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# An in-house multiplex PCR test for the detection of *Mycobacterium tuberculosis*, its validation & comparison with a single target TB-PCR kit

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***Background & objectives*: The conventional techniques used in TB diagnosis like AFB (acid fast bacilli) smear microscopy lack sensitivity and the gold standard, culture test takes time. A test based on multiplex polymerase chain reaction (PCR) targeting the 38 kDa gene and *IS6110* insertion sequence, specific to *Mycobacterium tuberculosis* was developed to further increase the sensitivity of a TB-PCR kit targeting only 38 kDa gene developed earlier in the same laboratory. The multiplex test was validated using sputum samples from pulmonary TB (PTB) cases. The sensitivity and specificity were compared with AFB smear examination and Lowenstein-Jensen (LJ) culture test.**

***Methods*: Multiplex PCR amplifying 340 and 245 bp sequence of 38 kDa gene and *IS6110*, respectively was standardized and analytical sensitivity was verified. Sputum samples (n=120) obtained from PTB cases were subjected to AFB smear examination, LJ culture and a multiplex as well as single target PCR test. Additionally, 72 non-TB respiratory samples were included in the study as negative controls.**

***Results*: Analytical sensitivity of multiplex PCR was found to be 100 fg for 38 kDa gene and 1 fg for *IS6110*. Multiplex PCR, using both the targets, showed highest sensitivity of 81.7 per cent, followed by 69.2 per cent for L-J culture test and 53.3 per cent for AFB smear when clinical diagnosis was considered as a gold standard. The sensitivity of detection of *M. tuberculosis* in AFB smear positive and negative samples by multiplex PCR was 93.7 and 67.9 per cent, respectively. Sensitivity of 77.1 per cent observed for the detection of *M. tuberculosis* with single target PCR increased to 89.2 per cent with multiplex PCR in culture positive samples. Four samples showed positive PCR results only with primers for 38 kDa gene.**

***Interpretation & conclusions*: Multiplex PCR increased the sensitivity of single target PCR and will be useful in diagnosing paucibacillary smear negative samples. Further, it can also be used to detect samples with *M. tuberculosis* strains lacking *IS6110*.**

**Key words** BRIT TB-PCR kit - *IS6110* - 38 kDa gene - multiplex polymerase chain reaction - *Mycobacterium tuberculosis*

Tuberculosis (TB) remains a public health problem worldwide with a global mortality of 1.2 to 1.5 million in 2010[1]. The situation is further exacerbated with increasing incidence of multi-drug resistant (MDR) and extremely drug resistant (XDR) TB and emergence of HIV. India holds one-fifth of the global burden of TB with more than 350,000 deaths each year[2]. Though pulmonary TB (PTB) cases, account for the vast majority of the total TB burden, almost 10-15 per cent of total cases are extra-pulmonary[2].

Though early diagnosis is mandatory in control of TB, especially for pulmonary TB as it is transmissible, it has remained enigmatic. Although, the conventional acid fast bacilli (AFB) microscopy has high positive predictive value for detection of causative agent *Mycobacterium tuberculosis*, it lacks sensitivity. Lowenstein-Jensen (L-J) culture test though considered as a gold standard is labour intensive and slow. Thus, detection of *M. tuberculosis* in clinical samples containing small numbers of the organism is still a major challenge.

