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Comparison of Ziehl-Neelsen Staining, Fluorescent Staining and PCR in the Diagnosis of Pulmonary Tuberculosis

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Abstract

Introduction: Tuberculosis remains one of the deadliest diseases in the world. The traditional bacterial diagnostic methods are either slow or their sensitivity is very low. So, there is a need for rapid accurate diagnostic test like PCR (Polymerase Chain Reaction). *Materials and Methods*: 100 sputum samples were collected from 50 clinically suspected cases of Pulmonary Tuberculosis as per RNTCP guidelines. Each sputum sample was subjected to ZN (Ziehl-Neelsen) staining, fluorescent staining and PCR. The sputum sample found to be negative by direct smear was then subjected to concentration technique by NALC-NaOH (N-acetyl-L-cysteine-sodium hydroxide) method and staining methods were repeated. *Results*: Out of 100 sputum samples examined, 44 (44%) were positive and 56 (56%) were negative by ZN staining. 52 (52%) were positive and 48 (48%) were negative by fluorescent staining and 76(76%) samples were positive by PCR. *Conclusion*: The rapidity and more positivity rate in PCR for the diagnosis of Tuberculosis,encourages the routine use of this in clinical practices.

Keywords: ZN staining; Fluorescent staining; PCR; Tuberculosis.

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Introduction

Tuberculosis remains a major public health problem with approximately one-third of the world's population infected.[1] Tuberculosis can potentially involve any system or organ of the body, while pulmonary tuberculosis has its most common presentation.[2] When associated with HIV, Tuberculosis is one of the leading causes of death.[3]

In spite of numerous diagnostic methods, the early diagnosis of active tuberculosis still depends on the presence of Acid fast bacilli(AFB) in stained smears. The specificity of AFB microscopy is high but sensitivity varies.[4] Therefore, there is a need to improve the sensitivity of smear microscopy. The inclusion of concentration method enhances the sensitivity of the direct smear method.[5] Observation of Mycobacterium tuberculosis by fluorescent staining has been reported to have greater sensitivity than light microscopy due to larger field of view and the better contrast.[6]

Culture on LJ (Lowenstein Jensen) medium, though sensitive, takes 4-8 weeks and should be followed by identification tests such as niacin test.[7] In addition, culture may give false-positive result in 10-20% of cases.[8] So, the most promising diagnostic modality to address this problem is Polymerase

Chain Reaction (PCR). The major benefits of this rapid diagnostic test are improved patient care and reduced medical cost.[9]

Materials and Methods

The material for the study includes 100 sputum samples (spot & early morning samples) collected from 50 clinically suspected cases of pulmonary TB attending medicine, TB & chest medicine department at Chigteri hospital, Davangere.The Institutional ethical committee clearance was taken.

Any patient with cough lasting over 2 weeks along with sputum production and weight loss was suspected as a case of Pulmonary TB.[10] The patients were instructed to submit sputum samples in a clean, sterile, leak-proof, wide-mouth containers. One spot and one early morning sputum samples were collected from every case. Thin, clear saliva or nasopharyngeal discharge was rejected. Specimen were collected,transported & processed immediately in the laboratory. If a delay was unavoidable, the specimen was refrigerated till further processing.

The following investigations were done

Microscopy

RNTCP (Revised National Tuberculosis Control Programme) guidelines were followed for smear preparation.[10] Two smears were prepared from every sputum samples and subjected to ZN (Ziehl-Neelsen) staining[11] and Fluorescent staining (using Auramine O).[12]

Tubercle bacilli look like red colored fine rods, straight or slightly curved, more or less granular standing out clearly against the blue background in ZN stained smears (Fig. 1). Tubercle bacilli emitting a bright yellow fluorescence against a dark background were seen in fluorescent staining (Fig. 2). A minimum of 100 fields were examined before reporting as negative. Sputum samples showing negative result by direct smear examination were subjected to concentration technique by NALC-NaOH (N-acetyl-L-cysteine sodium hydroxide) method[13] and once again subjected to ZN staining and fluorescent staining and the same pellet was used for PCR.

