www.jmscr.igmpublication.org

Impact Factor 5.244
Index Copernicus Value: 5.88
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossref DOI: http://dx.doi.org/10.18535/jmscr/v4i4.01

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

Evaluation of In-House Multiplex Polymerase Chain Reaction (MPCR) For Diagnosis of Pulmonary Tuberculosis

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ABSTRACT

Background: *Diagnosis of tuberculosis is still an ongoing problem however nucleic acid amplification test has emerged as a promising tool for its diagnosis. Single gene target may result in false negative due to the absence or the presence of only few copies of target DNA in some M.tuberculosis isolates. The objective of this study was to evaluate a multiplex PCR (MPCR) for the detection of M. tuberculosis in sputum samples.*

Materials & Methods: *150 clinically suspected cases of pulmonary tuberculosis were processed for detection of mycobacterial infections by ZN staining smear examination, LJ culture and Multiplex PCR tests which comprised of genus specific primer targeting insertion sequence 6110 (IS6110), MPB64 (23kDa) and Protein b (38kDa).*

Results: *Multiplex PCR showed highest sensitivity of 100 %, followed by 65.2 % for AFB smear when LJ culture was considered as gold standard. Conclusion: Multiplex PCR increased the sensitivity and it can be used to detect samples with M.tuberculosis strains lacking IS6110.*

Keywords: *Mycobacterium tuberculosis complex (MTBC), IS6110, MPB64, 38kDa (Pab b), Multiplex polymerase chain reaction (MPCR)*

INTRODUCTION

One hundred and thirty three years after Robert koch recognized Mycobacterium tuberculosis (MTB) as the causative agent of tuberculosis (TB), it still poses an enormous global public health burden today[1] World health organization (WHO) reported that there were an estimated 9.6 million new TB cases and 1.5 million TB deathsin 2014[2]. In India, incidence (all cases) was estimated to be 167 person per 100,000 population and the prevalence (all cases) was 195 person per 100,000 population[2]. However on the other hand, the detection rate for all TB cases was 74%[2]. The main concern about TB control in India and elsewhere is the rapid and

sensitive diagnosis of the infection. In developing countries, the diagnosis of mycobacterial infection is not made timely leading to mismanagement and empirical therapeutic trials which gave rise to development of drug resistance[3].

Although the conventional technique of direct smear examination is cheap and easy to perform, its low sensitivity is a major drawback[1]. On the other hand, the molecular-based diagnosis by polymerase chain reaction techniques is faster but accuracy is determined by the choice of the target DNA. The PCR methods has been used as an alternative that presents high sensitivity and specificity for the rapid diagnosis of infectious diseases. However, the use of PCR in thedetection of MTBC has produced varying results, especially in relation to the sensitivity of the test[4,5,6]. Various targets sequences such as insertion sequence IS6110, 65kDa (GroEL), 38kDa (PhoS, CIE Ag78 or Pab) and MPB64 (23kDa), have therefore been used. Among these IS6110 is more commonly used because of its repetitive sequence nature. This property helps increase the sensitivity of PCR over that obtained in the amplification of single DNA sequence[7]. However, the absence or the presence of only a few copies of this sequence has been reported in some strains particularly those from Southeast Asia[8]. A large number of clinical isolates of M.Tuberculosis from South India[9] had either a single copy (40%) or no copy (4%) of IS6110. An alternative approach may be Multiplex Polymerase chain reaction (MPCR), in which several target genes for mycobacterium tuberculosis complex (MTBC) are amplified simultaneously to increase the sensitivity and specificity of the test (IS6110,MPB64 and Protein b). MPCR also has several strengths such as cost effectiveness and reduced possibility of PCR contamination[10].