Polymerase chain reaction (PCR) based assays offer high sensitivity by amplification of small amount of DNA, and have been extensively evaluated for the detection of *M. tuberculosis* from clinical samples[3]. Many of the tests described in the literature are based on amplification of *IS6110*, an insertion element that is believed to be restricted to members of the *M. tuberculosis* complex[4-6]. The presence of multiple copies of this element in the majority of *M. tuberculosis* strains undoubtedly enhances the sensitivity of PCR. However, the discovery of occasional *M. tuberculosis* strains from India lacking *IS6110*[7] implies that relying only on *IS6110*-based PCR is not advisable. To overcome these problems, initially, a PCR test was developed in our laboratory[8] targeting a house keeping gene of *M. tuberculosis,* for 38 kDa protein (RV0934), involved in phosphate transport[9]. The test after thorough validation with clinical samples from PTB as well as extra-pulmonary TB (EPTB)[10,11], was assembled in a kit form and was launched in the market in August 2009 in collaboration with Department of Atomic Energy's commercial department, BRIT (Board of Radiation & Isotope Technology). Independently, a PCR test targeting *IS6110* was evaluated in our laboratory for the diagnosis of tuberculous meningitis (TBM)[12] and TB osteomyelitis (TBOM)[13], both EPTB known for paucibacillary clinical samples. The aim of the present study was to develop a multiplex PCR targeting both 38 kDa gene (RV0934) and *IS6110* to increase the sensitivity of the existing TB-PCR kit especially for the samples with lower bacterial load and having *M. tuberculosis* without *IS6110*. The test was evaluated using sputum samples from PTB patients and non-TB controls. The PCR data were compared with conventional microscopy and culture technique and also with results from existing TB-PCR kit.

## Material & Methods

*Clinical specimens*: One hundred and twenty sputum samples were randomly collected from PTB patients visiting the TB clinic in KEM Hospital, Mumbai (during 2008 to 2010) and were mostly from fresh cases but also included some from patients at different stages of chemotherapy. In addition, 72 specimens (broncho alveolar lavages= 32, sputum= 40) from non-TB cancer patients received for various microbiological investigations in Microbiology department of Tata Memorial Hospital, Mumbai, were also included in the study as negative controls. All samples were AFB and culture negative.

*Processing of samples*: All samples were subjected to decontamination by modified Petroff's Method[14] and smear AFB and LJ culture test were performed using standard techniques[14]. The decontaminated pellets were preserved at -20ºC.

*DNA extraction*: The pellet obtained after the decontamination method was subjected to DNA extraction protocol using the DNA extraction kit (TB-PCR kit marketed by BRIT). The pellet was incubated at 80°C for 20 min for inactivation of mycobacteria and processed further as per the instructions given with the kit. Briefly, samples were incubated initially with lysozyme (2 mg/ml) at 37°C for 90 min and then with proteinase K (1 mg/ml) at 60°C for 2 h in presence of lysis buffer. The DNA released was precipitated using ethanol and loaded on a silica column. The adsorbed DNA was washed free of inhibitors and further eluted out in elution buffer. The extracted DNA samples were used for multiplex PCR as well as single target PCR test of the TB-PCR kit. All tests were performed in duplicate and one of the test samples was spiked with positive control DNA (extracted from MTB $H_{37}R_v$) to look for an inhibition.

*Polymerase chain reaction*: Single target PCR was performed using primer pairs KD1(5'- CCA AGC AAG ATC CCG AGG GCT-3') and KD2 (5'-TTG ATG ATC GGG TAG CCG TCC-3')[15] from the BRIT TB-PCR kit targeting 340 bp segment of 38 kDa gene and INS1(5' CGT GAG GGC ATC GAG GTG GC 3')

**Table I.** Combinations of different concentrations of two primer pairs used in standardization of multiplex PCR

| | Combination pairs | | | |
|---|---|---|---|---|
| Primer pairs | 1 | 2 | 3 | 4 |
| KD1/2 (µM) | 0.50 | 0.25 | 0.25 | 0.50 |
| INS1/2 (µM) | 0.25 | 0.25 | 0.50 | 0.15 |

and INS2 (5' GCG TAG GCG TCG GTG ACA AA 3')[16] targeting 245 bp segment of *IS6110* (primers were synthesized by Sigma Chemical Co, USA), to confirm the analytical sensitivities of both PCRs. Ten-fold serial dilutions ranging from 100 pg -1 fg of mycobacterial DNA were used as template for this purpose.

