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Clinical evaluation of an in-house novel primer sequence based PCR for conventional molecular diagnosis of pulmonary and extrapulmonary tuberculosis.

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Abstract

Background:Tuberculosis is one of the chronic infectious bacterial disease that still remains as major cause of morbidity and mortality in the world. The limited role of diagnostic methods for correct and early detection of bacilli in different types of clinical samples remains uncertain increasing prevalence of TB. Aim & Objective: The purpose of present study is to develop an in-house primer to PCR targeting the *fadE-20* gene for diagnostic applicability in detection of M.tuberculosis complex species from clinically suspected cases of pulmonary and extrapulmonary tuberculosis. Materials & Methods: This study was conducted in Genomix Carl Pvt. Ltd, Pulivendula, A.P during the period of 2 years 6 months from March 2017 to September 2019. A total of 233 different types of clinical samples were assayed in this study with optimized fadE gene primer and its accuracy was comparatively analyzed with conventional IS6110 sequence based PCR ,Acid fast Bacilli(AFB) smear microscopy and LJ-culture tests results. Results: In the present study The highest overall positivity rate of 85.4% was observed by using in-house *fadE-20* PCR whereas overall positivity with IS6110 PCR was found to be 82.4%(192). The in-house fadE PCR test resulted in sensitivity of 100% & 96.1% for pulmonary and extrapulmonary culture positive specimens whereas IS6110 PCR resulted in sensitivity of 98.8% and 93.5%.respectively.Furthermore both the amplification tests gave consistently negative results from the negative control group therefore showing overall specificity of 100%. Conclusion: By comparative analysis of overall data from both amplification tests,It can be concluded from this study that the *fadE-20* PCR appears to be a reliable tool for accurate identification of Mycobacterium tuberculosis complex in different types of pulmonary and extrapulmonary samples obtained from tuberculosis patients.

Key words- Pulmonary and extra pulmonary tuberculosis, in-house primer, Polymerase chain reaction, *Mycobacterium tuberculosis complex.*

Introduction

Tuberculosis still continues as one of the world leading air borne chronic communicable mycobacterial infection causing severe health threat to humans despite the existence of laboratory methods for diagnosis of tuberculosis(TB) cases and effiicious chemotherapy treatments(ATT). TB globally leads to almost 9,0 million new infections each year and death toll of approximately 1.5 million per year (Kiran Chawla et al., 2015; Scherer et al. 2011).It is reported that India accounts for one quarter of the worlds TB buden sharing prevalence rate of 30% and mortality of almost 350,000 annually (S Kulkarni et al., 2012; Paras Singh et al., 2013). Eventhou,tuberculosis most commonly affects lungs(90%) but can also be reactivated by immune compromisation and disseminate outside lungs to other body sites leading to manifestation of Extrapumonary TB(EPTB 15- 20%)(S Kulkarni et al., 2012; Shavi Nagpal et al., 2016). In clinical practice by most of clinicians the corner stone for the confirmation of mycobacterial infections on patients with clinical signs, symptoms & radiographic image changes suggestive of tuberculosis are still made by conventional microbiological techniques like Acid fast Bacilli(AFB) Ziehl-Neelsen (ZN) staining smear microscopic examination and culture of clinical specimens on Lowenstein-Jensen(LJ) medium for identification of mycobacteria. But the limited role of conventional microbiological methods which are inadequate in sensitivity, specificity and lengthy for definitive identification of tuberculosis infection have made the disease undiagnosed. The emergence of molecular methods which provides higher degree of positive results with good specificity, reliability and fast in detection of *Mycobacterium tuberculosis* (MTB) from pulmonary and extrapulmonary samples have made suitable choice alternative to the traditional diagnostic methods. Among molecular methods,Polymearse chain reaction(PCR) targeting amplification of specific mycobacterial DNA sequence directly from variety of clinical specimens(both pulmonary and extrapulmonary) have attracted considerable importance for use in clinical practice in rapid diagnosis of mycobacterium tuberculosis infection with high sensitivity and specificity. Although primers targeting for *IS6110* gene sequence present as multiple copies in genome which is mostly used in many laboratories that is considered to be specific but there are many literatures describing the presence of this insertion sequence in some bacteria species other than tubercle bacillus and its absence in some *M.tuberculosis* strains across the geography, reducing the sensitivity and specificity of PCR tests (S. Narayanan, 2013; T. Moatter et al., 1998).Hence the development of a robust genetic marker is importantly needed to establish a rapid and reliable molecular based PCR test for diagnosis of tuberculoss. With this background,in the current study we aimed to design and develop an In-house primer to PCR assay based on hypothetical novel *fadE-20* gene sequence for rapid identification of *M.tuberculosis complex* in various clinical specimens obtained from suspected cases of pulmonary and extra-pulmonary tuberculosis.In addition,we also evaluated its potential diagnostic value by comparing with molecular *IS6110* based PCR assay and conventional acid fast bacilli (AFB) smear microscopy and LJ medium culture methods results.*fadE* genes of *mycobacterium tuberculosis complex* encoding for acyl coenzyme A enzyme is reported (predicted) to be associated with host cell entry and suppressing immune defense mechanism to enable their increased survival causing host pathogenecity (M. Kaley et al., 2018; Rochelle van Wyk et al., 2019; F. Matthew et al., 2013). So with this baseline we focused at studying the clinical implications of *fadE-20* gene that may lead to be a diagnostic marker for rapid diagnosis of tuberculosis.

