Depiction of new mutations in the *rpoB* gene associated with rifampin resistance in clinical isolates of *Mycobacterium tuberculosis*

Muhammad Mumtaz Khan[1], Maria Silvana Alves[2], Sajid Ali[3], Sikandar Khan Sherwani[4], Mirza Adnan Baig[1], Afaq Ahmad Siddiqui[5], Mustafa Kamal*[1]

Department of Biotechnology, University of Karachi, Karachi-75270, Pakistan[1], Department of Pharmaceutical Sciences, Faculty of Pharmacy, Federal University of Juiz de Fora, Minas Gerais, Brazil[2], and Provincial Reference Laboratory, Peshawar, Pakistan[3], Federal Urdu University of Arts, Science and Technology, Karachi, Pakistan[4], Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Karachi, Karachi-75270, Pakistan Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Karachi, Karachi-75270, Pakistan[5]

Abstract— Tuberculosis (TB) is an alarming infectious disease well spread in the world. This serious illness is responsible for millions of deaths every year in many countries. The Pakistan position in the ranking of TB cases is 8th among the 22 high burden countries, whereas average multiple drug resistance is 15%. Resistant strains to rifampin are commonly found worldwide, especially in developing countries, and this phenotype is associated with more than one mutation in the *rpoB* gene, which encodes the bacterial RNA polymerase β-subunit. The aim of the present study was to detect the probable cause of resistance against rifampin through the sequencing of the *rpoB* gene in a well-established bacterial collection obtained from Pakistani MDR-MTB patients. After culture procedures and a drug resistance investigation, we selected samples, isolated and sequenced the DNA from all rifampin resistant strains. To better understanding of this event, we made multiple alignments to clarify the correlation between the sequenced *M. tuberculosis* isolates and the *M. tuberculosis* reference strain H37Rv. Based on our results, consecutive mutations in amino acid of different codons in specific and highly sensitive regions of the *rpoB* gene were observed. The following new mutations detected here were earlier not reported from Pakistani clinical isolates: codons 509, 511, 513, 518, 519, 521, 522 and 526.

Key words — mutations; *rpoB* gene; MDR-MTB; clinical isolates; Pakistan.

1 Introduction

TUBERCULOSIS (TB), a severe infectious disease caused by *M. tuberculosis*, is a global health problem. Since last decade this illness has undergone a remarkable resurgence in incidence. In 2008, the estimation of TB was 8.9-9.9 million and 9.6-13.3 million for incident and prevalent cases, respectively [1]. Briefly, a global overview showed that a small number of cases were observed in the regions of the Eastern Mediterranean (7%), Europe (5%) and Americas (3%). On the other hand, most of the estimated number of cases occurred in Asia (South-East Asia and the Western Pacific WHO regions) (55%) and Africa (30%). Therefore, considering the geographical location and according to the indicators reported by the World Health Organization, Pakistan is in a high-risk area for TB (Asia). Unfortunately, TB has been neglected by health services of Pakistan, although this disease has been more prevalent in this country. According to a WHO report, the Pakistan position in the ranking of TB cases is 8th among the 22 high burden countries. Considering the Eastern Mediterranean Region, Pakistan contributed around 44% of the TB burden [1].

Nowadays, we are facing a new challenge around the entire world and a real threat, to control the tuberculosis, specifically the drug resistant strains of *M. tuberculosis*. In this context, intractable TB can be considered a deadly disease in recent times. Even with intensive therapy, the observed rate of mortality is 20%, and the resistance rate found in the high burden countries is 15% [2]. Emergence of multidrug resistance is due to resistance against the major anti-TB drugs, at least to rifampin and isoniazid, and these strains are called MDR-MTB [3]. Resistance against isoniazid (INH) is due to changes in coding of the regulatory region of *inhA* and due to mutations mainly in the *katG* gene but also in the *ahpC*, *oxyR*, *ndh*, and *kasA* genes [4]. Considering the resistance against rifampin (RIF), this event occurs due to one or more alterations in any base of codon located in the hyper-variable region of *rpoB* gene, which encodes the bacterial RNA polymerase β-subunit [5].

