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Original Research Article

Rapid Detection of Mycobacterium Tuberculosis Complex with IS6110 Marker Based on Real Time PCR High Resolution Melting Analysis

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ABSTRACT

Mycobacterium tuberculosis *(MTB)* is a major agent of infection for human tuberculosis (TB) worldwide. *Although T.B. treated by a combination of six-monthly course of antibiotics, the rise in prevalence of drug-resistance TB (MDR-TB, XDR-TB) makes the it global health problem.* Molecular methods have a significant role to play in MTB and drug resistance TB. *Recently, high-resolution melting (HRM) Real time PCR analysis was used for amplicon genotyping and mutation scanning.* The present study aims to develop a diagnostic method for MTB detection based on HRM analysis that does not require labeled oligonucleotides. Polymerase Chain Reaction was performed for MTB detection with IS6110 as a target gene. Derivative melting curves of the IS 6110 specific target amplified duplexes were characteristic of the genotype of MTB and non tubercular clinical isolates. On fluorescence analysis, Light cycler 480 gene scanning software analyzes and distinguishes presence or absence of MTB depending on the shape of the curve. We collected 100 suspected clinical isolates, including 10 non tuberculosis cases. These samples were analyzed for acid-fast bacilli (AFB), culture & Real Time PCR. The sensitivity and specificity of detecting MTB by HRM Real Time PCR was 98.51% and 93.94% respectively. This technique requires the unlabeled primers specific to the target sequence and a DNA specific intercalating dye which can prove a promising method for detection of Mycobacterium tuberculosis.

Keywords: *Mycobacterium tuberculosis*, HRM, RT PCR, IS6110.

INTRODUCTION

Tuberculosis is known to cause significant mortality all over the world. World Health Organization has recognized India as a serious hot-spot region for tuberculosis and as the leading cause of death. [(1,2)] Detection of MTB in clinical isolate is important for the definitive diagnosis of tuberculosis. Microscopic examination needs a comparatively detectable amount of bacterium in specimens. The gold standard method of identifying mycobacteria is through mycobacterial culture. Though culture based detection is a sensitive method; a period of 4 to 8 weeks is necessary for definite diagnosis. [(3)] The development of diagnostic tests that are rapid, sensitive and specific for recognition of the causative agent of tuberculosis is essential to successfully control the disease. Conventional MTB PCR, targeting IS6110, TRC4, or hsp60 genes sequences have been extensively evaluated as an alternative for rapid diagnosis of tuberculosis. [(4)] To demonstrate the diagnostic potential of real-time PCR for detecting MTB in sputum specimens, we

performed AFB staining, Culture and real-time MTB PCR assays using 100 Clinical Isolates. The sensitivity and specificity of these methods were calculated using mycobacterial culture results as the reference point. [5] High-resolution melting (HRM) curve study is a novel, accurate and simple technique for analyzing the genotype of *Mycobacterium tuberculosis* without the need for specific probes. The dye LC Green, SYTO9, or Eva Green saturates amplified DNA, SYBR green dye, during identical melting curve analysis. [6-9] HRM curve analysis generates a difference plot curve, which analyzes nucleic acid sequences with high accuracy. Application of genotyping by HRM curve analysis has been followed for detection of much drug resistance case. [10] The aim of the study described here was to develop a useful molecular technique for the detection of M. *tuberculosis* in an accurate, fast and cost effective manner. [6-9] HRM dye could successfully detect IS6110 amplified product; a signature for MTB and at the same time did not inhibit or adversely affect PCR. In this article, we showed the usefulness of applications of HRM dye in MTB detection that involve high-resolution melting curve analysis of HRM RT PCR for detection of MTB. We also demonstrate the fluorescence normalization. Difference plot analysis was distinguished by shape of the curve and/or specific position as a useful method of differentiating MTB and non-MTB cases.