The multiplex PCR was standardized using different combinations of primer concentrations of two primer pairs (Table I). Each combination was checked with different concentrations of *M. tuberculosis* DNA used as template such as 100, 10, 1 pg, and 100, 10 fg to check the analytical sensitivity of the multiplex PCR. A PCR reaction (25 µl reaction mixture) was set up containing, 2.5 µl of 10 x buffer, 1.5 µl of 25 mM $MgCl_2$, 200 µM (each) of the four deoxyribonucleoside triphosphate (available in TB-PCR kit) 1.0 U of Taq polymerase and forward and reverse primers at final concentrations as mentioned in Table I. The combination of the concentrations of two primer pairs exhibiting clear amplification even at the lower template DNA concentrations of 1 pg and 100 fg was selected for further evaluation of clinical samples. Amplification cycle used for all single target or multiplex PCR consist of 1 min at 94°C, 1 min at 65°C, and 72°C for 1 min and after 40 such cycles, 10 min of extension at 72°C was given. PCR products were electrophoresed on a 2 per cent agarose gel in 0.5 x TBE buffer containing ethidium bromide at 10 mg/ml concentration. Samples showing the presence of both 340 and 245 bp bands or any of the two, under ultraviolet transillumination (Biovis, India) were considered positive. In each experiment, positive (1 ng of *M. tuberculosis* DNA) and negative control (distilled water) were included.

## Results

*Analytical sensitivity of single target PCR test*: The detection limit for 38 kDa PCR was found to be 100 fg (Fig. 1) whereas *IS6110* PCR showed amplification with even 1 fg of template DNA (Fig. 2), which is theoretically equivalent to 30 and 0.3 organisms, receptively.

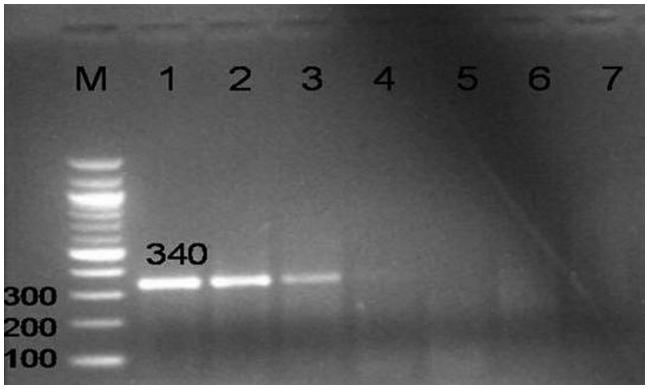


**Fig 1.** PCR amplification of 340 bp region in 38 kDa gene of *M. tuberculosis* using KD1 and KD2 primers exhibiting the analytical sensitivity of PCR. Lane M- DNA ladder, Lanes 1 to 6 - 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg of MTB DNA, respectively, Lane 7- Neg control.

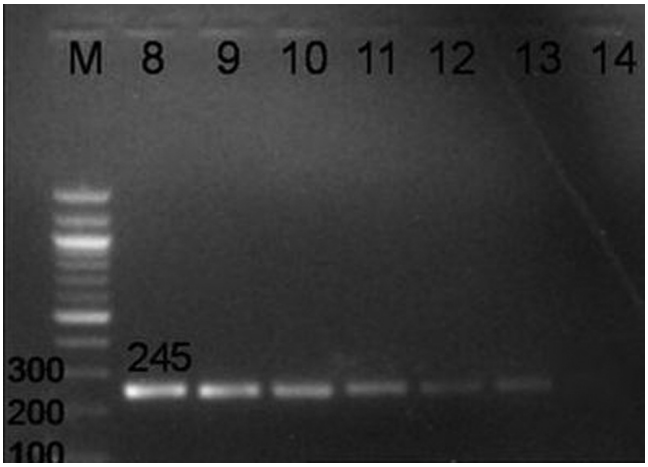


**Fig. 2.** PCR amplification of 245 bp region in insertion sequence, *IS6110*, of *M. tuberculosis* using INS 1 and INS 2 primers exhibiting the analytical sensitivity of PCR. Lane M- DNA ladder, Lane 8 to 13- 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg of MTB DNA, respectively, Lane 14- Neg control.

