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RELATIONSHIP BETWEEN RPOB MUTATIONS AND MINIMUM INHIBITORY CONCENTRATIONS OF RIFAMPICIN IN MULTI DRUG RESISTANT STRAINS OF MYCOBACTERIUM TUBERCULOSIS

Shashikant Vaidya
Department of Clinical Pathology, Haffkine Institute for Training, Research and Testing, Mumbai, India
shashikantvaidya@hotmail.com

Jinesh Maniar
Department of Clinical Pathology, Haffkine Institute for Training, Research and Testing, Mumbai, India
ginesh51@gmail.com

Shreyasi Muley
Hafting Bio-pharmaceutical Corporation Ltd. Mumbai, India
shreyasi.mulye@gmail.com

Geeta Koppikar
Breach Candy HospitalTrust, Mumbai, India
info@breachcandyhospital.org

Mohan Kulkarni
T.N. Medical College and B.Y.L. Nair Charitable Hospital, Mumbai, India

Abhay Chowdhary
Grant Government Medical College and Sir J.J. Hospital, Mumbai, India
abhaychowdhary@yahoo.com

Abstract

Tuberculosis is an infectious disease caused by obligate intracellular bacteria Mycobacterium tuberculosis, which has created a health problem worldwide. Incidence of Multi-drug resistance tuberculosis creates the scenario dangerous, which is resistant to both the first line drugs namely Rifampicin and ionized. Mutation in rpoB and inhA confers resistance to rifampicin and ionized respectively. In the present study we studied the relationship between rpoB mutations and minimum inhibitory concentrations of Rifampicin in Multi drug resistant (MDR) strains of Mycobacterium tuberculosis. Total 20 MDR strains were selected for the study. The mutations in the rpoB gene were identified by sequencing the PCR products. The minimum inhibitory concentrations of Rifampicin, was determined by Absolute Concentration method. Mutations conferring resistance to Rifampicin in Mycobacterium tuberculosis occurs in the defined region of 81 base pair of rpoB gene. The most frequent mutation found in Rifampicin resistant strain is at position 532, 526 and 516. Mutation occurring at this position results in high level of Rifampicin resistance. Mutation at 511, 518 and 522 is associated with low level of Rifampicin resistance.

Keywords

ROPB, Rifampicin, Multi Drug Resistancem, Tuberculosis

1. Introduction

Tuberculosis (TB) remains a major health problem in developing nations. Here TB infections are responsible for one in four avoidable adult deaths (Cornwall, 1997).In India about 2 million cases occur every year. In India one person dies every minute due to TB and two persons become sputum positive cases. Prevalence of TB in India is fairly high. About 40% of population is infected and from this pool, cases with clinically active disease continue to develop all the time. The incidence of TB is a function of the extent of infection in the community. This renders the provision of permanent diagnosis and treatment facilities as an absolute necessity. The most significant emergence has been that of the multi-drug resistant (MDR) strains, which is resistant to Ionized (INH) and Rifampicin (RF) with or without being resistant to other drugs

(Rosha & Kataria, 2001). Detection of Multi Drug resistant (MDR) TB strain would not only eliminate non-essential use of antibiotics, but would also help in selection of the most effective drug regimen and guide therapy in chronic cases. Conventional culture methods using egg- or agar- based Medicare still the most utilized ones in many countries. Although the long turnaround time of drug susceptibility testing (DST) results displeases physicians for the purpose of case management, it is suitable for Drug Resistance Surveillance.

Molecular diagnostic technology allows rapid detection of Mycobacterium tuberculosis (M. tuberculosis) in clinical specimens (Bodmer et al., 1995).Also method using a readymade DNA probe reduces the time required for detecting drug resistant M. tuberculosis. This method is based on the culture of the microorganisms (Kawa et al., 1989).There are numerous reports on molecular techniques to detect gene mutations related to resistance, including hybridization of amplified gene segments or other PCR-based method. However, not all resistance-related genes for the different ant tuberculosis drugs and their sites of mutation have been found, except for rpoB gene mutations, which lead to RF resistance which encodes beta subunit of RNA polymerase (Mitchison, 2005).More than 95% of these mutations occur on an 81-bp fragment of the gene between bases 1276 and 1356(codon 432-458 in the rpoB gene of M. tuberculosis and codon 507-534 in the Escherichia colirpoB gene. This region is known as the RF resistance determining region (RRDR), or hotspots and is used as a target for direct sequencing and commercial line probe assays (Jenkins et al., 2005). The rapid detection of RF resistance is of particular importance, since it is a key element in the treatment of TB and it also represents a valuable surrogate marker for MDR, which is a tremendous obstacle to TB therapy. (Heep et al., 2001)