Materials and Methods:

We performed this study in the Molecular Biology laboratory at GENOMIX-CARL, Research centre,Pulivendula, during the period of 2 years 6 months from March 2017 to September 2019. The samples undertaken for the current study were obtained from Genomix Molecular diagnostics Division,Hyderabad, that were received for the diagnosis of *M.tuberculosis* infection collected from different categories of TB cases attending the referred Hospitals.

Study subjects and clinical specimens collection: A total of 233 clinical samples were investigated in the study having high clinical suspicion of Tb/ confirmed by other laboratory investigations and Radiographic evidences in conjuction with strong response to chemotherapy treatment from patients with either pulmonary or Extra-pulmonary tuberculosis infection. Out of total 233 samples, 114 of them were Pulmonary ,while 119 of the samples were Extrapulmonary.The Pulmonary samples comprised sputum & bronchoalveolar lavage fluid,while the Extra-Pulmonary samples included Lymph nodes, Synovial fluid, Breast tissue, gastric aspirates,pleural fluid ,Ascitic fluid and cerebrospinal fluid.On the other hand 60 samples from nontuberculous subjects recieved for other bacteriological tests referred from hospitals and confirmed negative to TB by ZN smear microscopy and

radiological scans were included as Negative Controls. All the clinical presentations history, radiological scan findings, cyto-pathological details and response to chemotherapy treatment reports for each case were collected from the medical report files that was recorded from received samples investigation form of the referred hospitals.

Processing of Clinical specimens: All the Pulmonary and extrapulmonary specimens received from referring hospitals were collected in MC-Cartnet tightly capped bottles accompanied with requisition form. The Sputum Samples were mechanically subjected to homogenization and decontamination within 24 hours after collection by using standard CPC-NaCl protocol.Briefly this protocol included addition of optimum concentration of Cetyl pyridinum chloride(CPC) in sodium chloride(Nacl) to sputum samples,centrifugation and the resulting deposit was divided in aliquots by collection in sterile vials for microbiological and PCR assays.For aseptically collected Extrapulmonary specimens(EPTB), such as body fluids were processed by standardized sulphuric acid method. Briefly this protocol involved addition of sulphuric acid(5%) to fluids and the resulting deposit divided into alliquotes for bacteriological identification and PCR tests.In case of tissue specimens, they were homogenized with a sterile Teflon tissue grinder and then processed by sulphuric(5%) as for other body fluids.An aliquot from each category of processed Pulmonary and Extrapulmonary specimens was used for ZN staining microscopy and culture on LJ medium. The other aliquots from processed specimens were stored at −70°C until DNA extraction which was used further for PCR amplification Assay.

