MOLECULAR CHARACTERIZATION AND COMPARISON OF MULTI DRUG RESISTANT STRAINS OF MYCOBACTERIUM TUBERCULOSIS BY PHENOTYPIC AND GENOTYPIC METHOD

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INTRODUCTION

The complete genome of M. tuberculosis strain has been mapped as a length of about 4.4 Mb. However, each gene of M. tuberculosis may have separate function including the specific complex formation with important drugs being used as tuberculosis treatment regime. Unusual genetic alteration of bacterial genes leads to the development of drug resistance.

The KatG encodes catalase-peroxidase which is necessary to activate INH to a toxic substance in the bacterial cell.1 This toxic substance subsequently affects intracellular targets such as mycolic acid biosynthesis which eventually results in loss of cellular integrity and the bacteria die.2 Middlebrook et al. initially demonstrated that a loss of catalase activity can result in INH resistance.3 The majority of INH-resistant clinical isolates become resistant by losing or altering KatG activity, nevertheless, only KatG mutations do not account for all observed INH resistance, but mutations in another putative INH

ABSTRACT

Introduction: Drug resistant tuberculosis is a significant threat to tuberculosis control because only a few effective drugs are available against M. tuberculosis. The aims of this study were to compare multi drug resistant (MDR) strains of tuberculosis by phenotypic and genotypic method and determine type, location and frequency of rpoB and KatG gene mutations.

Methodology: Anti-tuberculosis drugs susceptibility test of M. tuberculosis grown on Lowenstein Jensen medium was performed by proportion method. MDR cases were analyzed for mutation of rpoB and KatG genes. The regions of these genes were amplified by polymerase chain reaction (PCR) and sequenced.

Results: Two different mutations were identified in rifampicin resistant strains. The most common point mutations were in codons TCG 531→TTG (85%) and GAC 516→TTC (15%) of the rpoB gene. Two different mutations in KatG gene were detected. The most common KatG point mutations were AGC 315 ACC (Ser→Thr) (85%) and CGG 463 CTG (Arg→Leu) (10%). In this study DNA sequencing analysis did not find mutation on KatG gene of one of the strain tested. Male and female were equally affected by MDR tuberculosis and majorities (35%) of them were found in 21-30 years age group.

Conclusion: The present investigation agrees that genetic mutation is responsible for change in phenotypic characteristics of M. tuberculosis.

Key words: Mycobacterium tuberculosis, Multi drug resistant, KatG, rpoB

INTRODUCTION

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The KatG encodes catalase-peroxidase which is necessary to activate INH to a toxic substance in the bacterial cell.1 This toxic substance subsequently affects intracellular targets such as mycolic acid biosynthesis which eventually results in loss of cellular integrity and the bacteria die.2 Middlebrook et al. initially demonstrated that a loss of catalase activity can result in INH resistance.3 The majority of INH-resistant clinical isolates become resistant by losing or altering KatG activity, nevertheless, only KatG mutations do not account for all observed INH resistance, but mutations in another putative INH

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target called inhA also. The Ser315Thr substitution is estimated to occur in 30–60% of INH resistant isolates. The KatG (CGG463CTG) (Arg-Leu) amino acid substitutions is the most common polymorphism found in the KatG gene and is not associated with INH resistance.

Rifampicin (RIF) binds to the β-subunit of DNA-dependent RNA polymerase hindering transcription and thereby killing the organism. Extensive studies on the rpoB gene in RIF resistant isolates of M. tuberculosis identified a variety of mutations and short deletions in the gene. A total of 69 single nucleotide changes; 3 insertions, 16 deletions and 38 multiple nucleotide changes have been reported. More than 95% of all missense mutations are located in a 81bp core region (Rifampicin resistance determining region) of the rpoB gene between codons 507–533 with the most common changes in codons Ser531Leu, His526Tyr and Asp516Val. These changes occur in more than 70% of RIF resistant isolates. Drug susceptibility testing (DST) of M. tuberculosis in clinical specimens is time-consuming. INH and RIF are crucial elements of the standard treatment regimen of tuberculosis, and resistance to these drugs requires extension of therapy. Proportion method is a widely used method, especially in resource-limited settings (RLS). It uses solid media, Lowenstein-Jensen to determine the proportion of resistant mutants to a given drug. Its turnaround time (TAT) is between 4-6 weeks. The vast majority of RIF resistance is caused by mutations located in the 81-bp region of the rpoB gene. INH resistances are more complex, as the mutations conferring resistance are located in several genes and loci. INH resistance has been associated mainly with mutations in KatG, inhA, ahpC, and kasA. Sequencing of PCR-amplified products of rpoB and KatG has become the most widely used genotypic method for detecting drug resistance in M. tuberculosis; it is accurate and reliable and it has become the reference standard for mutation detection. DNA sequencing has been widely used for characterizing mutations in the rpoB gene in RIF-resistant strains and to detect mutations responsible for resistance to other anti-tuberculosis drugs.

Aims of this study were to compare multi drug resistant cases of tuberculosis by phenotypic and genotypic method and determine types, location and frequency of mutation on rpoB and KatG.

**METHODOLOGY**

This study was carried out at the GENTUP Kathmandu and HNB Garhwal University during January 2008 to December 2008. Ethical approval was taken. Data on the MDR-TB patients were collected and recorded on a standardized form. The data were collected on age, gender and type of disease (new or old). This study included 9 primary and 11 acquired drug resistant cases. The research objectives and methods were explained to the patients and verbal consent obtained from them before the sputum samples were collected. The cases were selected using random sampling technique. Drug susceptibility test was performed on M. tuberculosis isolates by proportion method as standard protocol. M. tuberculosis strains were tested against four antibiotics used in DOTS program of Nepal such as isoniazid, rifampicin, ethambutol and streptomycin.

