



RAPID DETECTION OF MULTIDRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* BY REAL-TIME PCR BASED ASSAY IN INDIAN POPULATION

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Abstract

The current study evaluates a rapid Real-Time Polymerase Chain reaction (RT-PCR) based assay for the detection of *Mycobacterium tuberculosis* complex (MTC) strains by targeting the IS6110 insertion element. In addition, hydrolysis probes were used, in this investigation for the diagnosis of Drug Resistance to genotype specific point mutations in *rpo B* and *kat G* genes in *Mycobacterium tuberculosis* that confer resistance to rifampin (RIF) and isoniazid (INH) respectively. This assay demonstrated high degree of sensitivity for detection *Mycobacterium Tuberculosis*. This method provides a rapid, sensitive and robust protocol for diagnosis of Multi Drug Resistant (MDR) MTB and offers several advantages over current molecular and culture-based techniques.

Keywords: *Mycobacterium tuberculosis*, Multidrug Resistance, RT-PCR, Hydrolysis probes, Rifampin, Isoniazid, Indian Population

Introduction

TB is one of the leading causes of mortality in India, killing two persons every three minutes, thus nearly 1,000 per day (Directorate of Health Services, India, 2009). An estimated 2 billion people worldwide are infected with *Mycobacterium tuberculosis* (MTB), which remains a vast reservoir of potential tuberculosis cases. T.B is the major co infection in HIV patients (Steinbrook, 2007). At present, about 5% of new tuberculosis cases in India occur in people infected with HIV infection. An extremely worrisome aspect of MTB is a recent rise in Multi Drug Resistance (MDR) MTB cases in several countries. New MDR MTB cases according to WHO estimates, constitute about 5% i.e. half million of nine million all types of TB (WHO, 2009). In a study conducted on a cohort of patients from urban population near Mumbai recently, a very high (51%) incidence of MDR- MTB was reported (Almeida *et al.*, 2003). MDR has now been recognized as a major public health problem that threatens success of DOTS, the WHO-recommended treatment approach for detection and cure of TB, as well as global tuberculosis control (WHO, 2009).

Currently, the routine diagnosis of TB is based on microscopy (AFB- staining) and culture of MTB. The microscope based technique lacks sensitivity. The culture diagnosis though sensitive takes 2-3 weeks, with additional weeks for diagnosis of drug resistance. Such delayed diagnosis facilitates further transmission of T.B. If MDR remains undetected, the patient not only suffers from drug toxicity but also continues

transmission of MDR bacilli. A quick and reliable diagnosis can permit early chemotherapeutic intervention and hence interrupt transmission. This could improve prognosis and avoid improper, ineffective medication as well as drug toxicity. Quicker diagnosis and appropriate therapies should therefore be accorded top priorities in controlling this growing epidemic.

Molecular diagnostic methods such as conventional PCR, though faster than culture diagnosis, have variable success in diagnostic mycobacteriology (Brisson *et al.*, 1991). RT-PCR, the new innovation of PCR, which is faster, more sensitive, has now been accredited for diagnosis of H1N1. Its features such as High Resolution Melt analysis (HRM) have diverse, powerful applications. HRM of the amplified sequences can be used to identify mutations in the targeted region. RT-PCR involving fluorogenic probes, has been successfully used for rapid detection and identification of a variety of microorganisms, including MTB and their mutants (Yoshinob *et al.*, 2000, Bruijnesteijn van cooperate *et al.*, 2004 and Johansen *et al.*, 2004). Such detection of drug resistant MTB, carried out in some European (Spanish, Polish and Romanian) populations, (Toress *et al.*, 2000, Marín *et al.*, 2004, Sajduda *et al.*, 2004 and Hristea *et al.*, 2010) show immense promise. No such study has so far been reported in Indian population that harbors 20% MTB patients, and a significant proportion of MDR-MTB patients of world. Validation and application of such assay for routine diagnosis could thus have a very

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significant impact on the control of transmission of TB. In these studies we have evaluated a very rapid RT-PCR based assay for diagnosis of MTB, targeting IS6110, present in the MTB genome in multiple copies (Thierry *et al.*, 1991a, b).

