AMPLIFICATION OF rpoB, kat G & mab A (fab G1)- inh A PROMOTOR DNA SEQUENCES BY PCR IN MULTIPLE DRUG RESISTANCE TUBERCULOSIS

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ABSTRACT- Multiple Drug resistance (MDR) tuberculosis timely diagnose is of utmost clinical relevance and needs to be diagnose at initial stages for the proper treatment. The current study was done to detect the several genes for MDR tuberculosis (TB) in clinical isolates by molecular tools. 60 clinical isolates were collected and subjected for AFB smear preparation, Nested PCR (IS6110) for mycobacterium tuberculosis complex detection and MDR TB PCR targeting rpoB, kat G, mab A promoter. 12 came positive for AFB smears, out of which 08 were pulmonary and 04 were extra pulmonary. Nested PCR targeting IS6110 gene was amplified at 123 base pairs with 340 base pairs as IC (internal control) was seen in 25 cases which include 19 pulmonary and 6 extra pulmonary. The Positive TB PCR specimens were subjected for MDRTB PCR Only 06 cases yielded, an amplicon of 315 bp confirming the rpoB gene resistance for resistance for rifampicin drug. In any of the 06 positives none of the other resistance gene other than rpoB was amplified. Targeting multiple genes at once, additional information will be gained from a single test run that otherwise would require several times the reagents and more time to perform. Current study signifies the usage of quick, cost effective, DNA sequences based method for MDR TB detection where disease will be diagnosed earlier and hence treatment would be started at an early stage.

Keywords: Multiple drug resistance, amplicon, Polymerase chain reaction, Nested PCR, Rifampicin.

INTRODUCTION

Tuberculosis is a contagious disease caused by various strains of Mycobacterium tuberculosis (MTB). In India each year about 2 million people develop active disease and up to half a million die. About .5 million HIV patients and about 1.8 million of these are co-infected with tuberculosis [1]. The bacteria that cause tuberculosis (TB) can develop resistance to the antimicrobial drugs used to cure the disease. Multidrug-resistant tuberculosis (MDR-TB) is TB that does not respond to at least isoniazid and rifampicin, the two most powerful anti-TB drugs. Inappropriate or incorrect use of antimicrobial drugs, or use of ineffective formulations of drugs (e.g. use of single drugs, poor quality medicines or bad storage conditions), and premature treatment interruption can cause drug resistance, which can then be transmitted, especially in crowded settings such as prisons and hospitals [2,3]. Treatment options are limited and expensive, recommended medicines are not always available, and patients experience many adverse effects from the drugs.

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In some cases even more severe drug-resistant tuberculosis ay develop. The genetic basis of resistance to most anti-TB drugs is established. Resistance to rifampicin results from missense mutations in the rpoB gene, which encodes the β subunit of RNA polymerase [4]. Rifampicin specifically binds to the β subunit and prevents early steps of transcription that leads to the bacterial death. However, mutation in rpoB gene results in resistance by decreasing rifampicin (RIF) binding affinity. Mutation leading to resistance of M. tuberculosis to rifampicin is rare and occurs at a rate of 10^{-10} per cell division with an estimated prevalence of 1 in 10^5 cells in drug free environment [5]. However, it rapidly results in the selection of mutants that are resistant to other anti-TB drugs. Most commonly, it exists in conjunction with mutations in kat G. The rate of mutation for isoniazid is 10^{-8} resulting in resistance in 1 out of 10^8 bacilli [6]. A missense mutation of the inhA gene which encodes an enzyme involved in the mycolic acid biosynthetic pathway also causes resistance. About 20-34% resistant isolates have mutations in the promoter region of inh A, either alone or in combination with kat G. Isoniazid resistance following inh A mutation alone is rare. Mutations have been found in the ahp C promoter region of approximately 10% of isoniazid resistant isolates; however these mutations have always been found to occur in association with mutations in kat G. The rate of mutation for isoniazid is 10^{-8} resulting in resistance in 1 out of 10^8 bacilli [7]. Pyrazinamide is also a pro-drug that can be converted into an active form presumably by pyrazinamidase enzyme of susceptible organisms. The target for the active drug is not fully known. However, mutations in the gene pnc A, encod-
ing for the enzyme pyrazinamide is the major causes of pyrazinamide resistance. Between 72% and 98% of pyrazinamide resistance in clinical isolates is correlated with mutations scattered throughout the 558 bp pncA coding region and 11 promoter regions. The rate of mutation for pyrazinamide is 10^{-3} with a probability of resistance 1 out of 10^{8} bacilli [8]. The identification of specific mutations responsible for drug resistance has facilitated the development of novel, rapid molecular tools for Drug Susceptibility Test (DST). The detection of RIF resistance is traditionally used as a predictor of MDR-TB – its positive predictive value is a function of the sensitivity and specificity of RIF resistance testing and the prevalence of MDR and non-MDR RIF resistance, which is highest among previously treated cases in settings with high MDR prevalence and low non-MDR RIF resistance [9, 10]. Thus, the present study was carried out to study various genes responsible for multiple drug resistant tuberculosis which includes; genes sequences: rpoB, katG, mab A (fab G1) inh A promoter and were amplified by PCR (multiplexing) and drug resistance pattern were studied from clinical isolates.

