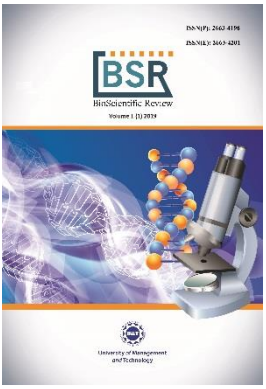

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Research Article

Molecular Characterization of Rifampicin Associated Mutations in *Mycobacterium tuberculosis* Isolates

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Abstract

Mycobacterium tuberculosis (MTB*)* is among the most lethal pathogens causing infection in 1.8 billion people, annually. *M. tuberculosis* causes pulmonary infection which spreads easily through the air. Rifampicin is a first-line drug used for the treatment of this disease. By binding with the 30S ribosomal subunit, it inhibits the protein synthesis. The misuse of this drug and incomplete treatment causes alteration at the genetic level of the *rpo*B gene of the MTB cells. The aim of our study was to find the mutations in the *rpo*B gene obtained from the clinical isolates of tuberculosis (TB) patients. We collected 412 sputum samples from the patients suspected of being infected with TB and cultured these samples in the microbiology laboratory of THQ Fatehpur, Layyah. Positive sputum cultures were analyzed for the drug susceptibility test. Genomic DNA was isolated through the sonication method using 20 Hz frequency of ultrasonic waves. Afterwards, primers were designed using bioinformatic tools for the amplification of the gene. Additionally, *rpo*B was amplified using a hemi-nested PCR technique. A total of 91 samples (54 males and 37 females) were positive for the sputum culture. Out of the 91 positive cultures, four samples showed rifampicin resistance. Two samples carried single missense mutation at positions 526 and 531 in the amino acid sequence of the *rpo*B gene, whereas two more carried a single missense mutation at position 516 in the amino acid sequence of the *rpo*B gene. Moreover, 22% of the tested sputum samples were positive for TB. This means that in the population of Tehsil Fatehpur, District Layyah TB is prevalent. Furthermore, 4.3% of the positive samples were found to be rifampicin resistant. In future researches, the harmful effect of the *rpo*B gene mutations associated with the function of the β-subunit of RNA polymerase in 16S rRNA and its interaction with rifampicin can be estimated by performing molecular docking.

1. Introduction

Mycobacterium tuberculosis (MTB) is the infectious pathogenic agent causing tuberculosis (TB) around the globe. Worldwide, 1.8 billion people are infected by this disease which is approximately equal to one-third of the world population. TB is the foremost cause of deaths globally and it spreads rapidly [1]. When an individual having pulmonary or laryngeal TB sneezes, coughs, laughs and shouts, tiny infectious water droplets are released into the air which infect other healthy individuals. This airborne pathogenic disease is primarily a pulmonary disease but may also affect other organs of the body [2].

The higher concentration of lipid in MTB cells makes them different from other bacteria and resistant to many antibiotics, gives them impermeability to stains and dyes, resistance to osmotic lysis via complement deposition, resistance to toxic oxidations and subsistence inside the macrophages [3]. In the developing countries of the world, malnutrition and TB are the most important problems. Indeed, malnutrition is the predisposed factor which leads a person to secondary immunodeficiency that raises the host's predisposition to the disease. Individuals with TB have a reduced appetite, micronutrient malabsorption, nutrient malabsorption and altered metabolism leading to metabolic wasting syndrome [4]. Another major risk factor for acquiring this disease is the excessive consumption of alcohol [5].

Clinical identification and diagnosis of TB includes the tuberculin or monteux test, sputum culture, chest X-ray and detection by Ziehl-Neelsen method[3]. Sometimes, administering a single drug to the patient leads to the development of resistance in the bacterial population to that drug. The current treatment of TB includes multiple drugs to which organisms are vulnerable. Administering multiple drugs simultaneously helps in preventing the development of resistance to other drugs. At least 6 to 9 months are required to cure drug-susceptible TB using rifampicin-based treatment [6].