DNA extraction from Sputum & other body fluid Specimens: DNA extraction from processed sputum and other body fluid specimens was performed by standardized CTAB-Nacl method. Briefly this protocol involved addition of equal volume of TE buffer to each alliquote of processed specimens sediment tubes and heat killed at 80°C. Lysozyme at a concentration of 2mg/ml was added and incubated initially at 37°C.Then Sodium dodecy sulfate(SDS-10%) ,proteinase-k(10mg/ml) was added to the mixture and finally incubated at 60°C.Thereafter a volume 100 microlitres each of 5M NaCl and Cetyltrimethylammonium bromide(CTAB-1%) were added to each sample and subjected to incubation at 65°C/15 min.

DNA was extracted by adding an equal volume of chloroform-Isoamylalcohol(24:1) and after being centrifugation.upper aqueous phase transferred to sterile tube.DNA was precipitated with ice cold isoproponal followed by overnight incubation at -20°C. Then the precipitated DNA was recovered by centrifugation and the pellet was washed with 70% ice cold ethanol. Finally the pellet was allowed to air dry until ethanol evaporated and later dissolved in 30-50 microlitres of 0.5X TE buffer.The Resulting final DNA was stored at -20°C until PCR assay.

DNA extraction from Tissue Specimens: The sediments obtained from the homogenized and decontaminated tissue specimens were initially subjected to incubation at 80°C/45 min for heat killing of mycobacterial cells. DNA extractions from these tissue specimens was then performed with a commercially available qiagen DNA tissue mini kit(Qiagen,Bangalore,India),as per to the procedure recommended in the protocol provided along with all the reagents in the DNA extraction kit.The resulting eluated DNA was freezed at -20 degree centigrade until further used for a PCR assay.

Amplification of In-house & IS6110 genes by single target PCR : The extracted DNA samples were used in a single target PCR assay for the identification of TB using specific oligonucleootide primer pairs forward 5'-AAACACCCAAGCCACGAC-3'and reverse 5'-ATGGGCAGTGCCACCAA-3'.amplifying 1161bp of inhouse *fadE-20* gene sequence.& IS-forward 5'-CCTGCGAGCGTAGGCGTCGG-3' and IS-reverse 5' CTCGTCCAGCGCCGCTTCGG-3'.amplifying 123bp of *IS6110* gene sequence.Both the inhouse *fadE-20* PCR and *IS6110* PCR were carried out seperately in a thermocycler(Bio-Rad,California,USA)with a total Reaction Volume of 50 µl containing *fadE-20* and *IS6110* forward and reverse primers each at a concentration of 20pmol & 25pmol.Each Amplification reaction consisted of 2x master mix (amplification buffer with Taq DNA polymerase,Tris-HCl,KCl,MgCl2,&dNTPs)(Banglore Genei, Bangalore,India),molecular grade water and 3 µL of each sample DNA.The Thermal cycling parameters programmed for amplifications of target *fadE*-20 gene sequence included 1 cycle at 95°C/4 min followed by 40 cycles of each of one min at 94°C, 66°C and 72°C then with a final cycle at 72°C/10 Minutes. For amplification of IS6110 gene sequence the PCR cycling conditions consisted of an initial denaturation step of 95°C/4 minutes followed by 35 cycles each of 1 min at 94 °C, 56 °C, and 72 °C with a final extension at 72°C/10 minutes. After the completion of PCR assay,the reaction mixture was electrophoresed on 2% agarose gel and the gel was visualized under a gel documentation system (Banglore Genei,Bangalore).. Samples showing the presence of amplicon size of 1161bp for *fadE*-20 and 123bp for *IS6110* were considered as positive result for detection of *Mycobacterium tuberculosis complex.H37Rv* strain. DNA template as positive control and Nuclease free water as negative grade were included in every set of amplification reaction for both PCR tests.