MATERIALS AND METHODS

Specimens: Clinical samples were obtained from patients with strong clinical symptoms of TB including clinical response to antitubercular treatment referred from different clinics like jay Clinic & MGM Hospital, Kamothe, MGM Central Research Laboratory. In this study, 90 sputum samples were obtained from adult pulmonary TB patients. 10 sputum samples were obtained from nontuberculous individuals (chronic asthmatics, chain smokers) initially screened by (-)ve AFB smear examination and chest X-ray were used as negative controls. Samples were processed immediately after collection. Samples were decontaminated and concentrated by modified Petroff's method. [11,12] Briefly, the samples were decontaminated by mixing 4% NaOH with an equal volume of sputum sample, mixed and placed in water bath (37°C) for 1 hour with intermittent shaking. The sample was then centrifuged at 3500 *g* for 30 minutes, the pellet was resuspended in 1 ml of 1× phosphate buffered saline (PBS). To inactivate the bacteria, the samples were kept at 56°C for 30 minutes in dry bath. From the pellet obtained, smears were prepared for AFB staining & other part of inactivation of samples was confirmed by culturing in LJ medium slant which were incubated at 37°C for 4-6 weeks and the inactivation found adequate. The inactivated sample was stored at -20°C until DNA extraction. DNA extraction was performed using QIAamp blood DNA extraction kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. [13,14]

DNA preparation: Processed sputum samples were used for DNA for positive control was obtained from solid cultures of a *M. tuberculosis* reference strain (H37RV) and *M. Bovis, M. kansasii* laboratory-identified strain grown on Lowenstein Jensen (LJ) agar medium. DNA extraction was performed using QIAamp blood DNA extraction kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. [13,14] DNA extraction was carried as described above.

Primers and PCR conditions: The primer sequences used are IS-F 5'-CCTGCGAGCGTAGGCGTCGG-3' and IS-R 5'-CTCGTCCAGCGCCGCTTCGG-3'.

Given Primer set was previously described for MTB detection. [15] Two primer sets, IS–F & IS-R and were used to amplify123 bp (65%GC). Genome DNA (5 ug) was added to 25 ul of a reaction mixture

containing 5 uM primers each of *IS-F & IS-R* and ingredients of the Light Cycler® 480 High Resolution Melting Master Mix. The PCR was performed on a LightCycler®480 Instrument II, 96-well. The Positive control for each reaction consisted of two tubes of DNA of *M. tuberculosis* H37Rv. The PCR cycles were run according to the following conditions: for amplification of the 123bp fragment of*IS6110*, 1 cycle of 95°C for 10 min and 45 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s; final elongation at 72°C for 7 min, and finally cooling to room temperature. Prior to HRM analysis, the products were heated to 95^0C for 1 min and then cooled to 40^0C for 1min. HRM was performed from 60 to 95^0C, rising at 1^0C/s with 25 acquisitions per degree. Light Cycler® 480 Gene Scanning Software was used for HRM curve analysis. The melting curves were normalized and temperature shifted to allow samples to be directly compared. Difference plots were generated by selecting a negative control/Positive control, converting the melting profile to a horizontal line and normalizing the melting profiles of the other samples against this sample. Significant differences in fluorescence from the horizontal baseline were indicative of nontubercular Isolates. Differences were judged as significant if the amplified DNA isolates fell outside the range of variation seen in the wild-type samples. The software analyzes the difference in the shape of the melting curve for a sample from the shape of the melting curve for the control strain (M. tuberculosis H37Rv) to detect sequence variants and generates a difference plot curve, which helps cluster samples into groups that have similar melting curves .The operator was blinded to the phenotype and genotype MTB Clinical Isolates data.

Statistical analysis: The data obtained were statistically analyzed for diagnostic values (Sensitivity, Specificity, positive predictive value, negative predictive value).The sensitivity, specificity, and the diagnostic odds ratio of different detection methods were calculated by MedCalc®. The performances of the HRM *IS6110* Real-Time PCR, Culture and AFB smear were compared statistically.

RESULTS

For optimization of PCR amplification, we used MTB H37Rv & *M. bovis* BCG genomic DNA as a template. For HRM Master Mix annealing temperature of 60^0C of stopped formation of primer dimers and improved the sensitivity of amplification to as much 100 copies of genomes. 5ug/ml DNA concentration was used in each reaction mixture. In that we tested a range of MgCl2 concentrations for each Master Mix (range of 2 to 5 mM tested in 0.5 mM increments) and finally decided on using 3 mM for all reactions. A primer concentration of 5 pmol per 20ul reaction mixture (5uM) (2.5 to 25 pmol tested), an elongation time of 40 s (20 to 60 s tested), an annealing time of 30 s (5to 50 s tested), and an annealing temperature of 60°C (58 to 62°C tested) allowed detection.

