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Received: 24 December, 2018

Accepted: 21 January, 2019

Published: 22 January, 2019

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Research Article

Detection of *katG* Ser315Thr substitution in *Mycobacterium tuberculosis* infected patients using PCR -RFLP method for screening Isoniazid-Resistance

In 2016 an average estimate of 28 lakh cases were occurred and 4.5 lakh people were died due to Tuberculosis (TB facts). In drug resistant TB person will not respond to one of the main TB drug. Mostly in India the drug resistant TB is due to failure of people to take their anti TB drugs properly. According to WHO TB statistics for India provides an estimated incidence of 2.79 million cases. In most of the cases the multidrug resistant TB is resistant to rifampin and isoniazid drugs. In rifampin the resistant due to the mutation in *rpoB* gene region while isoniazid resistant involves genes like *katG*, *inhA*, *kasA* and *aphC* but most frequently it is associated with the *katG* mutation [2]. Mutation in the region of *katG* gene occurs in higher fold frequency than *rpoB* gene mutations and is considered as a step in the evolution of MDR-Tuberculosis [3]. Although Ser315Thr mutation is commonly found mutation used for identifying isoniazid resistant isolates [4].

Isoniazid inhibits the process of biosynthesis of cell wall with mycolic acid and thereby making the mycobacteria more susceptible to the reactive oxygen radicals and along with environmental factors [5]. The isoniazid also requires the catalytic activation to be in active form and Catalase-peroxidase is the key product of the *katG* gene. The mutation in this specific gene will cause the mycobacteria contributes for isoniazid drug resistance. In this study we assess the PCR-RFLP technique to screen Ser315Thr substitution in *katG* region for isoniazid resistant *Mycobacterium* strains. In the treatment of tuberculosis Isoniazid is one most common first line drug if the resistant developed against this drug it will causes serious problems related to treatment. The most frequent mutation in *katG* gene is substitution from AGC to ACC at codon 315 [6]. The molecular method like sequencing is used for the detection of isoniazid resistant is time consuming therefore in the present study we evaluated the PCR-RFLP based rapid screening method. PCR-RFLP method is very quick for screening or detection of *katG* gene mutation in Isoniazid resistance Tuberculosis infected population.

Introduction

Currently mycobacterium tuberculosis is major health issue to the Indian population as well as global. According to WHO around 8 million cases of Tuberculosis occur each year, which resulting in approximately 3 million deaths and the situation is more complicated due to emergence of multidrug-resistant TB. It is the leading cause of death worldwide and results from a single infectious agent, ranking over HIV/AIDS. In 2017, Tuberculosis caused an estimated of 1.3 million deaths among HIV-negative people and additional there were 300 000 deaths from Tuberculosis among people living with HIV. Globally around 10.0 million people which ranges from 9.0–11.1 million were developed Tuberculosis in 2017 which accounts 5.8 million men and 3.2 million women and along with 1.0 million were children's. There were cases with all age groups reported worldwide but overall about 90% were adults (aged ≥ 15 years) and 9% were people living with HIV [1]. Rifampin and Isoniazid are two first line drugs involved in the treatment of TB when the resistance was developed against these two drugs they are termed as multi drug resistant (MDR) *Mycobacterium tuberculosis*.

The rate of spread of MDR strain directly affects tuberculosis control program so for that rapid screening or identification of MDR strain creates major help in the treatment and control of MDR strains in the population. There is an approximate estimation of 79,000 multi-drug resistant Tuberculosis patients notified cases with pulmonary Tuberculosis each year.

Materials and Methods

A total of ten Sputum samples which are TB Confirmed positive samples were collected from primary health care centers Hyderabad. The patients are already confirmed with AFB and culture positive and these patients showed advance stage of immunological complications hence it is important to evaluate for multi drug resistance pattern for these patients for further effective therapeutic treatment. We have used the reference standards to evaluate the specificity and sensitivity of this assay. A result with reference and samples generated were compared with DNA sequencing result. The study was carried out to identify Ser315Thr mutation at specific position of *KatG* gene for isoniazid resistant *Mycobacterium tuberculosis* strains.

Genomic DNA extraction

DNA extraction was carried out from the sputum samples using QiaAmp DNA Mini kit as per the manufacturer's protocol. The purity of the DNA was quantified by A260/A280 ratio and the optical density for DNA was measured at 260 and 280 nm) using Nan drop ND-1000 spectrophotometer (Thermofisher Scientific) and the DNA was visualized on 0.8% agarose gel.

