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Effectiveness of Polymerase chain reaction in Ziehl-Neelsen stain negative body fluids samples in suspected cases of Extra Pulmonary Tuberculosis

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Abstract
Background: *Diagnosis of extra-pulmonary tuberculosis (EPTB) in smear-negative patients can be difficult. We assessed prospectively the performance of Real Time PCR in diagnosing smear-negative tuberculosis (TB), which represents the most common form of TB in a low incidence setting.*
Methods: *The present study was undertaken to find the effectiveness of Polymerase Chain Reaction targetting IS6110, MPB64 and 16SrRNA genes and culture on L–J media in Ziehl-Neelsen stain negative samples of body fluids for rapid diagnosis of suspected cases of Extra pulmonary tuberculosis.*
Results: *A total of 62 samples were taken of which 2 samples were not adequate in amount and hence were excluded. All 6 (10%) culture positive samples were PCR positive, in addition PCR could detect 5 out of 60 (8.3%) samples to be positive which were negative by culture. Of the 11 positive samples only MTB was reported by PCR and No NTM was detected.*
Conclusions: *PCR is a sensitive method for rapid diagnosis of TB compared to the conventional ZN staining. PCR can serve as a sensitive diagnostic method for microbiological diagnosis of smear-negative TB in countries with a low TB prevalence.*
Keywords: *extra-pulmoary, smear negative tuberculosis, sputum smear negative, tuberculosis.*

Introduction

Tuberculosis is a significant cause of morbidity and mortality all over the world and is one of the major airborne infectious diseases with a wide range of mortality ranging from 1.6 to 2.2 million lives per year(1). National Institute of tuberculosis and respiratory diseases in their annual report April 2015-March 2016 reported 6005 suspected cases and 3010 cases with the final diagnosis of extrapulmonary tuberculosis(2). Though pulmonary tuberculosis cases account for a vast majority of the total tuberculosis burden, almost 10-15% of total cases are extrapulmonary(3).

The diagnosis of tuberculosis (TB) still offers big diagnostic challenges related to the detection limit of smear microscopy, long time to culture-confirmation and variable sensitivity of molecular tests. The extrapulmonary tuberculosis diagnosis is lacking more in rural health facilities which are being used by about 70% of the population in developing countries. Here, the diagnosis largely depends on unreliable methods

such as ZN microscopy, histology, and tuberculin test. Although ZN staining microscopy is rapid and cheap; however, it is less sensitive for diagnosis of EPTB because of paucibacillary nature of samples and non-uniform circulation of MTB.

Mycobacterium culture is the reference method and is considered as 'gold standard' for detection of tubercle bacilli[4] but its long turnaround time of 2-6 weeks, as well as the complexity of procedure demanding highly skilled staff along with biosafety level III lab, limits its applicability for the routine use of a diagnostic test.[5] At present, nucleic acid amplification-based assays are the most suitable choices for the identification of Mycobacterium tuberculosis in smear-negative samples with the high degree of sensitivity and specificity.[6] The role of PCR in the diagnosis of tubercular pleural effusion has been evaluated extensively as an alternative diagnostic tool and has yielded variable results, with sensitivities ranging between 42 and 100% and specificities ranging between 85 and 100% using various PCR targets such as IS6110, 65kDa, TRC4, GCRS, etc. [6,7,8] The most common form of extrapulmonary tuberculosis is tuberculous lymphadenopathy[7,9] and its diagnosis remains a challenge since granulomatous lymphadenopathy has an extensive differential diagnosis. Several conditions, including sarcoidosis, fungal infections, and other inflammatory conditions, can present the same cytology and/or histopathology as tuberculous lymphadenopathy. A diagnosis of tuberculosis is then confirmed by the presence of AFB and/or isolation of M. tuberculosis on culture. However, owing to the paucibacillary nature of the specimens, the sensitivity of AFB smear and culture are low; cultures grow mycobacteria in 39 to 80% of cases.[10] Thus, there remain samples that are both AFB and culture negative, and increasingly, conventional methods and cytological investigations are being used in conjunction with PCR techniques to further help in the detection and characterization of pathogenic mycobacteria associated with human lymphadenitis[11]. Many reports have demonstrated the value of PCR in the diagnosis of extrapulmonary tuberculosis, including pleural effusion and lymphadenitis.[7,11,12] However, the presence of PCR inhibitors in clinical samples hampers the use of amplification techniques with full confidence and ease [7,13], and there is a pressing need for a robust, reproducible, and uniform method of inhibitor removal from clinical specimens (sputum, fluids, and tissues). The present study was undertaken to find the effectiveness of Polymerase Chain Reaction and culture on Ziehl-Neelsen stain negative samples of body fluids for rapid diagnosis of suspected cases of Extrapulmonary Tuberculosis.