Effective tuberculosis control requires that patients be identified and placed on proper anti tuberculosis therapy. The rapid, sensitive and specific test for detection of mycobacterium has been a long standing need. Thus, we standardized and evaluate the multiplex polymerase chain (MPCR) utilizing multiple targets (Insertion Sequence 6110, MPB64 and 38kDa Pab b.) for rapid detection of mycobacterium tuberculosis complex in routine diagnosis. The sensitivity and specificity of MPCR were compared with Ziehl-Neelsen (Z.N) staining microscopy and Lowenstein-Jensen culture.

MATERIAL AND METHODS

The study was performed in the Department of Microbiology, Santosh Medical College in collaboration with Subharti Medical College and was approved by institutional research ethics committee. A written consent was obtained from all patients who consented to provide sputum samples after understanding the objects and other details of the study.

Specimen Collection: Two consecutive sputum specimens (up to 5 ml each), one spot and the other early morning, were collected from 150 clinically suspected tuberculosis individuals.

Acid fast staining: A direct smear was made from each sputum specimen and stained by the Z.N staining method[11]. Both the sample (spot and early morning) collected from each patients were pooled and were then processed for concentration.

Processing of sputum sample: An equal volume of N-acetyle-L-cystein -NaOH (NALC-NaOH)[12] solution was added to the sputum sample and the content was shaken for 15-30 seconds and allowed to stand for 15 minutes. The digested/ decontaminated sputum sample was then diluted with phosphate buffer (pH 6.8) and centrifuged at 3600×g for 15 mins at room temperature. Supernatant was discarded in splash proof container. The sediment part was used for LJ culture as per standard protocol[11] and for DNA extraction.

Culture and recovery of Mycobacteria from sputum samples: LJ media, widely used for tuberculosis diagnosis, was used as a baseline test to access the diagnostic accuracy of in –house multiplex PCR. From the sediment, inoculated two slopes of LJ medium[11]. The inoculated LJ culture

bottle were incubated for up to 8 weeks with daily examination for first week and then weekly examination for evidence of growth. Colonies from culture positive L.J bottle were confirmed for presence of AFB by Z.N staining microscopy and biochemical tests.

Biochemical tests*:* The mycobacterial isolates obtained were subjected to niacin and heat-resistant catalase test to differentiate mycobacterium tuberculosis complex with nontuberculous mycobacteria[11].

DNA extraction: The sediment obtained after the decontamination method was subjected to DNA extraction. DNA was extracted using commercially available QIAmp DNA mini kit (QIAGEN) with one initial additional step. The sediment obtained after the processing of sputum sample were kept at 80^0C for 15 minutes for inactivation of possible mycobacteria. And then further processed as per the manufacturer guidelines.

Multiplex PCR: The primer used in the MPCR were targeted to detect M.tuberculosis complex based on amplification of the DNA sequence of 123 bp from IS6110[13], 240 bp from MPB64[14] and 419 bp from 38kDa protein[15]. Table.1 shows the oligonucleotide sequences used in the amplification. A 50µl reaction was setup containing 1.25unit Taq DNA Polymerase, 1x Buffer 0.2mM dNTPs, 1.5mM $MgCl_2$, 10 pmol of each forward and reverse primer, extracted DNA sample and deionized water. Positive (H37Rv strain DNA) and negative control (Nuclease free water) run in each experiment. Applied Biosystem 2700 model of thermal cycler was used. Temperature cycling conditions include 94^oC for 5 min, followed by 30 cycles at 94^oC for 30 sec, annealing of primers at 65^oC for 45sec and primer extension at 72^oC for 45sec and final extension at 72^oC for 10 min. The amplified product was stored at 4^0C until separated on 1.5% agarose gels. The agarose gel was subsequently stained with ethidium bromide and visualized on a UV-light trans illuminator (Bangalore Genie, Bangalore, India) to look for bands 123bp for IS6110, 240bp for MPB64 and 419bp for Protein b (Pab b) using a molecular marker of 100bp ladder. The sample showing the presence of all 123 bp, 240 bp and 419 bp band or any two or any one were considered positive.