Results and Discussion:

The present study compared the efficacy of *fadE*-20 gene In- house PCR primers with *IS6110* PCR primers over other conventional gold standard techniques for the early diagnosis of MTB complex in pulmonary and Extrapulmonary tuberculosis cases.The analyzed clinical specimens obtained from 233 suspected TB study subjects collected from different pulmonary and extrapulmonary parts were processed for the diagnosis of *MTB complex* The conventional Culture on LJ media was used as gold standard for the detection of mycobacterial growth.

Table 1: Sensitivity of in house fade-20 PCR compared with IS6110 in culture confirmed Mycobacterium tuberculosis clinical samples.

Specimens	No. of samples	IS6110 PCR (+)	fadE-20 PCR (+)
Lymp nodes	18	100%	100%
Synovial fluid	15	86.6%	93.3%
Breast tissue	13	100%	100%
Gastric aspirates	11	90.9%	90.9%
Pleural fluid	11	100%	100%
Ascitic fluid	07	85.7%	85.7%
CSF	03	66.6%	100%
Total EPTB	78	93.6%	96.1%
Sputum	52	100%	100%
BAL	34	97%	100
Total PTB	86	98.8%	100%
Total	164	96.3%	98.1%

Of the 164 culture positive specimens 106(45.5%) were smear positive on ZN smear examination.. In smear positive category both In-house *fadE-20* PCR and *IS6110* PCR detected mycobacterial DNA in all specimens showing sensitivity of 100%, PCR positivity of *IS6110* and *fadE*-20 gene targets was found to be 90% and 95% in smear negative & culture positive TB cases. In Paucibacillary Extrapulmonary smear negative & culture positive patients, PCR sensitivity of *fadE-20* and *IS6110* gene targets were found to be 92.1% and 86.8%. However PCR positivity was found to be 100% in smear positive & culture positive samples through both PCR gene target assays.PCR positivity in smear positive & culture negative samples was also observed to be 100% by *IS6110* PCR and *fadE*-20 PCR amplification assays The sensitivity values of conventional *IS6110* PCR and in-house *fadE-20* PCR for identification of M.tuberculosis complex in phenotypically negative samples were determined to be 47.76% and 53.7% respectively.Further the specificity was determined to be 100% in true negative healthy controls or non-TB subjects with *IS6110* and *fadE*-20 gene targets.Table-2 shows sensitivity of detection of *MTB* for both *IS6110* PCR and *fadE*-20 PCR test, in four different groups from TB patients (Group A- Smear & Culture positive, Group B- Smear negative & Culture positive, Group C- Smear positive & Culture negative and Group D- Smear and Culture negative).

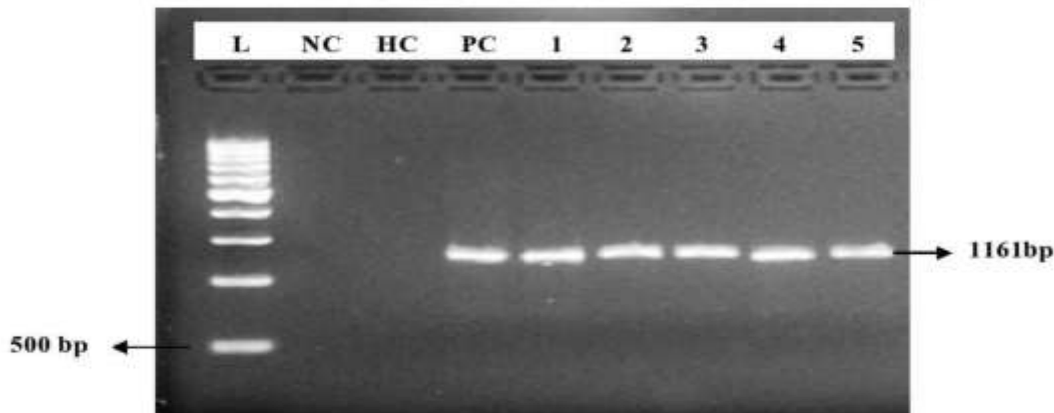
Table 2: Overall Positivity of IS6110 and in-house fade-20 PCR in categorized Pulmonary and Extrapulmonary TB samples.