Polymerase chain reaction (PCR) is a well-developed technique and has been extensively used for the diagnosis and detection of resistance markers of numerous infectious diseases including TB. Amplification through PCR and sequencing of PCR product detects the various mutations within the amplified fragment of *rpoB* gene, which predicts the cause of drug resistance, allowing the rapid detection of rifampin resistant strains of *M. tuberculosis* [6].

The aim of the present study was to detect the probable cause of resistance against rifampin by the amplification of the hot spot region of the *rpoB* gene through site specific primers in a well-established bacterial collection obtained from Karachi, Pakistani MDR-MTB patients.

2 Material and Methods

Bacterial Isolates: M. tuberculosis clinical isolates were sequentially recovered from pulmonary tuberculosis patients from Ohja Institute of Chest Disease (OICD), located in Karachi, Sind, Pakistan. Therefore, only one isolate per patient was included in the present study. The reference strain M. tuberculosis H37Rv was also included in this

*Corresponding author:
Dr. Mustafa Kamal
Department of Biotechnology, University of Karachi,
Karachi-75270, Pakistan.
Phone: +92-333-3613509
E-mail: mustafacamal@hotmail.com

International Journal of Scientific & Engineering Research Volume 4, Issue 11, November-2013
ISSN 2229-5518

investigation. All bacterial isolates were cultured on L-J medium and processed for DNA isolation and further analysis. Selection of the clinical isolates was based on macroscopic characterization (colony morphology) and microscopic presentation (AFB staining).

2.1 DNA Extraction:

From solid (L-J medium) 3 to 5 colonies were suspended in 1ml of sterile distilled water (molecular biology grade), incubated at 95°C for 30 min, kept for 15 min in ultrasonic bath. Tubes were centrifuged at 13000 rpm for 10 min at 4°C. Supernatant was transferred in a new tube, and 2µl of supernatant was used for PCR [4].

2.3 PCR Amplification:

157bp fragment of rpoB gene a hyper-variable region was amplified by using synthetic oligonucleotide primers; 5'-GCACGTCGCGGACCTCCA-3' and 5'-TCGCCGCGATCAAGAGT-3' [7-10]. The PCR amplification was carried out with an initial denaturation at 94°C for 10 min, followed by 40 cycles of 94°C for 1 min, annealing 45C for 1 min and extension 72°C for 1 min. The PCR product was finally extended at 72°C for 7 min for final extension for rpoB gene.

2.4 Gel Electrophoresis:

Gel electrophoresis was performed for visual analysis of amplified PCR product.

5µL of PCR products were separated through 2% agarose, horizontal gel electrophoresis by 80 volts of electric current. Gel was stained with 0.5µg/ml ethidium bromide, subsequently visualized by UV (Ultraviolet) and photographed [11].

2.5 PCR Product Sequencing:

The straightforward technology to detect the mutation is PCR-DNA sequencing [12, 13]. For sequencing purposes, PCR products were sent to the Macrogen Company, Korea. DNA sequence data were analyzed through applied bio system sequence Scanner v1.0 software, and mutations were observed by comparing with H37Rv (NC_000962.2) reference strain by using the NCBI web link (www.ncbi.nlm.nih.gov/BLAST).

3 Results

rpoB gene PCR product visualization:

The 157bp hyper-variable region of rpoB gene amplified product displayed a positive band when exposed to UV light for visual analysis (Figure 1).