Drug resistance in MTB has been characterized by a number of mutations in genes that are involved in drug targets/ metabolism. Mutations in *rpo B* and *kat G* genes of MTB have been shown to be responsible for resistance to rifampin (RIF) and isoniazid (INH) - respectively (WHO, 2008). Ninety-five percent of mutations for RIF resistance are located in 81-bp region of the *rpo B* gene that codes for the β subunit of RNA polymerase. Mutations in codon 315 of the *kat G* (Viedma *et al.*, 2002) seem to be associated with higher levels of resistance to INH in 60-70 % of cases. In present studies, hydrolysis probe based protocols are used to amplify mutation containing regions of *rpo B* and *kat G*, and characterize mutations with the help of Melting curve analysis (Toress *et al.*, 2000).

Materials and Methods

Study Group

Sputum samples were obtained from 43 (n = 43) patients (with appropriate consent) from MGM Hospital, Navi Mumbai and Jayalaxmi Clinic, Govandi, Mumbai. In 40 of these cases independent Microscopy and Culture diagnosis of sputum samples was available. In the other 3 naïve, untreated cases, culture diagnosis was not available.

Extraction of Mycobacterium DNA

Sputum samples were decontaminated by Petroff method (Groothuis *et al.*, 1991). DNA extracted by QIAamp nucleic acid mini kit (Qiagen, The Netherlands) was used for PCR as per manufacturer's instructions.

Real Time PCR

For diagnosis of MTB infection, RT-PCR based amplification was performed on Syber Green Detection format (Roche Light Cycler 480, Mannheim, Germany) using primers (ISF / ISR). The assay detects a 123-bp region from the *M. tuberculosis* complex-specific insertion sequence IS6110. RT-PCR of MTB with Syber Green detection involved following steps: Incubation at 95°C for 10 min; followed by 45 cycles, each one with :95°C for 10 s, 60°C for 20 s and 72°C for 10 s;. Since Syber Green intercalates with double stranded PCR products (Morrison *et al.*, 1998), melting curve analysis was performed following amplification, to characterize amplimers by their respective melting temperatures. Melt analysis included preincubation of amplimers at 95°C for 1min, 40°C for 1 min, followed by heating up to 95°C at a rate of 0.1°C/s with concomitant monitoring the decrease in fluorescence. Rate of decrease of fluorescence intensity (-dF/dT)

was plotted to obtain Tm value for each Melt curve. The total time for RT-PCR and subsequent Melting curve analysis was < 90 mins.

Separate Melt Curve Analysis were performed by adapting a previously described protocol and primer-probes (Torres *et al.*, 2000) designed for detection RIF and INH resistance mutations. The hydrolysis probes used in this study , spanned 81 bp region of *rpo B* and the 315 codon region of *kat G* that contain mutations associated with resistance to RIF and INH respectively. All primers and probes were synthesized by TIB Molbiol (DNA Synthesis Service; Roche Diagnostics, Berlin, Germany). The RT-PCR was followed by melting curve analysis was also performed on the Light Cycler 480. This takes total time up to 90min appropriate controls were included in each experimental run.

Results and Discussion

Diagnosis of MTB: PCR based amplification using primers specific to IS6110 and SYBR Green I detection format was indicative of positive diagnosis of MTB (Fig.1a, 1b) for all the 43 cases studied. The Cp values (inversely proportional to the initial concentration of MTB-DNA) ranged between 20-35 cycles, indicating wide range of concentrations of MTB in the respective sputum samples. Gradual heating of amplified products from 40-95°C following amplification, generated Melt curves, yielding Tm values, that depend on length and sequence of respective amplimers. These values provide (88.8°C $\pm$ 0.5) characterization of amplimers and confirmation of positive diagnosis of MTB. Amplification profile- Melt analysis based diagnosis was found to be in complete agreement with culture diagnosis available for 40 culture positive cases studied. No false positives or false negatives were observed in this cohort of cases. It was of interest that such high sensitivity was observed with syber-green platform, which does not require expensive fluorescently labeled probes. These quick procedures involves only about 1.5 hrs of RT-PCR and melt analysis. Prior decontamination (2-3 hrs) and extraction of DNA (0.25 hrs) together require less than about 3 hrs. In comparison, routine culture diagnosis of MTB takes 2-3 weeks, Bactec method requires about 8-10 days. It is claimed (John *et al.*, 2010) that Hybridization based diagnosis developed recently, requires up to 3-4 days (Boehme *et al.*, 2010). In the present study RT-PCR based detection using IS6110 specific primer pairs displayed sensitivity ~100%. Thus this RT-PCR based assay seems to offer a highly sensitive and rapid method for diagnosis of MTB.