Category	Number of samples	IS6110 PCR	In-house PCR
Smear (+)	106	100%	100%
Smear (-)	127	67.77%	73.22%
Culture (+)	164	96.34%	98.17%
Culture (-)	69	49.27%	55%
Smear(+)Culture (+)	104	100%	100%
Smear(+)Culture (-)	02	100%	100%
Smear(-)Culture (+)	60	90%	95%
Smear(-)Culture (-)	67	47.76%	53.7%
Total	233	82.4%	85.4%

In case of LJ medium culture methodology, 86 pulmonary TB samples (52 sputum and 34 BAL) and 78 Extrapulmonary TB samples were positive on LJ medium From 86 LJ medium positive pulmonary TB samples, *IS6110* PCR test was positive in 98.8% whereas *fadE*-20 PCR test was postive in 100%.Among 78 LJ medium positive Extrapulmonary TB samples, *fadE*-20 gene PCR test was positive in 96.1% and *IS6110* PCR test was positive in 93.5% culture positive samples.The overall diagnostic positivity of PCR with *fadE*-20 and *IS6110* gene targets were 91.2% and 88.6% for clinically diagnosed pulmonary tuberculosis and 79.83% and 76.4% for

extrapulmonary tuberculosis patients respectively. while the corresponding values for Smear microscopy and LJ culture methods were observed as 56.1% and 75.4%(pulmonary) and 35.3% and 65.5%(extrapulmonary) respectively. In total out of 233 clinical diagnosed samples,only 45.5% samples were positive with smear microscopy whereas 70.4% samples positive by LJ culture method.The overall positivity rate by using in-house PCR targeting *fadE-20* gene was found to be 85.4% while the positive rate with *IS6110* gene based PCR was observed to be 82.4% in suspected cases of Tb samples, the *IS6110* and *fadE-20* gene target amplification assays exhibited sensitivities of 96.3%(158) and 98.1%(161),respectively when the culture test was considered as a gold standard

Fig-1 : Gel Electrophoresis of *fadE-20* gene (1161bp).

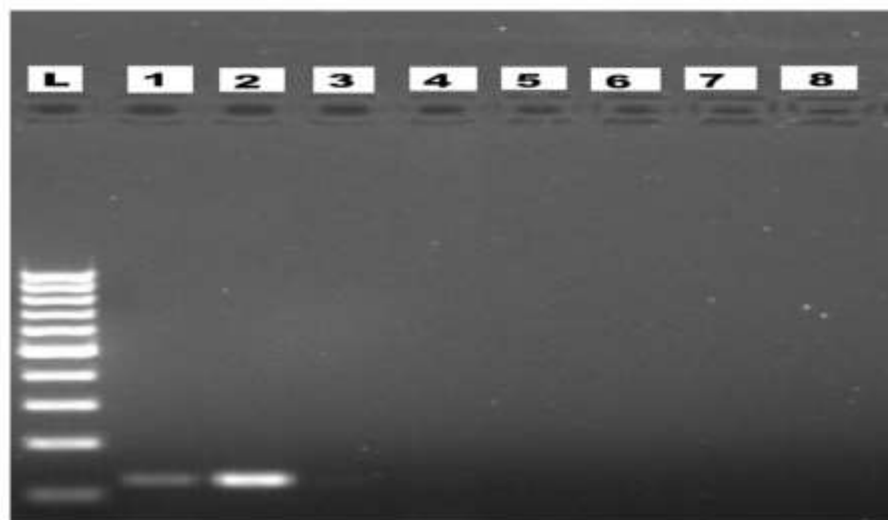


Lane- L:500bp DNA Ladder; Lane-NC: Negative control.

Lane-HC: Healthy Control sample; Lane-PC:Positive Control

Lane-1,2,3,4,5:PCR amplification of In-house *fadE* gene(1161bp)

Fig-2: Gel Electrophoresis of *IS6110* gene(123 bp)



Lane- L:100bp DNA Ladder; Lane-1:Positive Control

Lane-2,3,4: PCR amplified *IS6110* gene(123bp)

Lane-5,6:Healthy Control samples.