Polymerase chain reaction

This includes three steps

a) DNA extraction done by column method(NucleoSpin Tissue kit)
b) DNA Amplification
c) Identification of amplified products by gel electrophoresis

Amplicon information	
MTB ACE detection	**Size in agarose gel (bp)**
Internal control band	720
MTB band	360

Interpretation of results (Fig. 3)

	Internal Control	Target	Interpretation
Case 1	+	+	Specimen was positive for MTB (Mycobacterium tuberculosis)
Case 2	+	-	Specimen was a presumptive negative for MTB
Case 3	-	+	Specimen was a presumptive positive The results were confirmed by retesting
Case 4	-	-	Inhibitory specimen Test was repeated

Results

50 patients were enrolled in the study, 33(66%) were males and 17(34%) were females. Majority of the patients were in the age group between 51-60 years.

Of 100 sputum samples collected from50 patients, 44(44%) samples were positive and 56(56%) were negative by ZN staining. 52(52%) were positive and 48(48%) were negative by fluorescent staining. 76(76%) samples were positive by PCR and 24(24%) were negative (Table I).

Out of 52 smear positives, all 52 (52%) were positive by fluorescent staining and only44 (44%) were positive by ZN staining. That is 8 (8%) were positive by fluorescent staining but negative by ZN staining (Table II).

Out of 50 morning samples examined by ZN staining,23(23%) were positive and 27 (27%) were negative and of the 50 spot samples,21(21%) were positive and 29(29%) were negative. Two smears were positive from morning sample but negative by spot sample (Table III).

There was no difference in the result between morning and spot sample by fluorescent staining.

Table I: Findings of ZN Staining, Fluorescent staining and PCR.

Result	ZN Staining	Fluorescent Staining	PCR
Positive	44 (44%)	52 (52%)	76 (76%)
Negative	56 (56%)	48 (48%)	24 (24%)
Total	100	100	100

Table 2:Comparison between ZN Staining and Fluorescent staining.

ZN Staining result	FM Positive	FM Negative	Total
Positive	44 (44%)	00 (00%)	44 (44%)
Negative	08 (08%)	48 (48%)	56 (56%)
Total	52	48	100

Table 3::Comparison between morning samples and spot samples by ZN Staining

ZN staining result	Positive	Negative	Total
Morning sample	23 (23%)	27 (27%)	50 (50%)
Spot sample	21 (21%)	29 (29%)	50 (50%)
Total	44	56	100

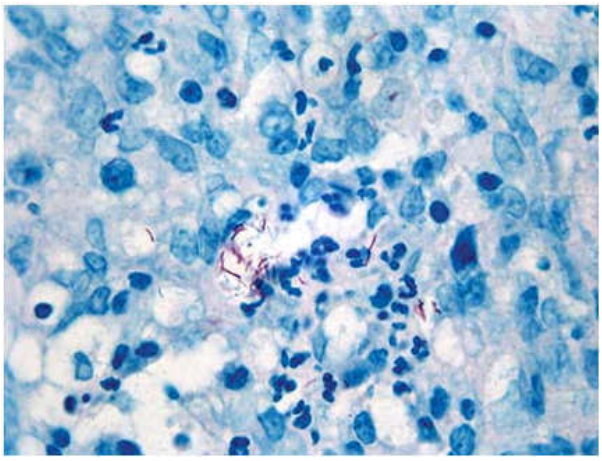

Fig. 1: ZN staining showing Acid fast bacilli.

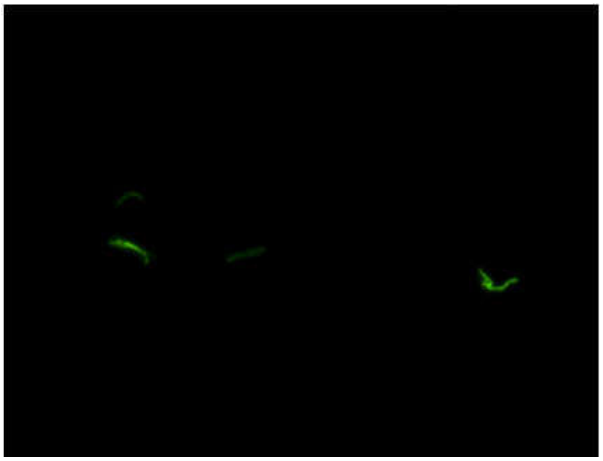

Fig. 2: Fluorescent staining showing Tubercle bacilli

Fig. 3: PCR results showing the internal control bands at 720bp andpositive bands (MTB band) at 360bp.

