

## Evaluation of in-house polymerase chain reaction assay sensitivity, can it be utilized in limited-resources settings?

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### Abstract

**Background:** Polymerase chain reaction (PCR) assay has widely used for the detection of tuberculosis (TB). This study tried to compare in-house PCR with some well-known commercial PCR kits for detection of TB agent.

**Methods:** Clinical samples obtained from 620 TB suspected patients were analyzed for the diagnosis of Mycobacterium tuberculosis complex (MTC) by in-house PCR. All samples were obtained through pulmonary specimens consisted of 384 sputum, 148 bronchial aspirates and 88 pleural effusions.

**Results:** Considering culture as a golden criterion, in which its diagnostic sensitivity and specificity of PCR assay were 87.7% and 85.6%, respectively. The findings of this study also indicate 22.1% (137/620) of the specimens were detected as MTC by PCR. Both PCR and culture confirmed presence of MTC in 57 of the samples. In comparison to culture, the diagnostic sensitivity of PCR for sputum was 87.5% (42/48), bronchial aspirates 100% (12/12), and 60% (3/5) for pleural effusions. The sensitivity of in-house PCR method is comparable with the sensitivity of Amplicor and Cobas TaqMan for MTC.

**Conclusion:** The study illustrates the in-house PCR assay for detection of MTC has high sensitivity and specificity versus approved commercial kits. This could be reliable test in the diagnosis of MTC in resource-limited countries.

**Keywords:** Mycobacterium tuberculosis, Polymerase chain reaction, Culture.

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### Introduction

The world today is facing with tuberculosis (TB) as one of the biggest health issues specifically since there are 8.8 million TB incidents worldwide and an estimated 1.7 million deaths in 2010 (1). According to WHO's report in 2011, TB incidence among Iranian populations is 16000 TB cases, approximately 21 per 100000 populations (2). Thus, early diagnosis of TB is

required to begin desirable anti-tuberculosis therapies. Conventional methods with rapid and accurate diagnosis of TB can take a very long time from a few hours to several weeks, as a result of these long delays, TB infection can continue to spread (3). Among TB diagnostic techniques, acid-fast bacillus (AFB) smear can show result within 24 h after admission of TB suspects. However, smear is fairly insensitive for de-

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tection of *Mycobacterium tuberculosis* complex (MTC) since it requires  $10^3$  to  $10^4$  organisms per ml of sputum and also needs expert specialists to report accurate diagnosis of TB in suspects. Furthermore, this technique is unable to distinguish *Mycobacterium tuberculosis* (Mtb) from *non-tuberculosis mycobacterium* (NTM) strains and cannot differentiate drug-susceptible from resistant species (4, 5).

Bacterial culture accepted as a golden standard is a diagnostic technique for detection of MTC and is much more sensitive and specific than direct smear. However, slow mycobacterial growth can cause a time delay for conclusive diagnosis of tuberculosis in TB suspects and is much more complex test than microscopic examinations, and also being an expensive diagnostic technique that requires appropriate biosafety conditions (6, 7). Detection of *M. tuberculosis* nucleic acid by amplification tests potentially helps us for early diagnosis of TB patients. A number of PCR-based methods, which are reliant on amplification of diverse target genes with pair probes, demonstrated high sensitivity and specificity for the early detection of *M. tuberculosis*, differentiate *tuberculosis* from NTM strains, and reduce the time for diagnosis of TB (6). However, these methods also need expert technicians and high technology process. Today, real-time PCR tools using commercial diagnostic kits and high-purity DNA extraction kits have great advantage over PCR methods. Studies showed that *IS6110/IS986* insertion elements from *M. tuberculosis*-specific gene sequences are two main target genes in many PCR diagnostic protocols (8). In this case, MTC group including *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. bovis BCG* contain copies of *IS6110* gene sequence (9). The current study was aimed to assess sensitivity of the in-house PCR technique for detection of pulmonary TB samples in resources-limited countries like Iran.