*Optimization of the concentrations of the two primer pairs in multiplex PCR*: It was observed that combination of 0.5 µM of KD1/2 primers (38 kDa gene) and 0.25 µM of INS1/2 primers (*IS6110*-insertion sequence) gave optimum results showing sharp amplicon bands for both targets in ethidium bromide stained agarose gel for 10 pg (Fig. 3a) and even for the lower template concentrations of 1 pg and 100 fg with prominent INS amplicon for 10 fg of template DNA (Fig. 3b). For 1 fg of template DNA, INS amplicon was faint but visible, thus the analytical sensitivity of multiplex PCR was found to be 100 and 1 fg for 38 kDa and *IS6110* gene targets, respectively.

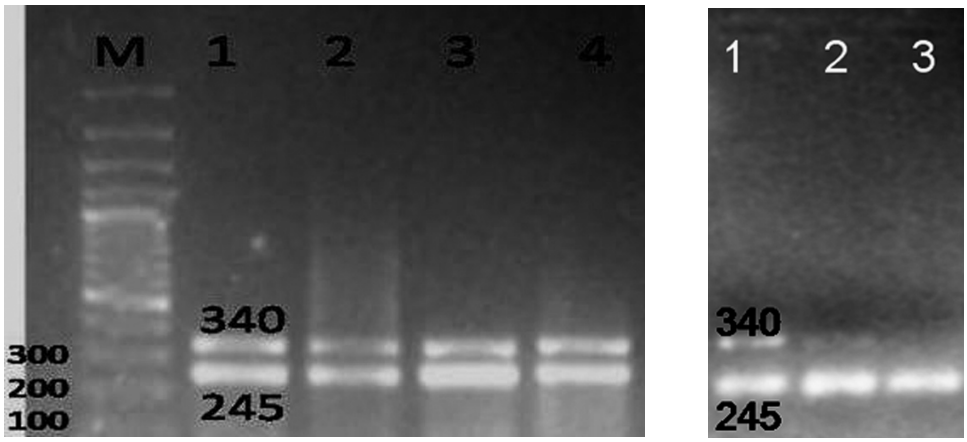


**Fig. 3.** PCR amplification of 340 bp region of 38 kDa gene and 245 region of *IS6110* of *M. tuberculosis* using different ratios of concentrations of KD1 & KD2 and INS1 & INS2 primers. Lane M- DNA ladder **(a)** Lanes 1, 2, 3 and 4 - $KD_{1/2}$ and $INS_{1/2}$ in ratios 0.5:0.25, 0.25:0.25, 0.25:0.5 and 0.5:0.15 (all in µM concentration**)** respectively with template MTB DNA concentration of 10pg. **(b)** Lanes 1, 2 and 3- template DNA concentration of 1 pg, 100 fg and 10 fg, respectively with $KD_{1/2}$ and $INS_{1/2}$ in ratios 0.5:0.25 in µM concentration per reaction.

The same combination of the primers concentrations was incorporated in PCR master mixture while evaluating the clinical samples.

*Comparison of smear, culture and multiplex PCR*: None of the samples from PTB or non-TB control group showed inhibition of PCR which was confirmed by amplification of standard *M. tuberculosis* $H_{37}R_v$ DNA in the spiked tube. Table II shows sensitivity and specificity of all individual tests used in diagnosis when clinical diagnosis was considered as the gold standard. AFB smear examination had a sensitivity of 53.3 per cent and a specificity of 100 per cent. For L-J culture test, sensitivity was 69.2 per cent and specificity was 100 per cent. In comparison, PCR test was found to have a much higher sensitivity of 81.7 per cent and a specificity of 97.3 per cent. The sensitivity of detection of *M. tuberculosis* in AFB smear positive samples by multiplex PCR was 93.7 per cent, whereas in smear negative specimens were 67.9 per cent.