MATERIALS AND METHODS

Specimen collection

A total of 60 Clinical specimens which includes Pulmonary such as Sputum, Pleural fluid, Bronchiolar alveolar lavage, bronchial secretions and extra pulmonary specimens such as Pus, Urine, Semen, Tissue, Endometrial biopsy, Cerebrospinal Fluid were considered for the study. Specimens were collected from patients attending Out Patient Department (OPDs) and In Patient Departments (IPDs) of different Departments of Shri Mahant Indresh Hospital, Dehradun, Uttarakhand, India. The current study was approved by institutional ethical clearance body and written consent was taken from patients. All the specimens were subjected for different parameters like Acid fast bacilli smear preparation, Nested Polymerase Chain reaction (N-PCR) and multiplex PCR targeting rpoB, kat G & mab A (fab G1) inh A Promotor DNA Sequences. The Nucleic Acid (DNA) was extracted from the clinical specimen by Spin Column based Nucleic Acid Extraction method (Genetix) and the template was used for the N-PCR and multiplex PCR.

Nested PCR for the detection of Mycobacterium tuberculosis complex targeting IS6110 gene

Further Nested PCR was performed on the DNA template isolated from various specimens. This test is based on the principles of single-tube nested PCR method, which is a powerful and sensitive diagnostic tool for the identification of Mycobacterium Tuberculosis complex. This assay is a two-step sequential assay. In the first step, the Insertion sequence region of Mycobacterium tuberculosis complex DNA sequence, a 220 bp is amplified by specific external primers. In the second step, the nested primers are added to further amplify a 123 bp amplification product. In this assay, false positive reactions that may be caused by previous ampiclon contamination are prevented by the use of uracil DNA glycosylase (UDG) and dUTP instead of dTTP added in the premix [11, 12]. Nested PCR. An amplimer of size 123 bp is indicative of infection with Mycobacterium tuberculosis complex. The amplification product of internal control DNA is 340 bp which is used for the validation of the results (as depicted in Figure1).

PCR for Amplification of rpoB, kat G & mab A (fab G1) - inh A Promotor DNA Sequences by PCR in Multiple Drug Resistance tuberculosis

Multiplex PCR involving amplification of rpoB, kat G & mab A (fab G1) inh A Promotor DNA sequences [13] were done for 25 nested TB PCR positive cases for which master mix was prepared using 5 µl 10X PCR buffer, 5 µl, deoxynucleotides (2 mM) 5 μl magnesium chloride (25 mM), 0.5 µl primers (25 µM) , 0.25µl Taq DNA polymerase (5 unit/µl) & nuclease free water was added (6.75µl) to make the total volume upto 25µl. Add 25µl of template DNA amplification was done on Benchtop 9600 thermocycler, Germany involving following parameters ; initial Denaturation at 94°C for 5 minutes followed by 35 repetitive cycles of denaturation at 94°C for 30 seconds, annealing at 55.5°C for 30 seconds & elongation at 72°C for 30 seconds with final elongation at 72°C for 7 minutes. After completion of amplification post amplification was done using 1.6 % agarose gel electrophoresis. The PCR will yield an amplicon of 315 b.p, 2223 b.p and 1362 b.p respectively for rpoB, kat G and mab A Promotor respectively (figure 2). Primers used are tabulated in table 1.
Results

Sixty samples were collected from patients attending Out Patient Department (OPDs) and In Patient Departments (IPDs) of different departments of Shri Mahant Indiraksh (SMI) Hospital, Dehradun, Uttarakhand, India. Out of sixty clinical isolates collected for the purposed study, twelve came positive for AFB smears, out of which eight were pulmonary and four were extra pulmonary. Further the DNA from all the samples were isolated and subjected for N-PCR targeting IS6110 gene and MDRTB PCR targeting rpoB, kat G , mab A promotor for N-PCR IS6110 gene was amplified at 123 base pairs with 340 base pairs as IC (internal control) as shown in figure 1, it was seen that 25 cases which include 19 pulmonary and 6 extra pulmonary were TB PCR positive. The specimens were further subjected for MDR. TB PCR which will yield an amplicon of 315 b.p, 2223 b.p and 1362 b.p respectively for rpoB,katG and mabA promotor respectively. Only 06 cases yielded, an amplicon of 315 b.p. confirming the rpoB gene resistance for rifampicin drug. In any of the 06 positives none of the other resistance gene other than rpoB was amplified (Table 2).