The nature and frequency of mutations in the rpoB gene of RF resistant clinical isolate of M. tuberculosis varies considerably according to the geographical location (Ahmad et al., 2000).Few reports have described the relationship between the Minimum Inhibitory Concentrations (MIC) of antituberculosis agents and genetic alterations. Although DNA sequencing method has not been used routinely for detecting resistance of M. tuberculosis to ant tuberculosis drugs, it is suggested for the investigation of gene mutations causing resistance (Bodmer et al., 1995). There is very scanty information available on the specific mutations patterns and its detection in the M. tuberculosis strains isolated from our country. In this study, we examined the relationship between MIC's of RF and alterations in the rpoB gene of M.

tuberculosis strains in order to elucidate the exact alterations or amino acid substitutions producing the resistant phenotype.

2. Materials and Methods

2.1 Mycobacterium Strains

Total 102 Clinical isolates of M. tuberculosis were collected from Department of Microbiology of P.D. Hinduja Hospital and Medical Research centre. All the strains were grown in Sterile Lowenstein Jensen Medium (LJM) slants with 2% glycerol (HiMedia Laboratories Pvt. Ltd., India). The clinical isolates were defined as M. tuberculosis according to their biochemical characteristics. Drug susceptibility testing of 102 M. tuberculosis isolates was done by using BACTEC 460TB system. (S. Siddiqi et al., 1981)

2.2 Determination of MIC of RF against MDR strains by Absolute Concentration method

The MIC of RF (Lupin Pharmaceuticals, India) against 40 MDRM. tuberculosis strains was determined by Absolute Concentration method (Canetti et al., 1963).Concentration of RF used for the experiment were 1µg/ml, 2µg/ml, 4µg/ml, 8µg/ml, 16µg/ml, 32µg/ml, 40µg/ml, 64µg/ml, 128µg/ml and 256µg/ml. Inoculated slants were incubated for 4 weeks at 37º C. Growth was defined as 20 colonies or more.

2.3 Extraction of DNA from MDR strains of M. tuberculosis

Out of the 40 MDR strains detected 20 strains were processed for molecular characterization. 20 MDR M. tuberculosis cultures were inoculated in Sterile Dubos broth with glucose& albumin supplement with 0.05% tween80 (HiMediaPvt. Ltd, India) and incubated at 37ºC for 14 days with intermittent shaking. A uniform cell suspension of 108cells/ml of test culture was prepared in saline using McFarland standard number 1. 100 µl of the cell suspension was added in a sterile micro-centrifuge tube to which 150 µl of lysis buffer Proteinase K (Sigma Co. USA) was dissolved in 100mM Tris-HCL, pH 8.5 to obtain a final concentration of 10 mg/ml, To make lysis buffer 1ml of Proteins' K solution was added to 1ml of 0.05% Twine 20 and 8ml distilled water was finally added was added. The suspension in lyses' buffer was overlaid with two drops of mineral oil. The micro centrifuge tubes (Labor Co.) were placed in floats and kept in shaking water bath at 60ºC for 18 hours, overnight. After incubation for 18hrs, the leases were incubated at 95ºC for 15mins to terminate the activity of Protein's K present in

lysisbuffer. The mixture was then centrifuge at 12000×g and the supernatant was used as sample for PCR (Van Embden et al., 1993).

2.4 Polymerase Chain Reaction of rpoB gene

The primers used for amplification of the rpoB gene were, forward primer: TCG CCG CGA TCA AGG AGT TCT TCG GC and reverse primer: TGC ACG TCG CGG ACC TCC AGC CCG GCA C. (Gene Bank Accession No: BX 842574, Sigma, & HPLC purified). The PCR was performed in 50 µl of reaction tube which contains 25 µl of 2X PCR Master Mix (Gene's et al), 50ng of each primer and 2 µl of extracted DNA. PCR amplification was performed using following protocol in thermal cycler(HybaidOmn-E.): single cycle of initial denaturation at 95º C for 10 minutes, followed by 40 cycles of Denaturation at 94ºC for 1 minute Annealing at 72ºC for 2 minutes Elongation at 72ºC for 2 minutes and then single cycle of final extension at 72ºC for 7 minutes. The size and the amount of amplified DNA were verified by electrophoresis on 2% (w/v) agarose gel (Sigma Co.) containing ethidium bromide. Documentation of the gel was done on alpha imager gel documentation system (Van Embden et al., 1993).