PCR-RFLP for *katG*

This PCR-RFLP method was designed to easily detect the *katG* specific codon mutation from AGC (Serine)→ ACC (Threonine) which leads Isoniazid resistance mycobacterium strains. The PCR product of 200 bp fragment was amplified for identifying *katG* codon 315 was carried out with the specific primers *katG* F and *katG* R as reported [7], in a 25ul of a PCR mixture containing 0.4 picomoles of each primers and Taq polymerase 2X master mix (G-Bioscience in which Taq polymerase supplied with 2X Taq buffer, 0.4mM dNTPs, 3.2mM MgCl₂ and 0.02% bromophenol blue and the PCR was performed in thermal cyler with the following conditions, initial denaturation at 95°C for 5min and 30 cycles of 94°C for 1 min with 61°C for 1 min and 72°C for 1 min and a final extension was carried out at 72°C for 4 min. The reaction controls were used to detection false positive results due to contamination a negative control (distilled water) was included in each PCR reaction. A reference control H37Rv was used in each PCR run which shows a clear band at position of 200bp in the agarose gel electrophoresis (Table 1 and Figure 1).

Agarose gel electrophoresis for MTB detection

The PCR products were detected by 2% agarose gel electrophoresis with stained ethidium bromide. *Mycobacterium* strain H37Rv used as a reference control and negative control was also used in the PCR reaction.

Restriction enzyme digestion for Ser315Thr *KatG*

The *katG* amplicons were further used for RFLP analysis using *MspI* digestion. To each of the PCR mixtures add restriction enzyme mixture containing a final concentration of 0.5U of *MspI* enzyme (NEB Biolabs), 1X NEBuffer used for reaction according to manufacturer's instructions. The mixture incubated at 37°C

Table 1: Primer Sequences used for *katG* Amplification.

Target Gene in Mycobacterium	Primer	Primer Sequence (Blast Confirmation)
<i>katG</i>	<i>katG</i> Forward	5'AGCTCGTATGGCACC GGAAAC3'
	<i>katG</i> Reverse	5'AACGGGTCCGGGATGGTG3'

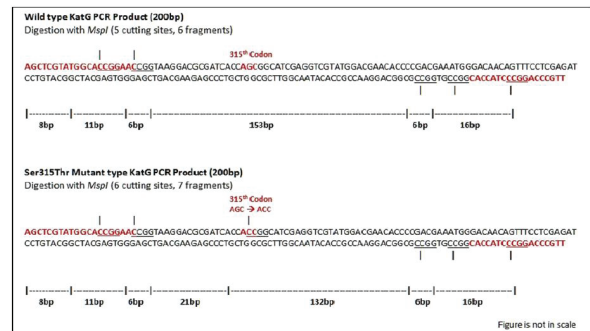


Figure 1: In the above 200 bp wild type *katG* amplified product absence of *MspI* restriction site (CCGG) at specific position 315th codon (AGC) yields the long fragment of 153 bp, while in the mutant type the presence of *MspI* restriction site at position 315th codon (ACC) yield long fragment of 132 bp [7].

for 1 hr. The restriction fragments were visualized by running on 2% agarose gel. This is very simple and rapid RFLP method used to detect the *katG* codon mutation from AGC to ACC, in which mutation creates an additional site for *MspI* (CCGG) and therefore it is cleaved by restriction enzyme. This mutation from AGC to ACC creates isoniazid drug resistant phenotype. As a result, 153 bp longest fragments indicates the wild type *katG* PCR product, while 132 bp long fragment indicates the presence of Ser315Thr *KatG* mutant along with shorter bands sizes (21 bp–8 bp products) which cannot be resolved clearly on gel due to smaller fragment size. MTB strain H37Rv (Accession Number X68081) used as a reference control in the restriction digestion.

Results

DNA extracted from ten isolates were visualized on 8% agarose gel displayed positive band (Figure 2). Further extracted DNA used for *katG* amplification, after amplification the amplified product were observed on 2% agarose gel for confirmation of 200bp positive amplification (Figure 3). After confirmation the *katG* amplicons were further used for RFLP analysis using *MspI* digestion. After digestion the electrophoresis of *katG* digested product shows out of 10 samples only one sample shows 132bp region results occurrence of Ser315Thr mutation while the other 9 samples shows 153bp region results no mutation. The (Figure. 4) shows that the products obtained by *MspI* digestion out of which lane 2 (sample 9) with undigested product or wild type; lane 4 (sample 10) with mutated *katG* (315ACC); last well is control; M represents the DNA ladder (G-Bioscience) lane 1 (20bp), lane 3 (50bp), lane 6 (100bp) respectively.