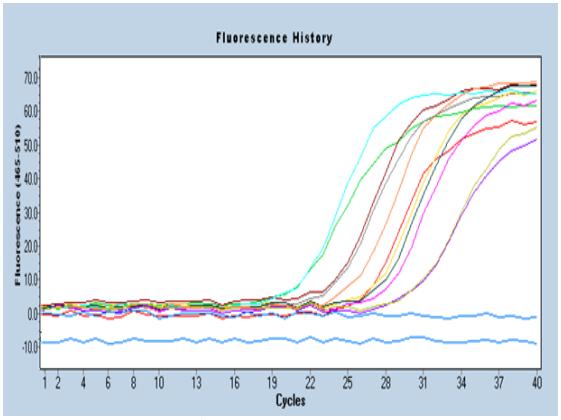

Figure 1-Detection of *IS6110* gene using HRM technique in LC480II. Amplification curves indicate positive control with a number of positive *IS6110* gene detection samples. Negative control did not show any amplification curve.

High-resolution melting curve analysis (HRM) is a recently developed technique for rapid and efficient screening of mutations in nucleic acid samples. This post- PCR method requires only the usual unlabeled primers and a ds DNA binding dye which detects subtle genetic

alterations in PCR- amplified samples based on their strand dissociation behavior. For further confirmation, samples were analyzed by 10% Poly-Acrylamide Gel Electrophoresis (PAGE) followed by silver staining of gel after run using standard methods.

For the HRM master mix kit, detection of only a single product, as analyzed by Tm, was evident. The Tm values for HRM Roche Kit were 88.5+/- 0.5^0C (Fig.2-A). This Tm value corresponds to the IS6110 gene product (123bp) as determined by melt curve analysis (Figure 2A). Analysis of PCR products of IS6110 by PAGE demonstrated that HRM master mix for IS6110 gene targets were being amplified (Figure 3).The peaks showing the melting curve profiles suggested that only the IS6110 gene was being amplified for most of the reaction conditions tested.