Twenty multi drug resistant strains of M. tuberculosis were screened for mutations of rpoB and KatG gene associated with resistance to rifampicin and isoniazid respectively by PCR-DNA sequencing method. Spin column method was used for extracting Mycobacterium tuberculosis DNA following the manufacturer instructions. The estimation of the extracted DNA from the samples was carried out using spectrophotometry method. Twenty 210-bp and 750-bp segment of the katG gene and 411-bp fragments of the rpoB gene, containing the sequence of the 157-bp fragment were amplified by standard polymerase chain reaction (PCR). PCR amplified products were sequenced directly on an Applied Bio-systems ABI Prism 3100-Avant automated DNA sequencer. Sequencing was done with big dye terminator cycle sequencing kit from ABI, following the manufacturer’s instruction.
RESULTS

Age wise distribution of MDR cases is shown in figure 1. Majority, 7 (35%) of the MDR cases of tuberculosis were found in 21-30 years age group. Similarly gender wise distribution of MDR cases of tuberculosis were found equal in both the gender. Among twenty strains of MDR M. tuberculosis 15% and 40% were sensitive to streptomycin and ethambutol respectively. PCR-DNA sequencing results of MDR strains found 100% and 95% mutation in rpoB and KatG respectively and they correlated well with the phenotypic method (Table 1).

![Figure 1. Age wise distribution of MDR strains](image)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Age</th>
<th>Sex</th>
<th>rpoB gene</th>
<th>KatG gene</th>
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<tr>
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<td>S 315 T</td>
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<tr>
<td>5</td>
<td>15</td>
<td>F</td>
<td>GAC516 TTC</td>
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<td>Old</td>
</tr>
<tr>
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<tr>
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DISCUSSION

The mycobacterium uses various mechanisms to evade killing by drugs, including mutations in genes that code for drug target proteins, a complex cell wall which blocks drug entry, and membrane proteins that act as drug efflux pumps.24, 25 Along with HIV/AIDS, MDR-TB is the most important threat to TB control. Countries with a high MDR-TB prevalence generally have a history of poor TB control. There are both preventive and restorative strategies to combat resistance –DOTS and DOTS-Plus. The major barrier to MDR-TB treatment is the high cost of second line drugs which are at least 300 times more expensive than first line drugs based on Green Light Committee (GLC) prices and between 1000-3000 times more expensive when market prices are used. Additional barriers include extensive laboratory requirements to conduct culture and drug susceptibility testing (DST), severe adverse events associated with second-line drugs and fear of development of resistance to second line drugs. The misuse of second line drugs could lead to the creation of TB strains resistant to all known anti-TB drugs.26

Twenty MDR cases of M. tuberculosis were included in this study. Two loci associated with drug resistance were selected for characterization viz., rpoB (RIF) and KatG (INH). Two different mutations were identified in rifampicin resistant M. tuberculosis strains. The most common point mutations were in codons 531 (85%) followed by 516 (15%) of the rpoB respectively. Similar study conducted by Sajduda et al. (2004)27 in Poland showed that nineteen different mutations were identified in 64 rifampicin resistant M. tuberculosis strains. The most common point mutations were in codons 531 (41%), 516 (15%), and 526 (9%) of the rpoB gene. These findings are in agreement with those reported by Spindola de Miranda et al. (2001)28 the later showed that among rifampicin resistant strains a double point mutation which had not been reported before was detected in one strain from France. The mutations were found in codons 531 (31.2%), 526, 513 and 533 (18.7% each). In Brazilian strains the most common mutations were in codons 531 (72.2%), 526 (11.1%) and 513 (5.5%). The heterogeneity
found in French strains may be related to the fact that most of those strains were from African or Asian patients.

PCR-DNA sequencing of isoniazid resistant *M. tuberculosis* strains identified two different types of mutations in KatG. The most common point mutations were in codons 315 (85%), AGC→ACC (Ser→Thr) followed by 463 (10%) CGG→CTG (Arg→Leu) of the KatG. Mutation were not found in 1 (5%) in KatG of the isolate. Similar study conducted in Bostanabad et al. (2008) showed that most mutations were in KatG gene codons 315, 316 and 309. Four types of mutations were identified in codon 315: AGC→ACC (85%), AGC→AGG (2.3%), AGC→AAC (4.7%), AGC→GGC (2.3%). The highest frequency of mutations sharing between primary and secondary infection was found in codon 315. Another similar study conducted by Sajduda et al. (2004) in Poland showed that six different mutations in the KatG gene of 83 resistant strains were detected. Fifty-seven (69%) isolates exhibited nucleotide substitutions at codon 315. The majority of hot mutations in *katG* gene of *M. tuberculosis* have been reported in codon 315 (Ser→Thr) and less in other codons. Most reports suggest that resistance of *M. tuberculosis* to isoniazid mostly corresponds to changes in codon 315.

In this study sequencing analysis did not find mutation on KatG gene of one (5%) of the strain tested, although that strain was resistant to isoniazid as determined by the proportion method. Other study revealed that a mutation associated with isoniazid resistance can also be located outside the KatG gene such as *inhA* gene, *kasA* gene, *ndh* gene and *ahpC* gene. Although this does not occur so frequently. In this case mutation may present to other genes. Other possibilities are that in this resistant strain other mechanism of resistance may be involved.

**CONCLUSION**

PCR-DNA sequencing shows MDR strains of *M. tuberculosis* isolates has mutation on *ropB* gene and 95% strains had mutation on KatG gene. The most common point mutations were found in codons 531 (85%) of the *ropB* gene and codons 315 (85%) of the KatG gene. This study showed that genetic changes in the *ropB* and KatG genes were more consistently associated with resistance phenotype.

**REFERENCES**


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