## Methods

The current study was conducted at

Masih Daneshvari Hospital, a referral TB center in Tehran, Iran. From 2011 to 2012. 620 pulmonary specimens including 384 sputum, 148 bronchial aspirates and 88 pleural effusions were examined through in-house PCR and TB culture.

### DNA preparation

Genomic DNA was extracted from the clinical samples by either, salting out method or classic phenol-chloroform procedure.

The salting out protocol developed by Miller and collaborator in 1988 (10). For salting out, 500  $\mu$ l of a decontaminated specimen was gently transferred to a 1.5 ml eppendorf tube and boiled at 84°C for 20 minutes. Sucrose with the final concentration of 50% was added and the tube was spin down at 14000 rpm for 15 min at 4°C. The supernatant was discarded and the sediment was suspended with 100  $\mu$ l of phosphate buffer saline (PBS) and centrifuged at 8000 rpm for 1 min at 18°C. The obtained pellet was mixed with 50  $\mu$ l of deionised water (dH<sub>2</sub>O).

Phenol-chloroform procedure was done according to Barker et al in 1998 (11). The sample was first poured to a 1.5 ml eppendorf tube and centrifuged at 12000 rpm for 10 min. The sediment was mixed with 150  $\mu$ l of lysing buffer and incubated at 80°C for 20 min. The sample was then treated with the aliquot of proteinase K (Fermentas Company, Germany) and incubated at 56°C for 30 min. To optimize pH, 50  $\mu$ l of neutralizing buffer was added. Next, an aliquot of equilibrium phenol was transferred to the tube and centrifuged at 5000 rpm for 10 min. The upper aqueous layer containing the DNA of interest was then mixed with 200  $\mu$ l chloroform followed by centrifugation at 12000 rpm for 10 min. The upper aqueous layer was suspended with 120  $\mu$ l of isopropanol and 20  $\mu$ l of sodium acetate and finally incubated overnight at -20°C. The mixture was first spun down at 12000 rpm for 10 min at 4°C. The supernatant was discarded and 200  $\mu$ l of 70% alcohol poured to the tube and centrifuged at 12000

rpm for 4 min at 4°C. The supernatant was removed and 50 µl of distilled water added to the tube.

### PCR

The protocol: (MTC) was rapidly detected by designed pair primers, which targeted a segment of 190 bp from IS6110 gene sequence: TB-sense 5' ATCCTGCGAGCGTAGGCGTCGG 3' and TB anti-sense 5' CAGGACCACGATCGCTGATCCGG 3'. In each experiment, genomic DNA was qualified by amplification of a 330 bp segment using designed pair primers: β-actin-sense 5'-TCCTGTGGCATCCACCAAAC T-3' and β-actin anti-sense 5'-GAAGCATT TGCGGTGGACCAT-3'. The amplification of all PCR reactions was performed in 200 µl micro-tubes and cycled in the program temp control system PC-320 thermo cyclers (ASTECC). The micro-tubes contained 25 µl PCR reaction consisted of 5 µl of isolated DNA, 1 X Buffer (Containing 20mM NH<sub>4</sub>SO<sub>4</sub>, 7 Mm Tris-HCl pH=8, and 0.1% Tween 20), 2mM of MgCl<sub>2</sub>, 0.2mM of each dNTP, 2 Unit/µl of Taq DNA polymerase (Fermentas Company, Germany), and 10 pmol/µl of each forward and reverse primers of MTC and β-actin. Thermo cycler condition was as follows: denaturation for 2 min at 96°C and 35 cycles with 30 second at 96°C, 1 min at annealing temperature, and 72°C for 30 seconds followed by the extension step at 72°C for 5 min. Only 10

µl of the amplified products were loaded on a 2% agarose gel. The DNA band corresponding to 190bp was visualized by a Gel Doc Viber Trans illuminator.

The PCR inhibitor reported, in such that genomic DNA was undetectable by β-actin primers.

### Statistical analysis

The sensitivity and specificity, positive and negative predictive values of in-house PCR versus bacterial culture was analyzed by Microsoft office excel 2010 with 95% confidence interval (CI).