Materials and Methods

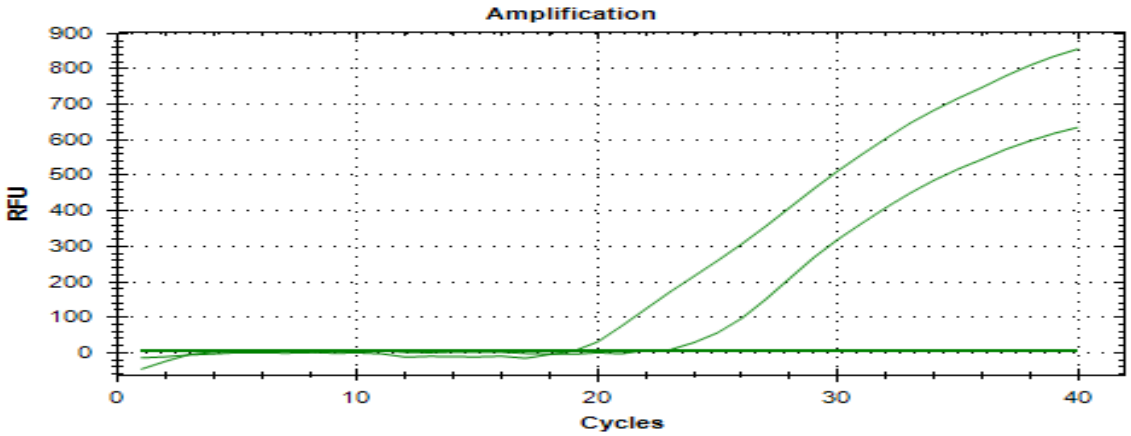
Extrapulmonary samples were collected under strict aseptic condition. A total of 60 Ziehl-Neelsen stain negative samples of body fluids (pleural fluid, pus aspirate, cerebrospinal fluid, urine, blood, peritoneal fluid, gastric aspirate) were included in the study. Samples were decontaminated, homogenized and cultured on LJ medium by the Petroff technique. Two drops from concentrated and homogenized samples were used for indirect smear preparation. Smear preparation, ZN staining and slide reading were done. Samples containing 1 mL body fluid were centrifuged at 1800g for 15 minutes; supernatants were discarded, and pellets were used for DNA isolation.PCR PROTOCOL: PCR was performed on all the samples targetting IS6110, MPB64 and 16SrRNA genes with the limit of detection of 40 threshold cycles (ct) and viral nucleic acid. DNA was extracted by using commercially available extraction kit QIAamp DNA blood mini kit. For reagent preparation, the master mixture was made which contained 10µl of TB & NTM reaction mixture (DNA polymerase, Uracil-N-glycosidase, buffer containing deoxynucleotides) and 5µl of TB and NTM probe mixture (oligonucleotides for amplification and detection of target and plasmid for internal control). To this 5µl of extracted DNA was added and the mixture was distributed in a 96 well plate on which PCR program was run till 40 threshold

cycles and results were noted. Presence of any of the sequences was considered as a positive result with typical S-shaped growth curve. Quality control Tests were also monitored for contamination using water as a negative control in each test run. Each PCR run included ATCC standard M. tuberculosis strain H37Rv as a positive control.