Table 1. Primers used to detect for MDR study in the study.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer set (direction)</th>
<th>Nucleotide sequence</th>
<th>Positions (bp)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB</td>
<td>PR1(forward) PR2 (reverse)</td>
<td>5'-CCGCCGATCAAGGAGTTGCTTC-3' 5'-CCGCCGATCAAGGAGTTGCTTC-3'</td>
<td>1256 to 1275</td>
<td>315</td>
</tr>
<tr>
<td>katG</td>
<td>PR3(forward) PR4 (reverse)</td>
<td>5'-TGCCCACGACCAACCCCCATTACAGAAC-3' 5'-TCAGCGCACGTCGAACCTGTCGAG-3'</td>
<td>1-32</td>
<td>2,223</td>
</tr>
<tr>
<td>mabA promoter</td>
<td>PR5(forward) PR6 (reverse)</td>
<td>5'-ACATACCTGCTGCGCAATTCC-3' 5'-GCATACGAATACCGCGAGAT-3'</td>
<td>217 to 198</td>
<td>1,362</td>
</tr>
</tbody>
</table>

Table 2. Positivity rates for different diagnostics assays for tuberculosis.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Positive</th>
<th>Negative</th>
<th>Positivity percentage</th>
<th>Negative percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested TB PCR</td>
<td>25(60)</td>
<td>35(60)</td>
<td>41.7%</td>
<td>58.3%</td>
</tr>
<tr>
<td>AFB smear</td>
<td>12(60)</td>
<td>48(60)</td>
<td>20%</td>
<td>80%</td>
</tr>
<tr>
<td>MDR TB PCR</td>
<td>06(25)</td>
<td>06(25)</td>
<td>24%</td>
<td>76%</td>
</tr>
</tbody>
</table>

Discussion and conclusion

The resurgence of tuberculosis has been accompanied by high frequency of drug resistant strains from all over the world. In most TB patients drug resistance predominantly arises as a result of multiple interruptions of treatment [14]. To avoid these problems, fixed-dose combinations (FDCs) tablets are now recommended by WHO. However, in FDC formulations the bioavailability of the component drugs, and especially of rifampicin, may be reduced. Simple, rapid and inexpensive methods of detecting drug resistant tuberculosis are also essential for effective treatment. Multidrug-resistant tuberculosis (MDR-TB) caused by Mycobacterium tuberculosis resistant to both isoniazid and rifampicin with or without resistance to other drugs is among the most worrisome elements of the pandemic of antibiotic resistance. Globally, about three per cent of all newly diagnosed patients have MDR-TB. The proportion is higher in patients who have previously received anti tuberculosis treatment reflecting the failure of programmes designed to ensure complete cure of patients with tuberculosis. While host genetic factors may probably contribute, incomplete and inadequate treatment is the most important factor leading to the development of MDR-TB. The definitive diagnosis of MDR-TB is difficult in resource poor low income countries because of non-availability of reliable laboratory facilities. Efficiently run tuberculosis control programmes based on directly observed treatment, short-course (DOTS) policy is essential for preventing the emergence of MDR-TB [15,16]. Management of MDR-TB is a challenge which should be undertaken by experienced clinicians at centers equipped with reliable laboratory service for mycobacterial culture and in vitro sensitivity testing as it requires prolonged use of expensive second-line drugs with a significant potential for toxicity. Judicious use of drugs, supervised individualized treatment, focused clinical, radiological and bacteriological follow up, use of surgery at the appropriate juncture are key factors in the successful management of these patients. In conclusion among the 60 samples, 12 were positive for AF und TB PCR and out of them 6 for rpoB gene. Isolates resistant and partially resistant to rifampicin were found to be 24%. We standardized a multiplex In-house PCR based protocol for detection of Multidrug Resistance in M. tuberculosis from clinical specimens on molecular basis in our laboratory. The utility of multiplex PCR is that it consists of multiple primer sets within a single PCR reaction to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information will be gained from a single test run that otherwise would require several times the reagents and more time to perform. This method will detect the very less number of infectious mycobacteria present in clinical specimens and hence the treatment will be started accordingly immediate after the diagnosis and detection. In the proposed study, we targeted rpoB, katG and mabA promotor gene for the detection of Multidrug Resistance in M. tuberculosis in the clinical specimens. The
significance of the proposed study includes quick method, reduction in cost of test, use of DNA sequences for the detection of Multidrug Resistance in M. tuberculosis depends on the right choice of the target sequences. Disease will be diagnosed earlier and hence treatment would be started at the early infection [17].

Conflict of interest None

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REFERENCES