2.5 Purification and sequencing of amplified DNA samples of MDR strains

Purification of amplified DNA samples from 20 MDR strains was done using Genes Quick Purification Kit. Briefly, 100 µl of binding buffer was added to 20µl of sample. The sample was vortexed thoroughly; the sample was loaded onto a spin column and centrifuged for 1 minute at 12,000rpm in a micro centrifuge. Spin column was placed back in the collection tube to which 500 µl of Wash buffer I was added and was centrifuged at 12,000 rpm for 1 minute. Then 700 µl of Wash buffer II was loaded on to spun column placed in the collection tube and incubated at RT for 5 minutes, the column was centrifuged at 12000 for 1 minute. After every centrifugation step the flow through was discarded and collection tube was used. In the last step 50 µl of elution buffer was added on the centre of the membrane and incubated at room temperature for 5 minutes. Spin column was centrifuged at 12,000 rpm for 1 minute to elude the DNA. Quantization of DNA was done spectrophotometrically (Schimadzu Co.) by measuring the ratio of absorbance at 260:280. Ratio at 260:280nm indicates the purity of DNA. The automated single pass sequencing of PCR product after purification was done in an Automated DNA sequence at Bangalore Genes using ABI Prism Automated DNA sequence. The sequence of the amplicon obtained was compared to published sequence of rpoB gene for H37Rv (GenBank

accession BX842574) to identify RF resistance in terms of any mutations, insertions, and deletions in Revamping Resistance Determining Region (RRDR) (codons 507 to 533). The nucleotide and amino acid numbering was based on homologous mutations in E. coli(Genei, 2005).

3. Results

Table 3.1: *MIC of RFagainst clinical isolates of MDR M. tuberculosis*

Sr.No	Clinical Isolate	MIC (µg/ml)
1	MDR-1	128
2	MDR-2	> 256
3	MDR-5	256
4	MDR-9	256
5	MDR-10	256
6	MDR-15	> 256
7	MDR-20	64
8	MDR-21	64
9	MDR-22	128
10	MDR-24	64
11	MDR-25	64
12	MDR-26	64
13	MDR- 27	128
14	MDR-28	64
15	MDR -29	256
16	MDR -30	256
17	MDR-31	256
18	MDR-32	128
19	MDR-33	64
20	MDR-37	> 256

Lowest MIC observed was 64µg/ml and highest was more 256 µg/ml MDR M. tuberculosis isolates. [Figure 3.1]

Table 3.2: *Mutation present in MDR TB isolates and its subsequent amino acid alteration with co-relation with RF MIC*

Sr. No.	**Clinical Isolate**	**Gene**	**Codon No.**	**Mutation**	**Amino acid Exchanges**	**MIC (µg/ml)**
1	MDR-1	*RpoB*	531	TCG→TTG	Ser →Leu	128
2	MDR-2	*RpoB*	531	TCG →TTG	Ser → Leu	> 256
3	MDR-5	*RpoB*	531	TCG→TTG	Ser→ Leu	256
4	MDR-9	*RpoB*	531	TCG→TTG	Ser → Leu	256
5	MDR-10	*RpoB*	526 531	CAC→TAC TCG → TTG	His →Tyr Ser→ Leu	256
6	MDR-15	*RpoB*	516 531	GAC→GTC TCG→TTG	Asp → Val Ser→ Leu	> 256
7	MDR-20	*RpoB*	516	GAC →GTC	Asp →Val	64
8	MDR-21	*RpoB*	526 516	GAC →GTC GAC →GTC	His → Tyr Asp →Val	64
9	MDR-22	*RpoB*	526	CAC →TAC	His →Tyr	128
10	MDR-24	*RpoB*	526	CAC →TAC	His →Tyr	64
11	MDR-25	*RpoB*	516 526	GAC→GTC CAC→TAC	Asp → Val His →Tyr	64
12	MDR-26	*RpoB*	516	GAC→GTC	Asp → Val	64
13	MDR -27	*RpoB*	531	TCG →TTG	Ser → Leu	128