Additionally, at the start of the therapy when the *in vitro* predisposition of an individual's isolate is unknown, choosing two or more drugs to which the patient's isolate is likely to be susceptible may be tough. So, the improper selection of drugs also leads to the development of drug-resistant *M. tuberculosis* [7]. Mismanagement and misuse of drugs against TB causes resistance in organisms [8], for instance when a patient does not complete their course of treatment, uses poor quality drugs, and suffer from the shortage of drugs and when the doctor prescribes the wrong treatment or duration of time for taking medicines [9].

TB can take the form of multidrug-resistant tuberculosis (MDR TB). In this form, MTB is resistant to at least two anti-TB drugs, that is, isoniazid and rifampicin. These drugs are considered as first-line drugs. Another rare type of MDR TB is extensively drug-resistant tuberculosis (XDR TB). This form of TB is resistant to isoniazid and rifampicin as well as to any type of fluroquinolone and also resistant at least to one of the second-line drugs, such as amikacin, kanamycin and capreomycin [10, 11]. Individuals having immune-deficiency like HIV [12] are at a higher risk for developing MDR TB and XDR TB and may die from the disease [13]. Mortality rate reached 71% within one year from MDR TB and XDR TB in patients having HIV [14].

First-line anti-TB medicine rifampicin is among the most effective drugs against it. Rifampicin binds to the beta-subunit of RNA polymerase and blocks transcription and RNA elongation. About 90% of clinical isolates which are rifampicin

Table 1. *rpoB* Gene Mutations in *M. tuberculosis* of Multi-Drug Resistant Samples

Mutation	Polymorphism (wild/mutant)	Codon position	Amino acid (wild/mutant)	Molecular detection method	References
1	GAC/TAC	516	Asp/Tyr	Sequencing	[19]
2	TCG/TTG	531	Ser/Leu	Sequencing	[19]
3	CAC/AAC	526	His/Gly	Sequencing	[19]
4.	GAC/TAC	516	Asp/Tyr	Sequencing	[19]

resistant are also resistant to isoniazid. Genotypic and phenotypic methods are used to detect resistance against rifampicin. About 95% of rifampicin resistance in patients is because of genetic alteration located at the center of the *rpo*B gene, shown by 81bp of rifampicin resistance-determining region (RRDR) [15, 16]. If we target a single amplicon using PCR, it is possible to test for both *M. tuberculosis* and rifampicin at the same time [17]. The *rpo*B gene is a crucial drug resistant gene and molecular target for the detection of TB. DNA fragment of *rpo*B of *M. tuberculosis* and its promoters were amplified. Similar condition was maintained for both the amplification and sequencing of *rpo*B gene [18]. In the sequence of mutation occurrence and genetic changes, *M. tuberculosis* carries a single mutation at 516, 526 and 531 positions in the *rpo*B gene as shown below in Table 1[19].

2. Material and Method

2.1 Study Design and Duration

This experimental study was designed for the identification of TB and of rifampicin mutation in its suspected patients at Tehsil Headquarter Hospital Fatehpur, Punjab, Pakistan. It was carried out at the University Institute of Medical Laboratory Technology (UIMLT), University of Lahore and accomplished at Tehsil Headquarter Hospital Fatehpur, Punjab, Pakistan. The current study took 3 months for completion and was conducted from October 2019 to December 2019.

2.2 Sample Size and Collection

Samples were collected from THQ Hospital Fatehpur, Layyah from patients who were suspected of having TB. Samples were cultured in the THQ microbiology laboratory in the biosafety cabinet and were further analyzed for their molecular characterization at the research laboratory of the University of Lahore.

These sputum samples were collected in sterile airtight containers. The collected specimen were labelled with patient name, sex, lab number, date, age, and with other useful information.

2.3 Culture and Microscopy of Smear

Sputum samples were cultured on a commercially prepared Lowenstein Jensen (LJ) media for 6 weeks. Positive samples were further analyzed for drug susceptibility tests.

For microscopic detection, the method previously used by Rasol et al. was used with little modification. The smear of the specimen was fixed either by heating or by alcohol fixation on a glass slide and staining was performed with methylene blue. Acid-fast bacillus may appear as pink colouredrod-shaped bacteria whereas the background may stain blue due to methylene blue [20].

2.4 Drugs Susceptibility Test

Sputum samples with positive culture were analyzed for rifampicin resistance by performing drug susceptibility tests [21].