Lane-7,8: Negative Control samples.

In the Present study the diagnostic sensitivity of in-house *fadE-20* PCR for identification of M.tuberculosis against clinically diagnosed (85.4%) and culture conformed tuberculosis(98.1%)patients was higher than *IS6110* PCR assay showing sensitivity of 82.4% and 96.3%.The overall positivity result of *fadE* PCR in clinically diagnosed samples was observed to be higher as compared to earlier reports with who obtained positivity of 83% (Minal Deshmukh et al., 2013; S.S. Negi et al., 2007) and 81% (Scherer et al., 2011; Sunil Sethi et al., 2012; S.S. Negi et al., 2007) using target *IS6110* based PCRs and in-house PCR methods. Moreover, the diagnostic sensitivity of *fadE-20* PCR and *IS6110* PCR against culture resulted in our study was also higher than previous reports showing 81-96% using various target gene primers (Portillo-Gomez L et al., 2000; Minal Deshmukh et al., 2013). In-house *fadE-20* PCR showed higher sensitivity of 100% as compared to *IS6110* PCR that resulted in sensitivity of 98.8%

for culture confirmed pulmonary specimens. Likewise, the sensitivity of In-house *fadE-20* PCR with culture as Gold standard is 96.1% which was significantly higher than *IS6110* PCR sensitivity of 93.5% for Extra-Pulmonary samples. In current study the analytical sensitivity of both In-house *fadE-20* PCR and *IS6110* PCR was found to be high in pulmonary TB as compared to extra-pulmonary TB specimens.Similar situations of higher sensitivity detection in pulmonary TB as compared to Extra-Pulmonary TB cases was also reported in previous studies using other target sites for diagnosis of M.tuberculosis infection (Minal Deshmukh et al., 2013; Sajjad Iqbal et al., 2011). Our sensitivity results with f*adE-20* PCR and *IS6110* PCR in Pulmonary TB and Extrapulmonary TB samples was also higher than that of earlier reports, who obtained 92% and 82.2%, 94.7% and 89.1% respectively (Kiran Chawla et al., 2015; Minal Deshmukh et al., 2013). The sensitivity value showed by In-house *fadE-20* PCR in smear negative(73,2%) samples was observed to be higher than the *IS6110* PCR(67.7%),emphasizing its efficiency in detection of Paucibacillary smear negative pulmonary and extra-pulmonary TB specimens. In 2 extrapulmonary patients, ZN smear microscopy for AFB and both amplification assays(in-house *fadE-20* PCR and *IS6110* PCR) were positive but were found to be negative for M.tuberculosis by LJ culture.This could probably be due to presence of nonviable or even low number of *Mycobacteria* in specimens of these patients as amplification tests are capable of identifying even a single copy of DNA found in a symptomatic individual (Parekh KM et al., 2006, Bechnoosh A et al., 1997).These results are unlikely to be false positives as the patients were found to be under long term surveillance for M.tuberculosis infection earlier and gave a positive response to anti-tuberculosis treatment and both amplification assays were observed to be repeatedly positive in these specimens.For Smear negative & Culture positive categorized pulmonary and EPTB samples with the use of Inhouse *fadE-20* PCR test the sensitivity of 95% was higher than achieved by other study(SS Negi et al., 2006).Amongst paucibacillary Extrapulmonary group specimens,one BAL sample and one specimen from each of CSF & Lymph nodes , where ZN smear and both amplification assays were negative but LJ culture showed positive result may be resulted due to fraction of sample tested contain less than target number of bacilli distributed non-homogenously in the suspension as explained by three previous studies for false negatives in culture positive specimens with other amplification tests (Aydan Ozkutuk et al., 2006; Paras Singh et al., 2013; Bechnoosh A et al., 1997). Three more false negative results obtained by *IS6110* PCR in culture positive samples where *fadE-20* PCR was positive could be caused by absence of *IS6110* sequence element as reported by earlier study (D. S. Chauhan et al., 2007; R. Balamurgan et al., 2006; Gomez-Marin JE et al., 2002). In our study presence of inhibitory substances was confirmed by the use of *M.tuberculosis* H37Rv as a positive control in the negative specimens. All the spiked negative specimens showed positive result indicating the absence of PCR inhibition. These results served as criteria