*Comparison of single target and multiplex PCR*: Table III shows sensitivity of detection of *MTB* for both single target and multiplex PCR test, in negative controls and in three different groups from TB patients (Group A- smear and culture positive, Group B- smear negative and culture positive, and Group C- smear and culture negative). The sensitivity of the detection in Group A increased from 84.3 to 93.7 per cent due to the multiplexing. The increase in sensitivity was more evident in Group B (52.3 to 73.6%) and Group C (21.6 to 64.8%). When the culture test was considered as a gold standard, the single target and multiplex PCR exhibited sensitivities of 77.1 and 89.2 per cent, respectively. When clinical diagnosis was used as a gold standard, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPP) obtained with multiplex PCR was 81.7, 95.8, 97 and 75.8 per cent, respectively. Further, with culture test as a gold standard, the sensitivity and NPV increased to 89.2 and 88.5 per cent, respectively (Table IV).

There were four samples in Group A which showed absence of INS and presence of 38 kDa amplicon in multiplex PCR indicating presence of *M. tuberculosis* strains lacking *IS6110*. Besides, some samples from Groups B and C which were negative with single target

**Table II.** Sensitivity and specificity observed with smear examination, L-J culture and multiplex PCR test when clinical diagnosis was taken as gold standard

| Test | Sputum samples from PTB patients N=120 | | Samples from non-TB negative controls N=72 (BAL=32, sputum=40) | |
|---|---|---|---|---|
| | Positive results | Sensitivity (%) | Positive results | Specificity (%) |
| AFB smear microscopy | 64 | 53.3 | 72 | 100 |
| L-J culture | 83 | 69.2 | 72 | 100 |
| Multiplex PCR | 98 | 81.7 | 69 | 97.3 |

**Table III.** Validation of multiplex PCR for detection of *M. tuberculosis* and its comparison with single target TB-PCR kit

| | PCR positives (%) | | |
|---|---|---|---|
| Total no. sputum samples from TB patients (N=120) | Test → | BRIT- RMC TB-PCR kit | Multiplex PCR |
| | Primers → | KD1& KD2 | KD1&KD2 and INS1&INS2 |
| | Target → | 38 kDa gene | 38 kDa gene & *IS6110* |
| Culture positive Group A | N= 83 | 64 (77.1) | 74 (89.2) |
| Smear +ve & Culture +ve Group B | N = 64 | 54 (84.3) | 60 (93.7) |
| Smear -ve & Cultue +ve | N = 19 | 10 (52.3) | 14 (73.6) |
| Culture negative Group C Smear -ve & Culture -ve | N=37 | 8 (21.6) | 24 (64.8) |
| Controls (Cancer patients) | N=72 | 3 (2.7) | 3 (2.7) |

Values in parentheses are percentages

**Table IV.** Statistical analysis of the results obtained with multiplex PCR used for detection of *M. tuberculosis*

| Patients/ Controls | PCR Pos | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|---|
| Clinically diagnosed TB patients (N=120) | 98 | 81.7 | 95.8 | 97 | 75.8 |
| Controls (N=72) | 3 | | | | |
| Culture positive TB patients (N=83) | 74 | 89.2 | 95.8 | 97 | 88.5 |
| Controls (N=72) | 3 | | | | |

PPV, positive predictive value; NPV, negative predictive value

(TB-PCR kit) and positive with multiplex PCR showed presence of only INS amplicon (Fig. 4).