Results

A total of 62 samples were taken of which 2 samples were not adequate in amount and hence were excluded. Of the 60 Zeihl-Neelsen stain negative clinical samples taken, maximum samples were of pleural fluid. Majority of the patients were males (68%) in comparison to females (32%). The maximum number of the patients belonged to the age group of 21 to 30 years followed by 31 to 40 years and 41 to 50 years. Of the 60 Zeihl-Neelsen stain negative samples 6(10%) samples were culture positive. Culture positivity rate was highest in samples of Pus aspirate i.e. 2 out of 9 (22.2%) followed by Pleural fluid i.e. 3 out of 15 (20%) and Urine i.e. 1 out of 10 (10%). No culture-positive samples were reported in Gastric aspirate, CSF, Blood and Peritoneal fluid samples. Of the 60 Zeihl-Neelsen stain negative samples 11 (18.3%) samples were PCR positive. (Figure 1) PCR positivity rate was highest in samples of Pus aspirate i.e. 3 out of 9 (33.3%) followed by Pleural fluid i.e. 4 out of 15 (26.6%), Urine i.e. 2 out of 10 (20%), Peritoneal fluid i.e. 1 out of 5 (20%) and CSF i.e. 1 out of 7 (14.2%). No PCR positive samples were reported in blood and gastric aspirate samples. Of the 11 positive samples, only MTB was reported by PCR and No NTM was detected. Of 60 clinical samples, 6 (10%) were culture positive and 11 (18.3%) were PCR positive. All 6 (10%) culture positive samples were PCR positive, in addition, PCR could detect 5 out of 60 (8.3%) samples to be positive which were negative by culture**.**

Figure 1: S shaped growth curve positive for MTB probe under the growth curve of positive control by Real Time PCR.(BIO RAD CFX96 Real Time system)



Discussion

In spite of the increased international focus on TB control programs, the incidence of TB continues to rise in several regions of the world due to various factors like rapid population growth, increased urbanization, civil wars and mass migration of populations, the HIV epidemic and the emergence of MDR-TB strains.

Males were more affected than females (2.1:1) which were in accordance with a similar study in which two-third of the patients were males and one-third of the patients were females.[8] This is in contrast to a study conducted by Amer et al were 48% were men and 52% were women.[14]

In one study, reported age of patients ranging from 1 year to 70 years with 79% of patients being less

than 50 years of age with a male to female ratio of 2.03:1 which was in accordance to our study, the age of patients ranged from newborn to 80 yrs with 68.3% of patients less than 50 yrs of age and male to female ratio was of 2.125:1.[15] In another study, the majority of the patients (48%) were between the age group of 21 and 40 years, followed by 11-20 years (17%) and 41-50 years (15%), with male to female ratio of 1.8:1 (64:36) wherein our study 50% patients were between 21-40 yrs followed by 41-50 yrs (18%) and 11-20 yrs (10%) with male to female ratio of 2.1:1(16). Our study has shown 10% positivity rate by LJ culture whereas high positivity rate of 42.1% was reported in the study conducted by Negi et al[17]. Different studies have culture positivity rate between 3.37% to 21.18% whereas in a study by a very less positivity rate of 0.66% was reported.[18,19,20,21] These results are in agreement with the worldwide use of M.tuberculosis culture as a gold standard. But conventional bacteriological methods are either slow or their sensitivity is quite low. In another study conducted by Iqbal et al (2011) culture positivity rate in samples of pleural fluid was 22.92%, CSF was 17.07%, pus aspirate was 35.29%, urine and peritoneal fluid was 11.11% which was in accordance with our study where culture positivity rate in samples of pleural fluid was 20%, pus aspirate was 22.2% and urine was 10%.[19] No culture positive result was reported in samples of CSF, peritoneal fluid, blood and gastric aspirate samples in our study. In a study conducted by Kaur et al (2016) culture positivity rate in samples of peritoneal fluid was 23%, pleural fluid was 25% and urine was 10% in comparison to our study where no culture positive result was reported in peritoneal fluid samples[22]. The poor performance of conventional microbiological techniques in extrapulmonary specimens has stimulated the increased use of PCR tests in the laboratory diagnosis of tuberculosis. In our study, PCR positivity rate was 18.3% as compared to the study of Ajantha et al in which PCR positivity rate was 10.9 %.In a study conducted by Makesh Kumar et al, Tiwari et al, Sid Siddiqui et al PCR positivity rate was 26.96 %, 62 %, 70% respectively wherein study of Hemal A K et al high positivity rate of 80.95% was reported by PCR.[18,23,24,25]