Fig. 1a: Accumulation of Green Fluorescence during RT-PCR using ISf-ISR6 primer pair with MTB-DNA extracted from sputum samples from different patients. Fig. 1b: Derivative melting curve of $-dF/dT$ versus Melting Temperature using SYBR Green detection format for MTB DNA extracted from sputum of a patient

Fig 1a

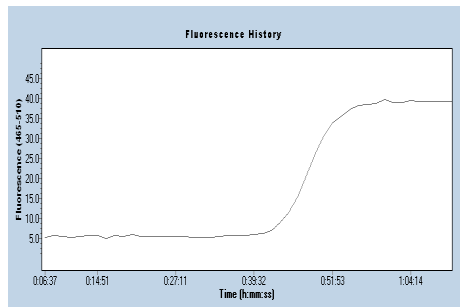
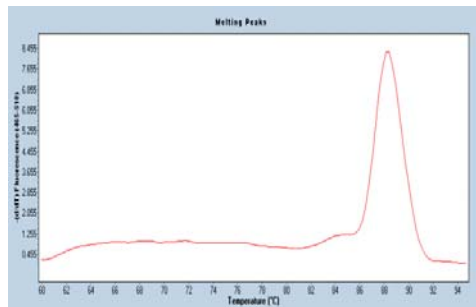


Fig.1b

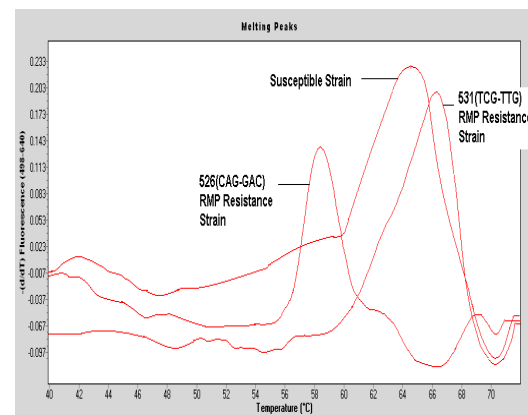


Diagnosis of MDR-MTB: For MDR diagnosis, specific sequences spanning mutation hot spots were amplified by respective primers and screened by fluorescently labeled hydrolysis probes. The amplification profiles for *rpo B* or *kat G* sequences have little implication for diagnosis, as these are not specific to MTB complex. Therefore only probe-amplimer melt profiles of *rpo B* and *kat G* have been included in the figures (Fig. 2a, 2b). T_m values obtained for these profiles are compared with corresponding data from Torres et al (Table-1a). In all, 16 cases displayed RIF resistance genotype, of which 4 indicated mutations at codon 526 and 12 at codon 531. 11 cases displayed susceptible genotype, with absence of any mutation. Similar comparison for INH resistance indicated presence of 7 cases each for AGC>AAC and AGC>ACC mutations in codon 315. No such mutations could be detected in 10 cases. This diagnosis of drug response for various categories based on RT-PCR-HRM was compared with corresponding culture diagnosis (Table-1b). These cases fall into of 3 categories: Resistance to RIF and INH, Sensitive to both drugs and sensitive to one of the drugs. There was complete agreement between diagnoses from RT-PCR and Culture in 38 cases out of 40 cases. Discordance was observed in only 2 RIF-

R/INH-S cases (Table-1a). While these cases were diagnosed as sensitive to both drugs by culture, they were found be resistant to RIF alone by RT-PCR assay. Assuming that the culture diagnosis was correct, this indicates 2 false positives for RIF resistance. No false negative case for RIF was observed. Interestingly no false positive or false negative cases were observed for resistance to INH in this study. Thus although the study is conducted on limited no of patients, yet it brings out high sensitivity of the RT-PCR based assay for detection of drug resistance.