### Results

Of 620 suspected TB samples, 361 (58%) and 259 (42%) were male and female, respectively. The mean age (SD) of TB patients was 22 which ranged from 1 to 96 years old.

The PCR assay detected MTC DNA in 137 (22.1%) of the clinical samples. The figure 1 shows MTC bands corresponded to 190 bp.

To express the sensitivity, specificity and predictive values, the results of PCR were compared with those of TB culture as a golden standard method. The PCR sensitivity was 87.7% (95% CI: 79-95) for both PCR and culture techniques which were positive for 57 clinical samples. The specificity of PCR against culture as a gold standard was 85.6 % (95% CI: 82-88) (Ta-

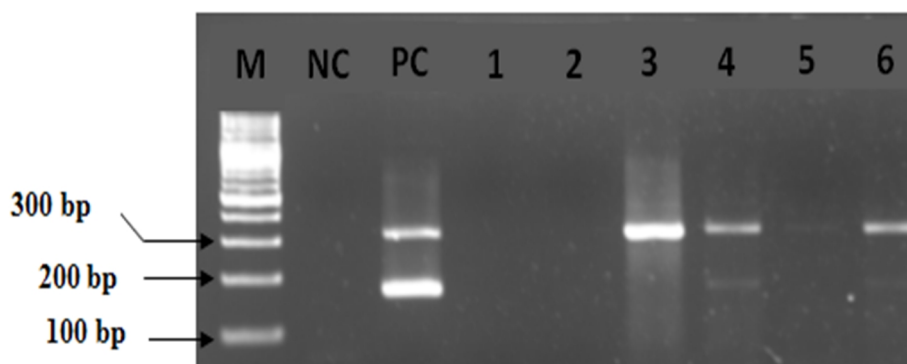


Fig.1. Analysis of 2% agarose gel. From left to right, lanes contain marker, negative control, positive control and following six clinical samples suspected of having pulmonary tuberculosis. The first sharp band in positive control shows B-actin corresponding to 330 bp and it was found positive in clinical specimen 3, 4, 5 and 6. The second down clear band in positive control lane presents the genomes of MTC identified at 190 bp, confirming that patients 4 and 6 are infected with TB. Both lanes number 1 and 2 have PCR inhibitors.

Table 1. Sensitivity, specificity and predictive value for in-house PCR

Variables	No. (%) of PCR-positive	No. (%) of culture-positive	Sensitivity (95%CI)	Specificity (95%CI)	Positive Predictive value (95%CI)	Negative Predictive Value (95%CI)
Sputum	114 (29.7%)	48 (12.5%)	87.5% (78-96)	78.6% (74-82)	36.8% (27.9-45.7)	97.7% (96-99.5)
Bronchial aspirates	18 (12.2%)	12 (8.1%)	100% (100-100)	95.6% (92-99)	66.6% (44.8-88.4)	100% (100-100)
Pleural effusions	5 (5.7%)	5 (5.6%)	60% (17-100)	97.6% (94-100)	60% (17-100)	97.5% (94.2-100)
Total	137	65	87.7% (79-95)	85.6% (82-88)	41.6% (33-49)	98% (97-99)

ble 1). The sensitivity and specificity of TB PCR were computed for clinical samples as: sputum (87.5%, 78.6%), bronchial aspirates (100%, 95.6%), and plural effusion (60%, 97.6%) (Table 1).

### Discussion

MTC can be accurately detected through the PCR technique (12, 13). These molecular techniques have been most often evaluated due to their variable rates of sensitivity and specificity (14). One approach might compare sensitivity and specificity of the in-house PCR assay with some commercial PCR systems like Amplicor and Cobas TaqMan for the detection of *M. tuberculosis*.

This study was performed to show the specificity and sensitivity of the PCR technique for detection of MTC. The results of PCR were compared with those of TB culture technique as the golden standard. Obviously, the sensitivity and specificity will increase when an optimal condition for DNA isolation and PCR processing considered. IS6110 target gene is an insertion sequence repeated in the genome of *M. tuberculosis*. The sequence is not found in NTM such as *M. avium* complex, *M. gastric* etc. Various types of natural and extraneous impurities that may result during DNA lysate preparation make inhibitory impacts on PCR reaction (14, 18).