In our study, we did not find any NTM positive case as all the suspected cases were MTB positive as compared to a study[26] where 6% were positive for NTM from pleural fluid samples and 25% were positive for NTM from lymph node aspirate. In our study highest PCR positivity rate of 33.3% was reported in samples of pus aspirate followed by 26.6% in pleural fluid samples, 14.2% in CSF samples and 20% in urine and peritoneal fluid samples. No PCR positive result was reported in samples of blood and gastric aspirate. An earlier Indian study by Narayan et al[27] has also reported 63% positivity by PCR using IS6110 element in specimens of extrapulmonary. Most of the studies which used IS6110 based PCR had reported 90% sensitivity in the Cerebrospinal fluid, pleural fluid, peritoneal fluid and other extrapulmonary specimens.[28,29] In a study of Siddiqui et al[15] positivity rate of 5.4% in samples of CSF,17.4% in samples of peritoneal fluid, 25% in samples of pleural fluid, 33.3% in samples of pus aspirate was seen in accordance with the present study. 0% positivity rate in urine samples was reported whereas in our study we found 20% positivity rate in urine samples. In the study by Nagpal et al [30], 1.4% samples were culture positive and 30.3% samples were PCR positive whereas in this study 10% samples were culture positive and 18.3% samples were PCR positive. These differences between the conventional methods and PCR were also reported by Negi et al (2007) and Hazia et al.[1,31] Because of the significant difference in the sensitivity rates of culture and PCR, PCR can be a very useful means of diagnosis of extrapulmonary tuberculosis. The common problem raised during the PCR assays is the high risk of false-positive results due to common laboratory contamination or presence of killed or dormant bacilli in the patient specimens[32]. Proper control checks and good laboratory practice can minimize the chances of false positive results. There are several other

possible reasons for false negativity viz., the paucibacillary nature of the disease, possible hypersensitivity mechanisms, or the availability of only one small amount or volume of a sample after it was distributed for various microbiological, pathological and biochemical investigations. The advantages of PCR are that it is very rapid, easy to perform method and result can be issued for early treatment and to prevent further transmission of tuberculosis infection. The drawback with PCR assay is that it is not able to differentiate live from dead organisms. In the present study, culture could detect 10% cases while PCR could detect M. tuberculosis in 18.3% of suspected extrapulmonary cases. Hence the proper utilization of PCR may give vital evidence in more number of cases as compared to the established conventional methods. Molecular techniques which are being simplified and improved continuously and rapidly, appear to be the future tests of choice for most of the infections, including tuberculosis 183 and can be applied where there is strong clinical suspicion, especially when the conventional techniques are negative. The rapidity, high sensitivity and simplicity of PCR may even compensate the higher cost of the test compared with less sensitive conventional tests in the diagnosis of EPTB[(12)]. When the results of various studies were compared, one fact became obvious that PCR is the method of choice for the diagnosis of mycobacterial infections in cases where the suspicion is high but Ziehl–Neelsen staining or culture is negative. Molecular diagnosis by PCR has a great potential to improve the clinician's ability to diagnose extrapulmonary tuberculosis.