## Discussion

PCR is theoretically capable of amplifying even a single copy of DNA. The present study was aimed to develop a multiplex PCR, by combining primers targeting *IS6110* with primers used in the existing

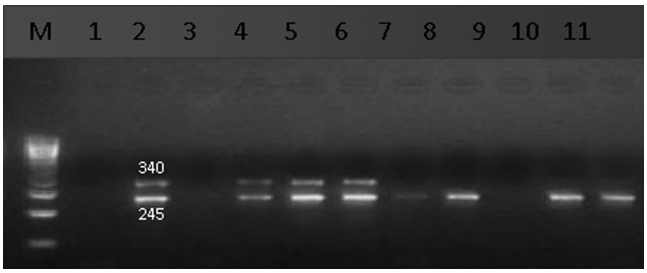


**Fig. 4.** PCR amplification of 340 bp region of 38 kDa gene and 245 region of *IS6110* of *M. tuberculosis* in multiplex PCR, representation of some clinical samples. Lane M- DNA ladder, Lane 1- negative control, Lane 2- positive control (MTB DNA), Lanes 3, 9- negative samples, Lanes 4,5,6- positive samples showing both the amplicons, Lanes 7,8,10,11- positive samples showing only *IS6110* amplicon of 245 bp.

TB-PCR kit, for further increasing the sensitivity. Additionally, the study was undertaken to explore the utility of multiplex PCR test for diagnosis of pulmonary TB and to compare the results with those from smear microscopy and L-J culture.

PCR targeting *IS6110* was proved to be more sensitive (1fg) than the one targeting 38 kDa gene due to the presence of multiple copies of this insertion element in *M. tuberculosis*. However, the absence of this element in some of the Indian strains may lead to false negativity and our study showed that this can be avoided by using two targets.

When the performance of various tests was compared for diagnosis of TB, multiplex PCR showed the highest sensitivity as compared to AFB smear microscopy and culture test (clinical diagnosis as Gold standard) as also observed in other studies evaluating in-house PCR tests[17-19]. None of the samples showed inhibition and spike test was positive in all the samples indicating efficient removal of inhibitors by the indigenously developed DNA extraction kit included in the BRIT TB-PCR kit. TB patients were assigned to three different groups based on the combinations of smear and culture results. Smear and culture positive group showed a sensitivity of 84.3 and 93.7 per cent for single target and multiplex PCR, respectively which were comparable to the sensitivity of 85 to 100 per cent achieved by other investigators[16,20], and was superior to a report showing lower sensitivity of 55 per cent[21]. Four samples from group A, which exhibited negative multiplex PCR test, were probably due to the lower bacterial count. A multi-centric blinded study by Noordhoek *et al*[22] showed that the sensitivity of positive detection by PCR for the samples with $10^3$ mycobacteria

was not reproducible and varied from 2 to 90 per cent while with $10^6$ cells, it was 90-100 per cent.

According to a meta-analysis[23], the diagnostic sensitivity and specificity of different in-house PCR tests for smear positive respiratory samples varied among different laboratories and was in the range of 80-95 per cent. The variation seen was attributed to improper reference test and inadequate sample size. Multiplex PCR in present study exhibited high specificity which was probably due to good laboratory practices while handling the samples and inclusion of well evaluated group of negative controls.

Though patients with positive smears are responsible for the maximum transmission of TB, Behr *et al*[24] demonstrated that smear-negative TB cases contribute much more to ongoing transmission than was previously believed. Thus early diagnosis of smear negative TB patients may prove to be a more pragmatic approach to control TB. For group B samples, the sensitivity of 73.6 per cent with multiplex PCR was comparable[18] or higher than the majority of the studies reporting it at around 60 per cent or even less[25].

Group C comprised 37 smear- and culture-negative samples which probably could be paucibacillary in nature and/or patients on ATT (anti-tuberculosis treatment) shedding noncultivable bacteria. Twenty four of these 37, were positive by multiplex PCR. Fifteen of these 24 were positive by in-house radio-respirometry method[26] indicating presence of live bacilli which were not detected by L-J culture test.

In summary, multiplex TB-PCR, amplifying two targets specific for *M. tuberculosis* was developed and its utility for TB diagnosis was evaluated in the present study. Because of higher specificity and sensitivity, our multiplex PCR can complement the tests in TB diagnosis. Because of high sensitivity observed in paucibacillary smear negative samples, this test may be used for diagnosis of EPTB as well as PTB which are difficult to diagnose with available standard methods.

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