14	MDR-28	*RpoB*	526	CAC→TAC	His → Tyr	64
15	MDR- 29	*RpoB*	531	TCG →TTG	Ser → Leu	256
16	MDR-30	*RpoB*	531	TCG →TTG	Ser → Leu	256
17	MDR-31	*RpoB*	531	TCG→TTG	Ser → Leu	256
18	MDR-32	*RpoB*	526	CAC→TAC	His →Tyr	128
19	MDR-33	*RpoB*	516	GAC→GTC	Asp → Val	64
20	MDR-37	*RpoB*	531 526	TCG→TTG CAC→TAG	Ser → Leu Asp → Val	□ 256

In our study mutations were detected at codon 516, 526 and 531, with frequencies of 30%, 40%, and 55%, respectively. MDR strains contained missense mutations that led to amino acid substitutions at the Ser-531, His-526, and Asp-516 residues. 15 isolates had single mutations. 8 isolates showed a mutation at codon 531, 4 at codon 526 and 3 at codon 516 out of these 15 isolates. While 5 isolates had mutations at 2 codons, Out of five, two isolates: (MDR 21) and (MDR25) showed mutations at codon 526 (GAC →GTC, His → Tyr) and at codon 516 (GAC →GTC, Asp →Val). Two isolates (MDR 37) and (MDR10) showed mutations at codon 526 (GAC →GTC, His → Tyr) and codon 531 (TCG →TTG, Ser → Leu. One isolate: (MDR 15), showed point mutation at codon 531 (GAC →GTC, His → Tyr) and codon 516(GAC →GTC, Asp →Val). The MDR 9, showed mutation at amino acid 531 just like other samples (ser 531 Leu) and also it had an insertion of a codon for Histidine (CAC) between amino acids 520 and 521.[Figure 3.2]

4. Discussion

Drug resistance in TB has become a major problem in the treatment and also in containment of TB epidemic. Drug resistance in M.tuberculosis in not mediated by mobile genetic materials like transposons and plasmids. The drug resistance arises by spontaneous mutation in its genome. Another main reason for the development of drug resistance is irregular treatment. INH and RF is the first line drug used for treatment of tuberculosis. Both the drugs are very effective in case of TB.

RF is an important first line drug for the treatment of TB. MICs of RF against TB ranges from 0.05 to 1μg/ml on solid or liquid media, but the MIC is higher in egg media (MIC = 2.5–

10μg/ml). Strains with MICs <1μg/ml in liquid or agar medium or MICs <40 μg/ml in Lowenstein-Jensen medium are considered RF-susceptible. RF interferes with RNA synthesis by binding to the β subunit of the RNA polymerase. The RNA polymerase is an oligomer consisting of a core enzyme formed by four chains α2ββ′ in association with the σ subunit to in order to initiate transcription from promoters. The RF binding site is located upstream of the catalytic centre and physically blocks the elongation of the RNA chain.RF is active against both growing and stationary phase bacilli. Mutation conferring resistance to RF in M. tuberculosis occurs in the defined region of 81 base pair of rpoB gene. The most frequent mutation found in Rifampicin resistant strain is at position 532, 526 and 516. Mutation occurring at this position results in high level of RF resistance. Mutation at 511, 518 and 522 is associated with low level of RF resistance.

In M.tuberculosis, resistance to RF occurs at the frequency of 10-7 to 10-8. Understanding the relationship between the mutation conferring resistance to RF and the minimum inhibitory concentration of RF against various M.tuberculosis strains possessing varied mutation is necessary to counter the drug resistance and for development of drugs against such resistance strains. In the present study we have compared the MIC against RF of various RF resistance M.tuberculosis strains with the specific mutation conferring resistance to Rifampicin.

In the present study we tested 20 MDR strains of M. tuberculosis to check the mutation in rpoB gene. We could detect mutations in all the 20 samples in the RRDR region. Point mutations were observed in an 81-nucleotide core region of the rpoB gene in the MDR strains of M. tuberculosis strains. Our findings of mutations in the progenies were comparable to those reported for strains from other parts of the world, especially the common mutations, which reflect a global pattern (Ramaswamy & Musser, 1998; Siddiqi et al., 1998). RF resistance is often regarded as an excellent surrogate marker for MDR tuberculosis and our study corroborates this hypothesis.