2.5 DNA Isolation

To isolate the DNA by disrupting cells, a sonication method was used. In the sonication procedure known as 8Ml sonication buffer, 50 mM Tris, pH 7.5, and 10 mM EDTA were used with two times centrifugation for 20 mins, one at 2000 × g and other at 2500 × g. The DNA extracted was 50µL [22].

2.6 Hemi-Nested PCR

Mutations in the *rpo*B gene were detected by HN-RT PCR [23]. In this technique, an amplified product is detected with the help of a fluorescent dye. For RNA isolation, master mix was added into the PCR tubes and then these tubes were placed in the thermal cycler for amplification. All chemicals and reagents used for PCR are shown in Table 2. HN-RT PCR program was set with one cycle of polymerase activation at 95°Cfor 5 minutes. It was followed by 30 cycles with denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute and final extension at 72°C for 10 minutes. For gene amplification, we used OligoCalc and a specifically designed primer 3 software to design the forward and reverse primer. The forward primer 3' CGATCACACCGCAGACGTTGA and the reverse primer 5' GCCACGCTCACGTGACAGACC 3' were used for amplification.

2.7 Statistical Analysis

Data was recorded and compiled in Microsoft Excel. The results were analyzed using the SPSS software (version 22). Frequencies were calculated and the association between gender and age was recorded.

3. Results

Strains of drug resistant *M. tuberculosis* (MTB) were grown for 6 weeks using Lowenstein Jensen media under normal conditions in THQ Fatehpur.These strains comprised brown granular colonies. Out of 412 tested sputum samples, only 91(22%) samples tested positive for *M. tuberculosis.* Out of the total 91 patients tested positive for TB, 54 were male and 37 were female (Figure 1). Only 4 samples were rifampicin resistant, 2 of them were collected from males and 2 from females (Figure 2). The age wise distribution of all the suspected patients is shown in Figure 3.

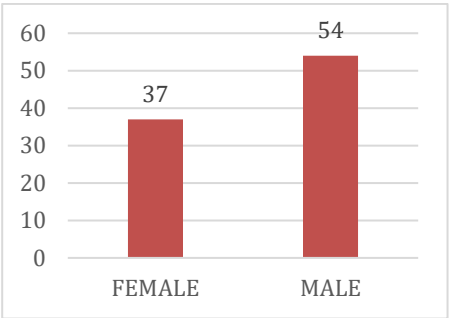


Figure 1. Gender wise distribution of TB in 91 patients. There were 54 male and 37 female patients

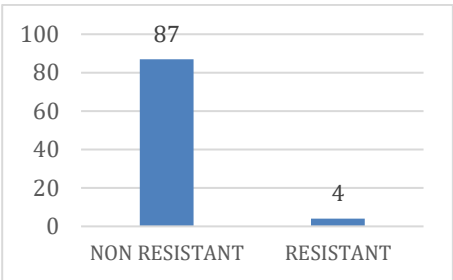


Figure 2. The number of rifampicin resistant and non-resistant patients. Out of the total 91 TB positive samples, only 4 samples showed resistance to rifampicin

The gene sequences of probes A, B, C, D, E are as follows:

Probe A:
GCACCAGCCAGCTGA
Probe B:
CTCGGTTAAGTACCTGGTCT
Probe C:
AACAACCCGCTGTCGG

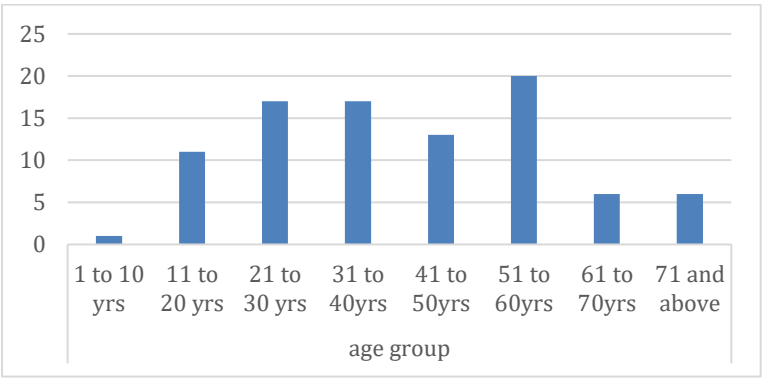
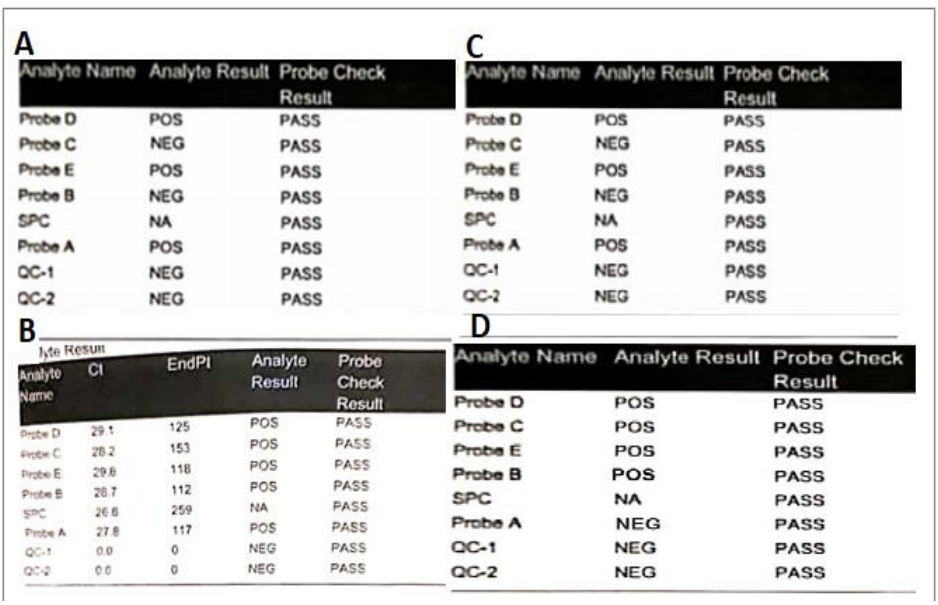


Figure 3. Age wise distribution of patients tested for *M. tuberculosis*



A

Analyte Name	Analyte Result	Probe Check Result
Probe D	POS	PASS
Probe C	NEG	PASS
Probe E	POS	PASS
Probe B	NEG	PASS
SPC	NA	PASS
Probe A	POS	PASS
QC-1	NEG	PASS
QC-2	NEG	PASS

C

Analyte Name	Analyte Result	Probe Check Result
Probe D	POS	PASS
Probe C	NEG	PASS
Probe E	POS	PASS
Probe B	NEG	PASS
SPC	NA	PASS
Probe A	POS	PASS
QC-1	NEG	PASS
QC-2	NEG	PASS

B

lyte Result

Analyte Name	CI	EndPI	Analyte Result	Probe Check Result
Probe D	29.1	125	POS	PASS
Probe C	28.2	153	POS	PASS
Probe E	29.8	118	POS	PASS
Probe B	28.7	112	POS	PASS
SPC	26.6	259	NA	PASS
Probe A	27.8	117	POS	PASS
QC-1	0.0	0	NEG	PASS
QC-2	0.0	0	NEG	PASS

D

Analyte Name	Analyte Result	Probe Check Result
Probe D	POS	PASS
Probe C	POS	PASS
Probe E	POS	PASS
Probe B	POS	PASS
SPC	NA	PASS
Probe A	NEG	PASS
QC-1	NEG	PASS
QC-2	NEG	PASS

Figure 4. Rifampicin resistance in TB positive subjects. There was one mismatching at probe C, as a result TCG changed into TTG (A). There was no mismatching and all the probes were attached to the *rpo*B sequence (B) point mutation 9 (TCG –to-TTG) in probe C, (C), mutation (CAC –to-AAC) in probe A (D).

Probe D:
GGGGTTGACCCACAAGCG
Probe E:
CGGCTGACAGCCGCGAC

Among 91 positive samples, 4 carried mutations in their sequence. One of the probes failed to hybridize and the mismatching of the probe indicated the presence of mutation in the sample. In the first positive sample, probe C was mismatched with the rpo*B* gene due to the mutation occurring at probe C. As a result, TCG changed into TTG, that is, Serine (Ser) switched into Leucine (Leu). In the second sample, probe A was mismatched with the sequence and mutation was present at position 526 in the sequence of probe A. As a result, CAC changed into AAC, that is, Histidine (His) switched into Glycine (Gly) amino acid. The sequence of probe A is (GCACCAGCCAGCTGA).