The Mutation in *katG* Ser315Thr observed in isoniazid resistant isolates. The drug susceptibility profile of these isolates were showed resistant to in isoniazid and rifampicin whereas 3 isolates were resistant to Ethambutol drug and Streptomycin respectively alternatively other Seven (7)

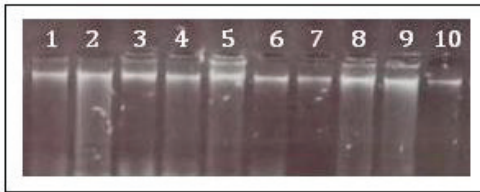


Figure 2: Representative of MTB-DNA Extracted from samples visualized on 0.8% Agarose gel.

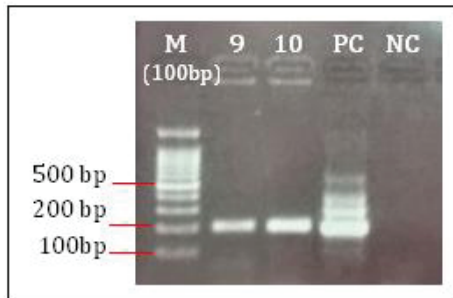


Figure 3: *katG* PCR amplification product visualized on 2% agarose gel.

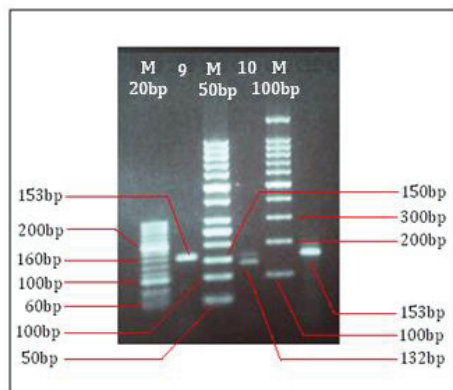


Figure 4: *MspI* digested *katG* PCR products.

patients were showing *katG* gene mutated strain in Ethambutol and Streptomycin resistant cases.

Discussion

We have observed out of 10 samples only one sample shows 132bp region results occurrence of Ser315Thr mutation while the other 9 samples shows 153bp region results no mutation. This suggests these remaining nine patients did not harbor drug resistance. The patients are put on regular treatment regimen except the one patient for multi drug resistance which follows standard treatment for MDR resistance and later follow up of the drug resistance indicates improved in his disease. PCR-RFLP based method for detection of drug resistance is rapid, easy to perform & interpret as compared to other techniques. Therefore this study was undertaken for rapid detection of Ser315Thr *katG* mutation in Isoniazid resistance population by PCR-RFLP assay. For quick and rapid identification of isoniazid many approaches were reported including real time PCR, single-strand-conformation polymorphism analysis and multiplex-allele-specific PCR but the RFLP approach has

several advantages over others methods because it is very cheap, robust, and simple to both perform and interpret, requires PCR equipment [8]. Early detection of resistance to drugs will helpful for treatment modalities and effective control measures to reduce the risk of further disease progression and transmission. The *katG* and *inhA* genes mutations account for up to 80% in resistance to isoniazid drug, however majority of mutations are associated with *katG* gene at specific codon at 315 an estimate of 30–90% of isoniazid-resistant isolates which spans across the world [3,9,10].

Conclusion

Isoniazid along with Rifampicin (RFP) is used as a combination chemotherapy course for tuberculosis (TB) treatment. Mutation at the specific codon position 315 of *katG* gene is most prevalent method to detect isoniazid resistant *Mycobacterium tuberculosis*, it will help to screen in high burden countries and in area with higher TB incidence. The technique is feasible for screening TB burden for drug resistance by RFLP method compared to other methods like Genexpert and Line probe assay where the assay require expertise, time consuming ,expensive reagents and expensive instruments.

Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) is a reliable and effective tool for the identification of the specific gene alteration in this specific region. The Ser315Thr *katG* mutation will be a reliable marker for the detection of Isoniazid resistance in *M. tuberculosis*. The research will also emphases on high prevalence of Ser315Thr mutation in *katG* in the study population helps in predicting MDR-TB by rapid RFLP Method.

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