References

1. Negi SS, Khan SF, Gupta S, Pasha S T, Khare S, Lal S. Comparison of the conventional diagnostic modalities, bactec culture and polymerase chain reaction test for diagnosis of tuberculosis. Indian J Med Microbiol 2005;23:29-33.
2. Index TB Guidelines. Guidelines on extra-pulmonary tuberculosis for India. World Health Organization; 2016. Available from: http://www.icmr.nic.in/guidelines/TB/IndexTB%20Guidelines%20-%20green%20colour%202594164. Pdf accessed on January 3, 2018.
3. World Health Organization. Global tuberculosis control. WHO report 2009; WHO/HTM/TB/2009.
4. Rattan A. PCR for diagnosis of tuberculosis: Where are we now? Indian J Tuberc2000;47:79-82.
5. Montenegro SH, Gilman RH, Sheen P, Cama R, Caviedes L, Hopper T, et al. Improved detection of Mycobacterium tuberculosis in Peruvian children by use of a heminested IS6110 polymerase chain reaction assay. Clin Infect Dis 2003; 36:16-2.
6. Kulkarni S, Singh P, Memon A, Nataraj G, Kanade S, Kelkar R, Rajan M. An in-house multiplex PCR test for the detection of Mycobacterium tuberculosis, its validation & comparison with a single target TB-PCR kit, Indian J Med Res 2012;135:788-94.
7. Parandaman, V., S. Narayanan, and P. R. Narayanan. Utility of polymerase chain reaction using two probes for rapid diagnosis of tubercular pleuritis in comparison to conventional methods. Indian J. Med. Res.2000;112**:**47-51.
8. Chakravorty S, Sen MK, Tyagi JS. Diagnosis of Extrapulmonary Tuberculosis by Smear, Culture, and PCR Using Universal Sample Processing Technology. Journal of Clinical Microbiology. 2005;43(9):4357-62.
9. Luca Norbis, Riccardo Alagna, Enrico Tortoli, Luigi Ruffo Codecasa, Giovanni Battista Migliori et al. Challenges and perspectives in the diagnosis of extrapulmonary tuberculosis, Expert Review of Anti-infective Therapy 2014;12:633-47.