In the third sample, two mutations were detected in probe D and probe E at position 516. Consequently, GAC changed into TAC, that is, Aspartate (Asp) switched into Tyrosine (Tyr). In the fourth sample, a single mutation was found in the region of probe E. Consequently, GAC changed into TAC, that is, Aspartate (Asp) switched into Tyrosine (Tyr). The sequence of probe E is (CGGCTGACAGCCGCGAC) (Figure 4).

4. Discussion

The diagnostic method of *M. tuberculosis* from sputum specimens recommended by WHO [17] is expensive and unaffordable in countries like Pakistan where TB is highly prevalent [24]. Among the 22 countries of the world where the prevalence of TB is high, Pakistan is ranked 5th in number. For doctors, MDR TB is a major challenge in countries like Pakistan [12]. The frequency of TB cases in Pakistan is approximately 342 per 100,000. In the past few years, lack of education, poverty and the absence of a TB control program has resulted in the high incidence of the disease [25].

This study enrolled individuals showing symptoms of TB. Sputum specimens were randomly collected from the population of Fatehpur, Layyah. All samples were labelled [26]. Clinical isolates of *M. tuberculosis* were grown under standard conditions on Lowenstein Jensen. It is a time-consuming microbiological technique which takes at least 4-6 weeks. However, it is considered as a highly specific and gold standard technique for the analysis of MTB [27]. Multi-drug resistant specimens which showed resistance against rifampicin were further examined for their molecular characterization. The analysis showed different missense mutations in MTB genes [28]. As described earlier, genetic alteration in the *rpo*B gene is responsible for rifampicin drug resistance. So, our study focused on the gene of interest, that is, the *rpo*B gene having the size 3519 bp. The area of interest of our gene was 81bp. Its specific region started from codon 506-533, respectively. Two primers were used which were designed using the primer 3 tool. The forward primer (CGATCACAC CGCAGACGTTGA) was positioned at codon 486-493 and the reverse primer (GGCACGCTCACGT GACAGACC) was positioned at codon 537-543, respectively [29]. A previous study showed that worldwide 96.1% of rifampicin resistant specimens have mutations on the *rpo*B gene. The drug susceptibility test revealed that 61.5% of samples have mutation in the region of the *rpo*B gene [30]. Another study conducted by Salma Hameed et. al in 2017 justifies our research that showed mutation in *rpo*B gene. The study revealed that out of 130 multidrug-resistant samples, 61.3% mutations were detected in the rifampicin resistance region (RRDR). At codon number 531, TCG was converted into TTG by the mutation. A single mutation at region 516 converted GAC into TAC and the mutation at position 526 converted CAC into TAC [31]. The mutation of serine to lucien is the most common point mutation reported in rifampicin resistant MTB [32]. The results of our work determined that in the series of mutation frequency, 4 samples of MTB carried a single mutation at locations 516, 526 and 531 respectively on the *rpo*B gene and this fact is significant for further study on TB in Pakistan

5. Conclusion

Our study confirmed the existence of mutations in the *rpo*B gene of MTB in MDR TB patients. A significant percentage (22%) was found to be infected by TB in the population of Tehsil Fatehpur, District Layyah, Punjab. Out of the infected individuals, 4.3% were found to be rifampicin resistant. Moreover, molecular analysis by hemi-nested PCR of

the resistant samples showed three-point mutations in the gene and subsequently three missense mutations in the amino acid sequence of the said gene. This result might help others in better understanding *M. tuberculosis*, the mutations in the *rpo*B gene and the resistance to the first line-drug rifampicin. In the future, the harmful effect of the *rpo*B gene mutations associated with the function of the β-subunit of RNA polymerase in 16S rRNA and its interaction with rifampicin can be estimated by performing molecular docking. The docking results may be used further to design better drugs which require less duration for treatment and provide quick diagnostic tools for TB.

Declarations

- **Funding**

No funding was received for this study.

- **Conflict of interest / Competing interests**

The authors declare no conflict of interest.

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