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Extended Abstract

Detection of mutations associated with isoniazid resistance in *M. tuberculosis* isolates by hybridization probe assay of real-time PCR

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Drug resistance in TB is an increasing problem because of the long therapy limitations. Anti-tuberculosis drug resistance is due to the spontaneous genetic events (Rusch-Gerdes 1999). The basic reasons of drug resistance are mutations in the target site, drug interaction and rendering the inefficient DNA (Ramaswamy and Musser 1998).

Real-time polymerase chain reaction is being globally used for rapid quantitative and qualitative detection of drug resistant *M. tuberculosis.* Real-time format allows the detection of PCR amplification during the early phases of the reaction and measuring the kinetics of the reaction in the early phases of PCR provides distinct advantages over traditional PCR detection (Parashar et al 2006). Sensitive and specific detection is possible with real-time PCR by using novel fluorescent probe technology probes. The high sensitivity (in some instances greatly exceeding sensitivity for conventional testing method) and specificity along with a short turn around time for results and ease of performance make real-time PCR an attractive replacement method for conventional culture and antigen based assays. Real-time PCR has been used to detect mutations in genes encoding resistance to rifampicin, isoniazid and ethambutol by using hybridization probe format.

In this study, we used inhouse designed probes and primers of *inhA* gene encoding 194 codon for direct detection of susceptibility in both sensitive and resistant culture and clinical isolates of isoniazid.

The system is based on modified protocol of Torres et al (2000) used fluorescently labeled shorter detection probe (Sensor 5' Red- 640 labeled) while the longer probe anchor 3' fluorescein labeled to detect mutation in *inhA* gene region of *Mycobacterium tuberculosis.* On the basis of fluorescence resonance energy transfer (FRET) system carried out by real-time

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PCR on Light Cycler allows rapid PCR cycling and continuous monitoring the product formation and analyses increase or decrease Tm. Fluorescence read at Channel F2/F1. F2 is the channel used by the Light Cycler to detect the Light Cycler-Red 640 light emission, F1 is the channel measuring the background.

Biochemically confirmed culture and clinical isolates of *M. tuberculosis* (A total of 26 culture isolates and 14 clinical isolates both resistant and sensitive to Isoniazid) were collected after processing from tuberculosis patients from Mycobacterial Repository Centre at Dept of Microbiology and Molecular Biology, NJIL & OMD, Agra, India and reference susceptible strain H37Rv as a control were included in our study. The selected isolates were cultured on Lowestein Jensen medium and incubated at 37°C until growth obtained. Susceptibility results were determined by MIC (minimum inhibitory concentration) method. MIC has been determined by using standard criteria of counting the colony forming unit as compare with culture control (Canetti et al 1969). Genomic DNA from each isolate was extracted as protocol described by using van Embden et al (1993).

Two primers sequence *inhA* F-CAAGAGCGCGTTGGAG and *inhA* R-GGCGTAGATGATGTCACCC spanning the region 194 codon were design and amplified and primer set with better PCR efficiency was selected. One sensor probe labeled with Red fluorophore (Light Cycler Red 640) at the 5' and phosphorylated 3´ and the other probe is labeled with fluorescein. In our study, we use two paired FRET probes of which one act as sensor and other as anchor and their standard designs are used. Mutation within the sequence covered by either of the two probes would lead to change in the Tm of probe. The Thermocycling protocol was 95°C for 10 min followed by 40 cycles of 95°C for 10sec, 56°C (annealing temperature for the *inhA* gene) for 10 sec and 72°C for 20 sec with continuous monitoring of fluorescence during the annealing phase, this is followed by a melting programme of 40 to 90°C at 0.1°C/s with continuous monitoring of the fluorescence (Torres et al 2000).

Twenty six *M. tuberculosis* culture isolates (resistant to isoniazid) and fourteen clinical isolates were amplified by using inhouse designed probes and primers. Both culture and clinical isolates were tested for isoniazid and we use

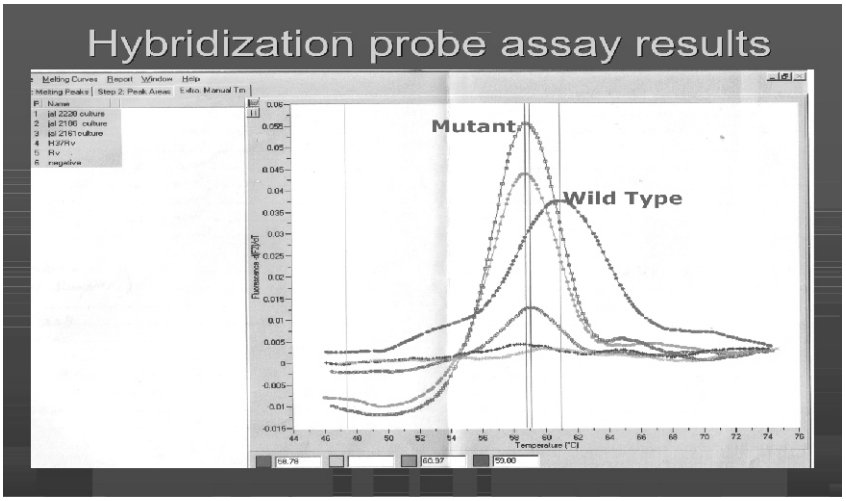

Figure 1 : Derivatives melting curves of Red 640 labeled *inhA* sensor probe at 194 codon.

reference susceptible strain H37Rv as a control. The temperature at which the probes melted as PCR products during the melting programme were calculated by Light Cycler software. The change in Tm for the products derived from resistance and susceptible *M.tuberculosis* strains. We were able to detect mutation in *inhA* gene of isoniazid all the susceptible *M. tuberculosis* strains determined by *inhA* sensor probe showed a Tm that corresponded to the susceptible strain used as control H37Rv and the resistance strain showed Tm variation from that of wild type were seen in Figure 1.

The designed assay is being evaluated for applicability of mutation detection directly from clinical samples which well enable us to identify the mutation at target sites rapidly without the need of having culture of pulmonary and extra pulmonary cases of tuberculosis. The detection of mutation with real-time PCR device is usually based on fluorescent probes that are optimized to detect specific mutation and another advantage of this technique is that it can be applied directly to the clinical samples, either in leprosy patient or in tuberculosis patient. Real-time PCR assay enables monitoring of the amplification and detection of mutations, so that appropriate treatment regimen for patients can be selected early in course of infection. As this technique can detect lower loads of organisms, it can be very usefull in *M. leprae* infections has feable bacilli load and cannot be propagated *in vitro*.

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