Table 1: Oligonucleotide sequences used in multiplex polymerase Chain reaction assay

Oligo. Name (Gene Target)	Primers Sequence 5'to 3'	Expected Amplicon Size (bp)
IS6110	Forward CCT GCG AGC GTA GGC GTC GG	123bp
	Reverse CTC GTC CAG CGC CGC TTC GG	
MPB64	Forward TCC GCT GCC AGT CGT CTT CC	240bp
	Reverse GTC CTC GCG AGT CTA GGC CA	
38kDa (Protein b)	Forward ACC ACC GAG CGG TTC GCC TGA ReverseGAT CTG CGG GTC GTC CCA GGT	419bp

STATISTICAL METHODS

The sensitivity, specificity, positive predictive value and the negative predictive value were calculated using the standard formulae. LJ culture was considered as the base line test.

RESULT

Of the total 150 pulmonary tuberculosis suspected patients, 112 were males with an average age of 37.1 yrs. and 38 were female patients with an average age of 36.9 yrs. 86% had complained of cough more than two weeks. Clinical data of patients in Table 2 also shows that, 80% had body temperature of $\geq 37.5\ ^0$C, chest pain 59% and loss of appetite 70%. Overall, 19.3% spot specimens were positive by smear compared to 20.6% being smear positive in the early morning specimens. The specimens positive for smear collected at spot were also positive for specimens collected early in the morning. Sensitivity of the Z.N staining method was 67% whereas specificity was 100%. The positive predictive value was 100% and the negative predictive value was 87.3%Table 3. Sputum culture

results showed 30.6% were found positive for growth of M.tuberculosis. All smear positive cases were also culture positive however, of the smear negative cases 12.6% were culture positive. Positivity rate of in-house MPCR was found to be the highest of all the diagnostic test used in this study. From150 pulmonary suspects, 40.6% were detected with MTB complex. The MPCR test was found to have a much higher sensitivity of 100% and specificity 85.5%. (LJ culture as Gold standard) whereas the positive predictive value was 75% and negative predictive value was 100%.Table 3

Table 2 Demographic and clinical characteristics of patients.

Demographic/Clinical		Total
Sex	Male	112 (74.6%)
	Female	38 (25.33%)
Mean Age	Male	37.43 Years
	Female	36.9 Years
Cough	>2 Weeks	129 (86.01%)
	<2 Weeks	21 (14.0%)
Fever	>2 Weeks	120 (80.0%)
	<2weeks	30 (20.0%
Chest Pain		89 (59.3%
Breathlessness		33 (22.01%)
Hemoptysis		28 (18.6%)
History Of Contact		67 (44.66%)
Loss Of Appetite		105 (70.0%)

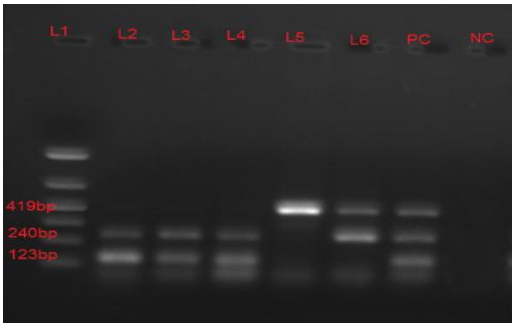

Figure 1: L1- 100bp Ladder, L2 to L4-Positive clinical sample with 123bp and 240bp band, L5-Positive clinical sample with 419bp band only, L6-Positive clinical sample with 240bp and 419 bp band. Lane PC –Positive control (H37Rv strain DNA) with all three 123bp IS6110, 240bp MPB64 and 419bp Pab b bands. And Lane NC-Negative control.