10. Sunnetcioglu A, Sunnetcioglu M, Binici I, Baran AI, Karahocagil MK, Saydan MR. Comparative analysis of pulmonary and extrapulmonary tuberculosis of 411 cases. Annals of Clinical Microbiology and Antimicrobials. 2015;14:34.
11. Shrivastava G, Bajpai T, Bhatambare GS, Patel KB. Genital tuberculosis: Comparative study of the diagnostic modalities. J Hum ReprodSci 2014;7:30-3
12. Eisenach, K. D., M. D. Cave, J. H. Bates, and J. T. Crawford. Polymerase chain reaction amplification of repetitive DNA sequence specific for Mycobacterium tuberculosis. J. Infect. Dis. 1990;161**:** 977-981.
13. Chitra, B. Usharani, S. Smita, C. K. Vidya Raj, S. Anbazhagi, M. Muthuraj. Diagnostic Performance of Polymerase Chain Reaction Targeting Insertion Sequence (IS6110) for the Detection of Extra Pulmonary Tuberculosis.American Journal of Infectious Diseases and Microbiology 2017, 5$^{(4),}$ 126-31.
14. Amer S, Hefnawy AE, Wahab NA, Okasha H, Baz A.Evaluation of different laboratory methods for rapid diagnosis of tuberculous pleurisy.Int J Mycobacteriol. 2016 ;5:437-45.
15. Siddiqui M, Anuradha PR, Nagamanik Vishnup H.Comparison of conventional diagnostic modalities,BACTEC culture with polymerase chain reaction for diagnosis of extra-pulmonary tuberculosis.J Med Allied Sci 2013;3:53-8.
16. Lakshmi KR, Kumari VS, Vasundhara N, Suresh K. Detection of Extrapulmonary Tuberculosis from Various Samples in Sputum Smear Negative Patients. Int J Sci Stud 2016;3$^{(10):}$63-6.
17. Negi SS, Anand R, Pasha ST, Gupta S, Basir SF, Khare S, 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 J Med Microbiol. 2007;25:43–49.
18. Kumar MV, Madhavan R, Narayanan S. Polymerase chain reaction targeting insertion sequence for the diagnosis of extrapulmonary tuberculosis. Indian J Med Res 2014;139:161-6
19. Iqbal S, Ahmed R, Adhami SU, Mumtaz A. Importance of polymerase chain reaction in diagnosis of pulmonary and extra-pulmonary tuberculosis. Journal of Ayub Medical College, Abbottabad: JAMC. 2011;23(1):73–76.
20. Sharma K, Appannanavar SB, Modi M, Singh M, Sharma A, Varma S. Role of multiplex polymerase chain reaction using IS6110 and protein b for the diagnosis of extra-pulmonary tuberculosis: North India. Indian J PatholMicrobiol 2015;58:27-30.
21. KhaledGhaleb,MagdyAfifi, and Mohamad El-Gohary Assessment of Diagnostic Techniques of Urinary Tuberculosis Mediterr J Hematol Infect Dis. 2013; 5:1.
22. Kaur J, Singh J, Mishra P. Comparative Evaluation of CFX96TM Real Time PCR with Conventional PCR for Rapid Diagnosis of *Mycobacterium tuberculosis* Complex in Clinical Isolates. Arch Clin Microbiol. 2016, 7:4.
23. Ganavalli S. Ajantha, Praveen C. Shetty, Raghavendra D. Kulkarni, UmeshBiradar. PCR as a diagnostic tool for extra pulmonary tuberculosis.J Clin Diagn Res. 2013; 7: 1012–5.
24. Tiwari V, Jain A, Verma RK. Applification of enzyme amplified mycobacterial DNA detection in the diagnosis of pulmonary and extrapulmonary tuberculosis. Indian J Med Res. 2003;118:224–8.
25. Hemal AK, Gupta NP, Rajeev TP, Kumar R, Dar L, Seth P. Polymerase chain reaction in clinically suspected genito-urinary tuberculosis: Comparison with intravenous urography, bladder

biopsy, and urine acid fast bacilli culture. Urology. 2000;56:570–74.

26. Chakravorty S, Sen MK, Tyagi JS. Diagnosis of extrapulmonary tuberculosis by smear, culture and PCR using universal sample processing technology.J Clin Microbiol. 2005; 43: 4357-62.
27. Narayanan S, Parandaman V, Narayanan PR, Venkatesan P, Girish C, Mahadevan S, Rajajee S. Evaluation of PCR Using TRC_4 and IS6110 Primers in Detection of Tuberculous Meningitis. Journal of Clinical Microbiology. 2001;39:2006-8.
28. Amin I, Idress M, Awan Z, Shahid M, Afzal S, Hussain A. PCR could be a method of choice for identification of both pulmonary and extrapulmonary tuberculosis. BMC Res Notes 2011; 4 : 332
29. Iqbal S, Ahmed R, Adhami SU, Mumtaz A. Importance of polymerase chain reaction in diagnosis of pulmonary and extra-pulmonary tuberculosis. Journal of Ayub Medical College, Abbottabad: JAMC. 2011;23:73-6.
30. Nagpal S, Chopra GS, Oberoi A, Singh N, Varghese SR. Conventional versus molecular methods for diagnosis of tuberculosis in a tertiary care center: A study from Punjab. CHRISMED J Health Res 2016;3:258-62.
31. Hajia M, Rahbar M, Amini R. Is PCR assay reliable for diagnosis of extrapulmonary tuberculosis; Afr J Microbiol Res. 2009; 3(12):877-881.
32. Beige J, Lokies J, Schaberg T, Finckh M, Fischer M. Mauch H, et al. Clinical evaluation of a Mycobacterium tuberculosis PCR assay. J ClinMicrobiol 1995; 33 : 90-5.