Fig. 2a: Derivative melting curves dF/dT of Red 640-labeled *rpo* sensor probe with samples containing mutations in codons. . Each trace shows the change in fluorescence ratio with time with respect to temperature, thus allowing calculation of the temperature at which the probe detaches from the PCR product. Susceptible refers to MTB that doesn't contain drug resistance point mutation. Fig. 2b: Derivative melting curves (dF/dT) of Red 640-labeled *kat* sensor probe with samples containing mutations in codon 315

2a



2b

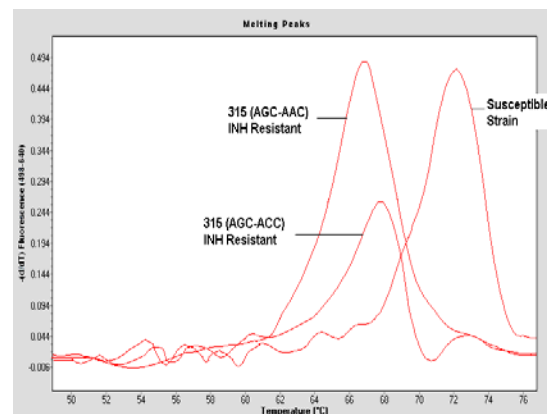


Table 1a: *T_m* values and corresponding Genotypes for RIF and INH mutations

Drug-Resistance Genes	Phenotype	Number of samples	Codon/ Mutations	T _m (Observed) This study	T _m (Reported) Toress <i>et al.</i>
<i>rpo-B</i>	Resistant to RIF	4	526 (CAC>GAC)	57.68± 0.21	58.16±0.2
	Resistant to RIF	12	531 (TCG>TTG)	66.7±0.52	66.96±0.5
	Susceptible to RIF	11	None	64.4±0.35	64.36±0.4
<i>kat-G</i>	Resistant to INH	7	315 (AGC>AAC)	67.81±0.1	67.9±0.07
	Resistant to INH	7	315 (AGC>ACC)	68.2±0.5	68.7±0.4
	Susceptible to INH	10	None	72.2± 0.4	72.4± 0.4

Table 1b: Comparison of number of cases diagnosed for Drug Sensitivity by RT-PCR and Culture Methods

Drug Response	No. of Cases	
	RT-PCR Diagnosis	Culture Diagnosis
RIF-R / INH-R (MDR)	11	11
RIF-R / INH-S	5	3
RIF-S / INH-R	3	3
RIF-S / INH-S	21	21
Total	40	38

(R-Resistance, S-Sensitive)

MTB Resistance to RIF is associated with 8 different point mutations clustered in 81bp region of *rpo B* gene (Toress *et al.*). Of these only 2 can be detected efficiently with the probes used in this study. It is possible that better probes, with more resolving power can improve specificity of this assay. In contrast, 2 mutations in *kat G* account for most of the cases of INH- resistance (Table-1a). The study indicates that both of these can be screened efficiently with the probe used in Indian context. The sensitivity and specificity of Probe based assays seem to depend on the design of the probes used. Thus it would be of interest to compare sensitivity of different combinations of primer-probes for detection of specific mutations in Indian population. In particular, sets of primers and probes that permit multiplex RT-PCR for detection of both MTB as well as MDR MTB would be desirable, as these would obviate need for separate PCR for MTB and MDR.

Very interestingly, in 3 naïve cases suspected of TB, the RT-PCR diagnosis was confirmed by RT-PCR that they were resistant to both RIF and INH. Culture diagnosis is not available for these cases so far. The present study is not designed to monitor incidence of various RIF and INH mutations in Indian population. However it does underline the usefulness of protocols to screen both naïve as well as treated cases. This is particularly important if the incidence of MDR is as high as 51% in urban Indian population as reported

(Almeida *et al.*, 2003). With diagnosis by a rapid assay, such as described here, appropriate treatment could be initiated promptly, in such naïve cases, to interrupt transmission to improve prognosis, and avoid unwarranted drug toxicity.

The study carried out for the first time in Indian context, brings out high sensitivity of RT-PCR based detection of MTB and resistance to RIF and INH.

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