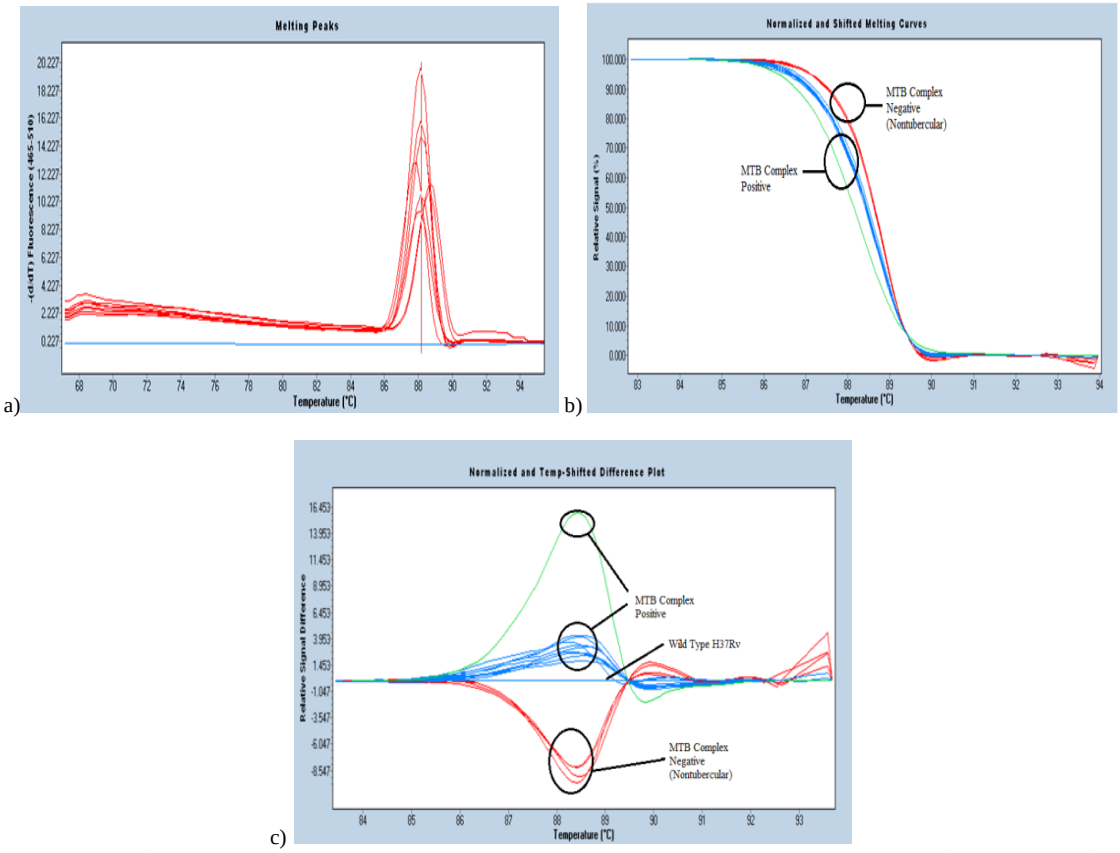

Figure 2-Detection of TB Positive *IS6110* gene by using HRM analysis in LC480II. The alternation in the shape of melting curves from the wildtype indicates the nontubercular isolates Amplification.2a)-Melting Peaks, 2b) Normalized Curve, 2c) Difference Plot Curves.

LightCycler® 480 Gene Scanning Software has module to process raw melting curve data to form Normalization Curve & a difference plot. The melting curve data at first glance does not appear to contain adequate information for detection. However, simple normalization of fluorescence before and after melting transition allows visual dissemination between MTB and non-tubercular Isolates (Figure 2B). The difference plot gives clearer representation of MTB and non-tubercular isolates. After normalization, the non-tubercular is easily distinguishable based on its temperature shifting and difference plot analysis. Automatic clustering algorithm could be devised for detection of MTB, simple visual clustering on difference plot appears simple & accurate (Figure 2C). Some cases Non-tubercular isolates showed amplification somewhere in range of Tm of desired gene sequence .This non specific amplification of undesired gene product is distinguished by differentiation curve. This differentiation between MTB & non-

tubercular isolates is concordant with Culture & AFB results. When HRM RT PCR amplified product was electrophoresis on PAGE, it was observed that few negative sample also resulted in an amplification product of 123bp. On primary observation based on amplification and melting curve, it is unable to distinguish between positive & negative samples as these isolates fall in the same melting peak area.

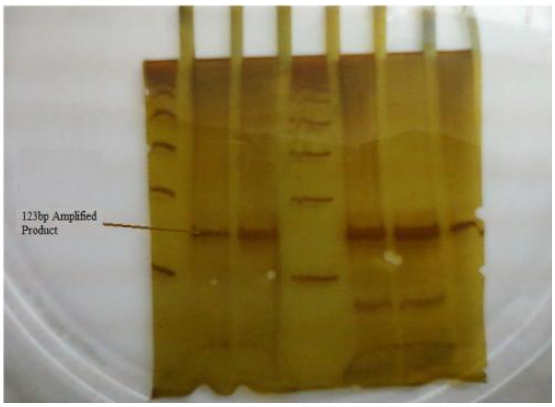

Figure 3- HRM RT PCR amplification of 123 bp region in IS*6110* targeting PCR for *M. tuberculosis* Complex Lanes 1&4: molecular weight marker; Lanes 2, 3, 7: positive for 123 bp MTB Positive; Lanes 5 and 6: MTB Suspected Samples Showing Amplification but in Difference Plot Showing Non tubercular Isolates.

Table 1-Comparison of sensitivity of PCR test with other Conventional Test

Conventional Tests	Total Clinical Isolates	PCR Sensitivity		
		+ve	-ve	Total Sensitivity
ZN positive	52	51	1	98.07%
ZN negative	48	17	31	35.41%
LJ positive	67	66	1	98.50%
LJ negative	33	2	31	0.6%
ZN positive LJ positive	52	49	3	94.23%
ZN negative LJ positive	16	16	0	100%
ZN negative LJ negative	32	11	21	34.37%

Table2: Summary of Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) results from different *Mycobacterium tuberculosis* detection methods

Method →			**Method to be compared with**			
			Culture		**ZN Smear**	
	Result →		(+)	(-)	(+)	(-)
	Total(n) →		67	33	52	48
IS6110 HRM	(+)	68	66	2	51	17
	(-)	32	1	31	1	31
				95% Confidence level		95% Confidence level
Sensitivity			98.51%	91.96% to 99.96%	98.08%	89.74% to 99.95%
Specificity			93.94 %	79.77% to 99.26%	64.58 %	49.46% to 77.84%
Positive Predictive Value(PPV)			97.06%	89.78% to 99.64%	75.00%	63.02% to 84.71%
Negative Predictive Value(NPV)			96.88 %	83.78% to 99.92%	96.88 %	83.78% to 99.92%
Disease Prevalence			67.00%	56.88% to 76.08%	52.00%	41.78% to 62.10%
AFB Smear	(+)	52	51	1		
	(-)	48	26	22		
				95% Confidence level		
Sensitivity			66.23%	54.55% to 76.62%		
Specificity			95.65 %	78.05% to 99.89%		
Positive Predictive Value(PPV)			98.08%	89.74% to 99.95%		
Negative Predictive Value(NPV)			45.83 %	31.37% to 60.83%		
Disease Prevalence			77.00%	67.51% to 84.83%		