Table 3 Diagnostic Comparison between Morning Sputum Smear, MPCR &Gold Standard Culture Test

Approach		LJ Culture (Gold Standard)		Sensitivity (%)	Specificity (%)	PPV	NPV
		Positive	Negative				
Z.N. Stain	Positive	31	00	67.3 9%	100 %	100 %	87.3 %
	Negative	15	104				
MPCR	Positive	46	15	100 %	85.5 7%	75.4 %	100 %
	Negative	00	89				

*PPV: Positive Predictive Value; NPV: Negative Predictive Value

DISCUSSION

Our result showed that, smear positivity rate was 20.6%, which is almost similar to a studies conducted in India by Myneedu et al[16], also in high prevalent countries like Nigeria and Ethiopia 21%, Yemen 24% and Nepal 25%[17] where sputum were examined for diagnosis of tuberculosis. The available limited data on the yield of smear positivity by examining spot verses morning sputum suggests that yield of single morning specimen is approximately higher by 12% over the yield of single spot specimen[18]. Higher yield in morning samples were also documented by Mase et al in his research publication[17].Sensitivity and specificity of smear examination method were 67.3% and 100% respectively (LJ culture as gold standard) which is comparable to a study conducted by Myneeduet al[16] and Hirao et al[19]. LJ medium culture method, which is considered as gold standard of M.tuberculosis detection, recovered 30.6% positive sputum specimens. Our data confirm that culture positive result was 10% more than the result of smear microscopy. This is comparable to Myneeduet al[16] whose findings confirmed that culture positive result was 13% more than the result of smear microscopy. Also, our data indicate that in the diagnosis of tuberculosis, culture had greater

sensitivity than Z.N staining microscopy method, in case of a single specimen, the diagnostic value of culture is quite significant. But these traditional bacteriological methods are either slow or their sensitivity is quite low.

PCR method has been used as an alternative that presents high sensitivity and specificity for the rapid diagnosis of infectious diseases. However, successful use of DNA sequence for the detection of mycobacteria crucially depends on the right and logical choice of the target sequences, which ideally should be present in all mycobacterial complex. The use of PCR in the detection of mycobacterium tuberculosis has produced varying results, especially in relation to the sensitivity of the test[4,5,6]. Most previous studies have generally targeted IS6110 as it is present in multiple copies in the MTB genome[20-24]. Kent and colleagues claimed to demonstrate the existence of homology between an IS*6110*-derived probe and DNA isolated from a variety of nontuberculous mycobacteria[25]. Also, absence or presence of only a few copies of insertion sequence IS6110 has been reported.[8,9] To overcome this problem we standardized and evaluated MPCR assay using three different gene i.e. IS6110, MPB64 and Pab b for detection of MTBC in sputum sample. Our study is unique in the fact that all genes were amplified together and were able to diagnose the cases which were missed by IS6110 alone or by MPB64 or Pab b in detection. MPCR method reduces errors, as well as cost and increases the sensitivity of the test[10].

Our result showed that the sensitivity and specificity of MPCR test for diagnosis of MTBC in sputum sample was 100% and 85.5% respectively (L.J Culture as Gold standard) which is comparable to K. Sharma et al who achieved 100% sensitivity and 100% specificity in a retrospective study[10]. We also noted a higher sensitivity when compared to uniplex PCR. Seth et al evaluated a uniplex PCR using MPB64 as a target reported a sensitivity of 85%[26], Negi et al evaluated Pab b as a diagnostic target alone using a uniplex PCR in pulmonary samples and reported a sensitivity of 74% and specificity of 100%[27]. MPCR method is not only a rapid and sensitive tool, but it also demands less skills. K Sharma et al, found that MPCR assay was as good as RT PCR[10] in the detection of MTBC. MPCR could be used for patient care in endemic resource poor countries, where RT- PCR technologies is not feasible due to cost and complexity.

CONCLUSIONS

Molecular diagnosis of tuberculosis by MPCR with more than 2-3 target genes may have a great potential to improve the clinicians ability to diagnosis of TB. This will ensure early treatment to patients and prevent transmission of disease.

ACKNOWLEDGEMENT- NONE
CONFLICT OF INTEREST: NIL

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