for confirming false negatives in culture positive specimens, 3 patients for In-house fadE-20 PCR and 6 patients for *IS6110* PCR assays. In clinically diagnosed paucibacillary samples negative by both the conventional tests,PCR test targeting *fadE-20* gene detected four additional patients while *IS6110* PCR missed these paucibacillary patients. Due to low detection rate by ZN smear and LJ culture in many forms of EPTB cases, many clinicians even considered clinical diagnosis as gold standard in diagnosis of extrapulmonary TB (Kibiki GS et al., 2007). So our study further investigated the same processed specimens from these four patients by repeat testing with *fadE-20* PCR test followed by laboratory and clinical features observations. Among these four cases, two were sputum samples with manutoux test positive, pleural fluid-1 with high level of ADA in pleural effusion and Ascitic fluid-1 with predominane lymphocytes. These specimens were repeatedly positive for *fadE-20* PCR and the patients had positive result for LJ culture earlier and were on the concurrent antitubercular treatment. So these results were considered as true positives with in- house f*adE-20* PCR and false negatives in both LJ culture and *IS6110* PCR. For Smear negative and culture negative cases, the overall sensitivity of 53.7% with *fadE* PCR in clinical suspicion TB cases was superior than previous published reports (Aroma Oberoi et al., 2007; Sunil Sethi et al., 2012). So *fadE-20* PCR can be opted as one of an alternative for accurate and sensitive confirmatory demonstration of MTB infections in clinical suspicion smear negative and culture negative paucibacillary patients which can often be not detected by conventional bacteriological tests. To Monitor false positive results that usually occurs due to the exogenous contamination of reagents with other target DNA besides template DNA.our study included PCR Grade water as negative control in every batch for DNA extraction and amplification reaction protocols. All the patients from control group in whom an alternative diagnosis with non-mycobacterial diseases was confirmed were found to be consistently negative with both amplification tests therefore resulting overall specificity of 100%.As,the specificity of PCR Reaction also depends on the choice of primer used in the study, the *fadE-20* primers designed for current study showed 100% homology with *fadE-20* complete gene sequence of *M.tuberculosis* and specificity of primer pair was confirmed with the predicted size of the amplified product.The *fadE-20* gene sequence was observed to be highly specific to *M.tuberculosis* Complex DNA and none of the genes from NTMs had homology with this particular sequence. Eventhough *IS6110* target gene has been used mostly in *M.tuberculosis* PCR for diagnosis of infection due to presence of this element in multiple copies,Absence of nucleotide sequence in insertion element(*IS6110*) or presence of a single copy within several clinical isolates of M.tuberculosis strains at a rate upto 20% in some

regions(India & Columbia) have been reported previously in several studies (D. S. Chauhan et al., 2007; R. Balamurgan et al., 2006; Gómez-Marin JE et al., 2002). Moreover,the use of this insertion element as target gene for PCR may lead to Poor sensitivity (T. Moatter et al., 1998) and false negativity so several different target genes are being now evaluated by researchers for optimal target gene identification to ensure for high sensitivity of M.tuberculosis DNA detection in clinical samples (K. Sharma et al., 2012; S. Sankar et al., 2011; D.Kumar et al., 1995; N. V. Bhanu et al., 2005). By overall data interpretation of our study, we found that as revealed in previous studies the sensitivity of both amplification assays was also higher than the conventional tests in identification of mycobacteria. We anticipated that *IS6110* PCR would show increased sensitivity than In-house fadE PCR due to repeat of insertion sequence as several fragments in M.tuberculosis DNA.The Inhouse *fadE-20* PCR test showed best performance with high diagnostic yield(sensitivity) as compared with *IS6110* PCR in the detection of M.tuberculosis from clinical specimens of patients with tuberculosis, past tuberculosis and clinical suspicion of tuberculosis.

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