacterium tuberculosis for detection of the organism in the leukocyte fraction of blood samples. *Indian J Med Microbiol*. 2010;28(3):227–32.
9. Varaine F, Henkens M, Gruzard V. Tuberculosis: Practical guide for clinicians, nurses, laboratory technicians and medical auxiliaries. Fifth revised edition .
10. Palomino JC, Leao SC, Ritacoo V. From basic science to patient care. In: Tuberculosis 2007; 2007. p. 25–48.
11. European society for Mycobacteriology. Manual of diagnostic and public health mycobacteriology. Edition 2. London: Bureau of Hygiene and Tropical Medicine; 1991.
12. Cohen RA, Muzaffar S, Schwartz D. Diagnosis of pulmonary tuberculosis using PCR assay on sputum collected within 24 hours of hospital admission. *Am J Resp Crit Care Med*. 1998;157:156–61.
13. Marais BJ, Brittle W, Paiczyk K, Hesseling AC, Beyers N, Wasserman E, et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. *Clin Infect Dis*. 2008;47(2):203–7. doi:10.1086/589248.
14. Forbes BA, Sahm DF, Wiessfield AS. Mycobacteria. In: Bailey and Scott's diagnostic Microbiology. USA: Mosby Elsevier; 2007. p. 478–508.
15. Khatun Z, Kamal M, Roy CK, Sultana T, Rahman MQ, Azad M, et al. Usefulness of light emitting diode (LED) fluorescent microscopy as a tool for rapid and effective method for the diagnosis of pulmonary tuberculosis. *Bangladesh Med res Counc Bull*. 2011;37:7–10. doi:10.3329/bmrcb.v37i1.7792.
16. Suria K, Chandrasekar C, Rajasekaran S. Comparison of conventional and fluorescent staining Methods in diagnosis of pulmonary tuberculosis among HIV Seropositive individuals. *J Evol Med Dent Sci*. 2012;1(4):463–6.
17. Ulukanligil M, Aslan G, Tasci S. A comparative study on the different staining methods and number of specimens for the detection of acid-fast bacilli. *Mem Inst Oswaldo Cruz*. 2000;95:855–8.
18. Acharya R, Vyas M. Comparative Study between FM Staining and Z-N Staining in Diagnosing Sputum Smear Positive PTB at S.P. Medical College and Hospital. *Int J Med Res Prof*. 2018;4(4):34–7. doi:10.21276/ijmrp.2018.4.4.032.
19. Dzodanu EG, Afrifa J, Acheampong DO, Dadzie I. Diagnostic Yield of Fluorescence and ZiehlNeelsen Staining Techniques in the Diagnosis of PulmonaryTuberculosis: A Comparative Study in a District Health Facility. *Tuberc Res Treat*. 2019;2019:4091937. doi:10.1155/2019/4091937.

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