We examined about 90 clinical isolates of MTB based on their clinical findings and other investigations performed at the time of diagnosis and 10 healthy non tubercular patients were included in this study. This study shows that 52 samples were positive for AFB, 67 were positive for culture and 68 were positive by HRM RT PCR. We evaluated the sensitivity & specificity of HRM PCR with two most widely used conventional method i.e. AFB staining & Culture. The highest PCR Sensitivity was found with culture about 98.5%, the sensitivity of PCR for smear positive isolates was 98% as summarized in table 1 & 2.

DISCUSSION

In India, mostly the diagnosis of tuberculosis is based primarily on clinical features, demonstration of acid fast bacilli and culturing of *Mycobacterium tuberculosis*. The direct AFB smear is cost effective; however, suffers from low sensitivity and specificity. [16]

Mycobacterial culture supposed to be a gold commonplace technique for detection of TB; however it takes a minimum of a pair of weeks, even with the recently introduced liquid culture system. [13] Nucleic acid amplification technique to detect Mycobacterium tuberculosis in clinical specimens is progressively used as a tool for TB identification.

Application of molecular techniques for routine diagnosis in country like India depends on varied factors like high value and trained personnel to perform the test & time to complete assay. [17-20] The Real Time LC480 instrument is a commercially available system designed to decrease the time of PCR by monitoring the intensity of amplification of the target sequences by use of fluorescent dyes. This technology is a significant get through in PCR amplification and target gene detection compared to conventional detection methods, and its benefits for clinical assays have been reported. [8, 21-24]

In this study, PCR was performed by amplification of IS6110 is a insertion sequence, that belongs to the IS3 family and was found in MTB Complex members. Most strains carry 2910 to 15 copies, which are present in a wide variety of chromosomal sites. [25-29] Previous studies have documented increased TB positive cases using the IS*6110* target in Pulmonary Samples. Negi *et al.* analyzed various targets precise for *M. tuberculosis* and reported the highest PCR positivity rates for pulmonary (90%) when using IS6110; their results further confirmed the poor sensitivity of smear microscopy for pulmonary (49%). The specificity, sensitivity and speed of PCR test in diagnosis of *M. tuberculosis detection* shown during this study ought to encourage to employ this technique in routine diagnosing of TB method in routine diagnosis of TB. [30-33]

With the use of HRM RT PCR test, we were able to detect *M. tuberculosis complex* in 98.51% smear positive samples found to be positive by the culture methods. HRM RT PCR test detected *M. tuberculosis complex* in less than one day, compared to 3.5-4 weeks required for detection by conventional (LJ) medium. In few samples, HRM RT PCR results were negative but AFB smear examination and culture reported positive. This could be due to PCR Inhibition & low bacterial load. Clinical isolates where PCR were positive, AFB smear and culture were negative may be due to occurrence of dead mycobacteria within the samples. The lower specificity rate during this study is also because of the use of complex genomic DNA (instead of plasmids) or to the high number of amplicons (+1 Sputum Sample) that we analyzed to screen the clinical isolates.

CONCLUSION

We conclude from our information that high-resolution melting analysis is as sensitive as alternative normally used pre-screening molecular methods. The key benefit of high resolution melting is no post-PCR processing demand, creating it a few manual work techniques whereas rising its cost-effectiveness, simple use, and high throughput. In summary, we are here with a fast and reliable *M. tuberculosis complex* detection strategy by high-resolution melting analysis. By introducing this method, our detection time for the *M. tuberculosis complex* has been reduced considerably (one third compared to Conventional PCR MTB detection) owing to the relatively low cost of the consumables (no need of fluorescence-labeled primers or special polymers) and the lower workload compared with other MTB Detection techniques. Thus proves to be a highly cost

efficient technology. We conclude that HRM analysis is a fast, economical, sensitive methodology, simple enough to be readily implemented in a diagnostic laboratory.

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