The findings of this study also indicate that the sensitivity of PCR for detecting the MTC was 87.7% and its specificity 85.6%. This rate of PCR sensitivity and specificity of PCR is comparable with the common ranges of 42% to 90.9% as clarified in most studies (19). Querol et al examined PCR

sensitivity for 314 respiratory specimens using IS6110 pair primers and showed PCR sensitivity about 97% (20). Another study by Thoe et al in Singapore used IS6110 primer and reported PCR sensitivity and specificity of 86.5% and 83.6%, respectively (21).

Our study indicate that 8 (12.3%) of total samples with PCR negative and culture positive results were expressed as PCR false-negative results due to, either, presence of NTM strains in the samples, or presence of PCR inhibitors, especially in sputum. In the current study, the PCR inhibitor was reported when genomic DNA from the samples was not detected using B-actin pair primers. A few review studies on diagnostic sensitivity of PCR have reported 5%-13% of PCR inhibitors in sputum samples (22, 23). Of 384 sputum samples, the inhibitor was detected in 12 (3.1%) of the samples. This rate of PCR false-negative can be also a reason for low copy numbers of MTC DNA in the samples.

The occurrence of PCR false-positive in our study was a matter of concern. In this case, 80 (14.4%) Of PCR-positive were negatively determined by culture. A few studies on diagnostic evaluation of PCR have reported up to 6% of PCR false-positive results (24, 25). This might involve the facts that, either, cross-over contamination in PCR processing, or some non-cultivable TB strains probably existed, or more importantly growth failure may be observed during TB culture (14). This issue can be a cause of low positive predictive value of PCR against MTC culture (totally 41/6 % (95% CI: 33-49)) (Table 1).

Diagnostic sensitivity and specificity of

in-house PCR would be reliable indicator and can be compared with those found in other experiments. Gomez-Pastrana et al in 2000 used 235 gastric aspirates and 16 bronchoalveolar lavage specimens to detect TB in children (26). They reported 60% and 96.8% for the in-house PCR sensitivity and specificity, respectively. In 2011, Hyun Kim et al evaluated Cobas TaqMan MTC PCR and Cobas Amplicor MTC PCR for detection of *Mycobacterium tuberculosis*. They collected 406 samples from 247 patients. Of these, 96 respiratory specimens were processed. The respiratory specimens were comprised of sputum, pleural and bronchoalveolar lavage (BAL) fluid. For respiratory specimens, they have also reported sensitivity and specificity of Cobas TaqMan for about 79.1%, 95.8%, and for Amplicor 58.3%, 98.6%, respectively (27).

Furthermore, Schirm et al assessed and compared Amplicor, in-house PCR, and bacterial culture for detection of *Mycobacterium tuberculosis* in five hundred four clinical specimens (337 sputum and 167 bronchial samples) from 340 patients. In-house PCR with the rate of 92.6% was relatively sensitive diagnostic technique compared to Amplicor *M. tuberculosis* test, culture, and microscopy 70.4%, 88.9%, 52.4%, respectively. Obtained specificities for all of those four tests were more than 98% (28). It can be concluded that accurate performance of the in-house PCR using designed pair primers for diagnosis of *M. tuberculosis* is a useful technique for diagnostic purposes. In the present study, sensitivity and specificity of in-house PCR were expressed in a valuable range corresponding to 87.7% and 85.6%, respectively with appropriate negative predictive value (98% (95% CI: 97-99)). Thus, it can be highlighted that in-house PCR assay provides valuable rate for sensitivity and specificity for diagnosis of TB in developing countries such as Iran where preparation of TB diagnostic kits and high technology PCR tools are economically limited.

## Conclusion

The study recommends that developing countries should apply the in-house PCR techniques for detection of TB. Proper performance of this technique needs to be considered in clinical laboratories. At the end, it seems that in-house PCR can be used as a rapid assay for detection of *M. tuberculosis* and should be required in conjunction with other routine TB detection techniques such as direct smear and TB culture in the referral health centers.

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