The rpoBcodons 531, 526, and 516 are the most frequently mutated codons worldwide, the mutation frequency of codon 531 (rpoB) was similar to that reported earlier (Ramaswamy & Musser, 1998; Siddiqui, 1981). The rpoB 526 codon mutations have also been reported in other similar studies (Valim et al., 2000, Mani et al., 2001, Jamieson et al., 2014). Mutations at codon 526 were found to be residue dependent in one of the study (Jamieson et al., 2014; Anna et al., 2009). The replacement of histidine with tyrosine or aspartic acid results in high levels of RF

resistance, but the replacement of the histidine residue with leucine results in low levels of RF resistance or susceptibility (Jamieson et al.,2014). There are variations in the relative frequencies of mutations in these codons have been described for M. tuberculosis isolates from different geographic locations.

Mutations in codon 516,526 and 531 predominate in most of the studies. Investigations have revealed that mutations in codons 516, 526, and 531 of the progeny are often implicated. Mutations at codon 531 occur at a relatively higher frequency, but different groups have reported disproportionate frequencies of mutations at these codons. Certain strains may be prone to develop specific mutations.

A direct correlation of mutations with MIC's was observed. Mutations at codon 526 and 531 conferred high-level resistance (MIC's $\geq$ 128μg/ml). Mutation at codon 516 conferred low-level resistance to RF. As reported by previous investigators (Telenti et al., 1993, Jamieson et al., 2014). A definite correlation was found between MIC's and type of mutation in the isolate. These results were comparable to earlier findings reported by Siddiqi (Siddiqi et al., 1998).

Thus degree of resistance exhibited by an isolate is related to the type of mutation in the rpoB locus. Thus this study provides data on different kinds of mutations occurring at various loci. A relationship between susceptibility to RF and alterations in rpoB gene is observed in 20 strains of MDR M. tuberculosis. However, relationship between gene alteration and drug-resistant phenotype is still unclear, further analysis of the relationship between MICs and gene alteration is necessary with more number of test strains.

5. Conclusion

In our study the mutation in 531, 526, and 516 codon were detected which is similar with the mutation pattern observed in other geographical location. The mutation in codon 526 results in high levels of MIC values and it is generally residue dependent. MIC pattern of MDRTB strains possessing mutation in codon 531 is less than mutation in codon 526. The mutation in codon 513 shows least MIC pattern and thus it confers low- level resistance. Mutation in codon 526 and 531 is distributed worldwide irrespective of geographical location, thus they are considered as mutational hotspots.

Mutation in ropy gene doesn't only confer resistance to RF but it is also related to resistance and susceptibility to Rifampicin. The proportion of RF resistance and RF susceptible

isolates is 13%-26%. The codon 526 and 531 mutation harbouring strains have found to be susceptible to RF. The mutation in codon 516 confers low level resistance to RF in vitro but is also necessary to evaluate its resistance to RF in vivo.

The primers used in the study amplified the region the rpoB gene which includes codon 516, 526, 531 which are the major mutational hotspot conferring resistance to RF and these hotspots are distributed worldwide, hence this primer can be used in laboratory to detecting the rpoB gene mutation by amplifying the gene using this primer and then subsequent sequencing of amplified product.

Mutation can that may be conferring high or low level resistance to rifampicin can be detected by using this method. This will help in organizing the specific treatment protocol for disease caused by that particular strain. In case of rifampicin mono resistance MDRTB which is caused by mutation conferring low level resistance, high dosage of rifampicin can be administered but this treatment procedure need to be properly evaluated in vivo. MDRTB cases detected in this study was 40 and out of 40 MDRTB strains only 20 strains were used in the study which is relatively less number of samples. This study is further carried out using more number of strains which are showing mono-resistance and strains that are resistance to both the first line drugs which will help in comparing the results between these studies.

In summary, we conducted the study to establish the relationship between various rpoB mutations and its respective MIC level. The study detected a strong relationship between various mutations in rpoB gene and its respective MIC levels. The study also identified the mutations which are present in M.tuberculosis strains circulating in Mumbai. The study provided the brief idea about the mutation which confers high and low level of resistance in M.tuberculosis.

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