Discussion

Tuberculosis is one of the most serious infectious diseases and a considerable public health problem due to its high risk of person to person transmission, morbidity and mortality. Early diagnosis followed by adequate treatment is essential to prevent both mortality and morbidity. We evaluated the performance of microscopic methods over PCR on all suspected cases ofPulmonary tuberculosis and new cases of Pulmonary tuberculosis.

The present study shows the male preponderance which is also seen in other studies.[14,15] This could be due to increased outdoor activity of men and hence they are more likely to come in contact with active case of TB.

The present study is comparable with the study done by Negi SS et al. in ZN smear positivity.[16] Smear positivity rate depends on the type, number and quality of sputum sample and as well as bacillary load (10^4 bacilli/ml is required to give smear positive result).[17] eight percent of samples were positive by fluorescent staining but negative by ZN staining. Fluorescent staining is a better method of microscopy for demonstration of acid fast bacilli. Many other studies have also shown the increased efficacy of fluorescent staining.[15, 18, 19] Consequently more smear positives can be put on treatment using Fluorescent staining, which may contribute to improved treatment outcome.[20] In a study done by Dzodanu EG et al early morning samples yielded more AFB as compared to spot samples.[21] The present study showed no much difference in the result between morning and spot sample in the diagnosis of pulmonary TB. This could be due to the small number samples and further evaluation is required.TB cultures give the highest sensitivity in the diagnosis of active pulmonary TB. Unfortunately, Mycobacterium tuberculosis takes 3-6 weeks to give visible colonies, showing a critical disadvantage to this method.[22]

PCR was positive in 76 (76%) cases. PCR is proven to be more sensitive and is capable of picking as few as 10-15 tubercle bacilli.[23] The PCR was based on the amplification of the IS 6110, which belongs to IS 3 family and is found in all members of Mycobacterium tuberculosis complex. Most strains of Mycobacterium tuberculosis carry 10-15 copies of this gene and are present in a wide variety of chromosomal sites. Significant difference in the sensitivity of smear microscopy and PCR have been documented in many other studies.[16, 24]

Conclusion

In a developing country like India, the confirmation of Tuberculosis is still based on the observation of ZN stained smears especially in a resource limited settings. Various studies including ours have shown that the technique of examining Acid fast bacilli by fluorescent microscopy is more sensitive and less time consuming than that of ZN staining method. But, the molecular methods like PCR are even more sensitive and specific compared to microscopic methods. The main disadvantage of PCR is high cost. Hence, application of PCR should be restricted to those cases, where the disease was clinically suspected but the microbiological or histological diagnostic test results were equivocal.

References

1. Caulfield AJ, Wengenack NL. Diagnosis of active tuberculosis disease:From microscopy to molecular techniques. Journal of clinical Tuberculosis and other Mycobacterial Diseases 2016;4:33-43.
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, 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 Medicine 2011:11:15
4. Oguz VA, Sezak N, Oztop A, et al. A comparison of two different fluorochrome stains for the detection of acid fast bacilli in sputum specimens. Turk J Med Sci 2011;41(3):411-17.
5. 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. Journal of Clinical Microbiology 2005;43(6):2703-08.
6. Torrea G, Chakaya J, Mayabi M, Deun AV. Evaluation of the Fluoreslen S TM and fluorescence Microscopy blinded rechecking trial, Nairobi, Kenya. Int J Tuberc Lung Dis 2008;12:658-63.
7. 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 sample. Indian Journal of Medical Microbiology 2010;28(3):227-32.
8. Carniel F, Costa ERD, Bello GL, Martins C, Scherer LC, Rossetti ML. Use of conventional PCR and smear microscopy to diagnose pulmonary tuberculosis in the Amazonian rainforest area. Brazilian journal of Medical and Biological Research 2014;47(12):1016-20.

9. D'amato RF, Wallman AA, Hochstein LH, et al. Rapid diagnosis of Pulmonary Tuberculosis by using Roche Amplicor Mycobacterium Tuberculosis PCR test. J Clin Microbiol 1995;33(7):1832-34.
10. Varaine F, Henkens M, Gruzard V. Tuberculosis: Practical guide for clinicians, nurses, laboratory technicians and medical auxiliaries. Fifth revised edition 2010:1-164.
11. Revised National TB control Programme. Manual for sputum smear fluorescent microsopy. Central TB division, Directorate General of Health Services, Ministry of Health and Family welfare.
12. Revised National TB control Programme. Manual for laboratory technicians. Central TB division, Directorate General of Health Services, Ministry of Health and Family welfare.
13. Winn WC, Allen SD, Janda WM, et al. Mycobacteria. In: Koneman's color atlas and textbook of diagnostic Microbiology. 6th edition, USA: Lippincott Williams ans Wilkins;2006:1064-125.
14. Cohen RA, Muzaffar S, Schwartz D, et al. 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.
15. Marais BJ, Brittle W, Paiczyk K, et al. Use of light-Emitting diode fluorescence microscopy to detect Acid-fast bacilli in sputum. Clinical Infectious Disease 2008;47:203-7.
16. Parvez MAK, Hasan N, Rumi MAK, et al. PCR can help in early diagnosis of pulmonary tuberculosis. South East Asian J Trop Med Public Health 2003;34(1):147-53
17. Forbes BA, Sahm DF, Wiessfield AS. Mycobacteria. Bailey and Scott's diagnostic Microbiology. 12th edition, USA:Mosby Elsevier;2007:p 478-508.
18. Ziaee M, Namaei M, Khazaei M, Azarkar G. Comparison of the value of two different sputum staining for diagnosis of acid-fast bacilli. Iranian Journal of Clinical Infectious Diseases 2008;3(2):99-102.
19. Padmaja GV, Srujana K, Sadhana C. Comparison of Ziehl-Neelsen's stain, fluorescent stain with CBNAAT of sputum for the diagnosis of pulmonary tuberculosis. Journal of Dr NTR University of Health Sciences 2019;8:238-43.
20. Kivihya-Ndugga LEA, Van clef MRA, Githui WA, et al. A comprehensive comparison of Ziehl- Neelsen and fluorescence microbiology for the diagnosis of tuberculosis in a resource-poor urban setting. Int J tuberc Lung Dis 2003;7(12):1163-71.
21. Dzodanu EG, Afrifa J, Acheampong DO, Dadzie I. Diagnostic Yield of Fluorescence and Ziehl-Neelsen Staining Techniques in the Diagnosis of PulmonaryTuberculosis: A Comparative Study in a District Health Facility. Tuberculosis Research and Treatment 2019. Article ID 4091937.
22. Che-Engku-Chik CEN, Yusof NA, Abdullah J, Othman SS, Zaid MHM, Wasoh H. Detection of Tuberculosis (TB) using Gold Standard Method, Direct Sputum Smears Microscopy, PCR, qPCR and Electrochemical DNA Sensor. Journal of Biochemistry, Microbiology and Biotechnology 2016;4(2):16-21.
23. Negi SS, Anand R, Pasha ST, et al. Diagnostic potential of IS6110, 38kDa, 65kDa and 85B sequence-based Polymerase chain reaction in the diagnosis of Mycobacterium tuberculosis in clinical samples. Indian Journal of Medical Microbiology 2007;25(1):43-9.
24. SS Lima, WT Calmente, M Palaci, et al. Conventional and molecular techniques in the diagnosis of pulmonary tuberculosis: a comparative study. J Bras Pneumol 2008;34(12):1056-62.