Amplified PCR product of *rpoB* gene fragment visualized in 2% agarose

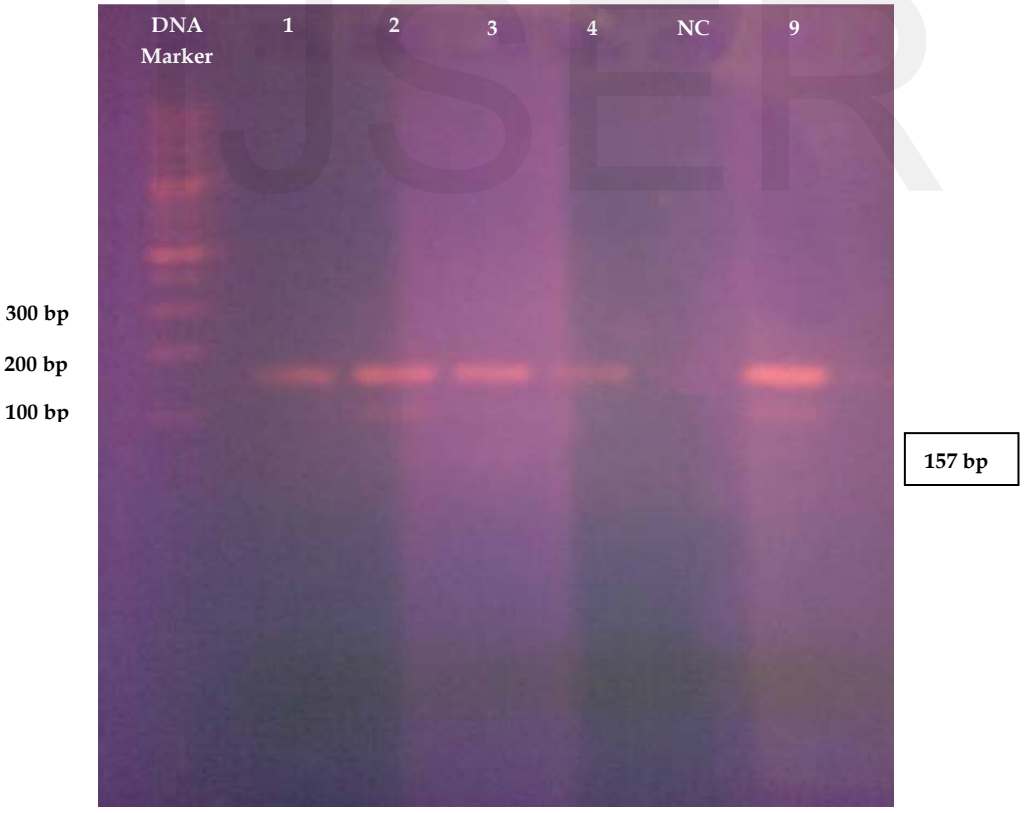

Fig. 1. 157 bp fragment of amplified product displayed a positive band when exposed to UV light, after gel electrophoresis.

- 1-5 represents the clinical isolate of *M.tuberculosis*.
- **NC PCR Negative control (PCR without *M.tuberculosis* DNA).

International Journal of Scientific & Engineering Research Volume 4, Issue 11, November-2013
ISSN 2229-5518

3.1 Sequence analysis:

We investigated mutations to confer rifampin resistance in pulmonary TB patients. All examined clinical isolates were resistant to rifampin. The 157bp amplified region were sequenced covering hyper-variable region from codon 507 to 533 [14]. Our investigations revealed new mutations when a comparative BLAST with H37Rv was run by the online NCBI BLAST tool. We detected 13 different combinations of mutations in the rpoB gene in examined MDR-MTB strains, 5 of them displayed silent mutations, and 4 previously reported and 4 non-reported mutations were detected (Table).Variability of mutations was also observed in codon 526 with 3 different combinations of mutations. Whereas a high frequency of mutation, significantly at codon 518, has been seen with 60% in all examined clinical isolates, all mutations at codon 518 of rpoB gene were to N518I (Figure 2). In addition, it has been noticed that more than 1 concomitant substitution mutations frequently observed in 8 clinical isolates (Figure 3). This subsequent study in rifampin resistant clinical isolates is an important addition to previous data; these new mutations can play significant role in the rifampin resistance event.

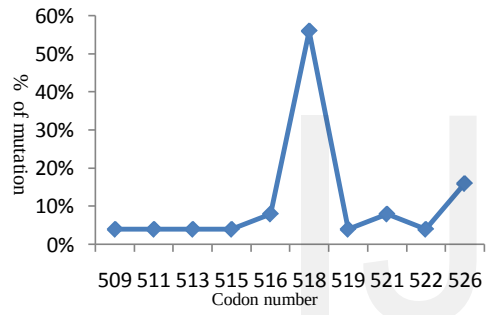

Fig. 2. Graphical peak shows the percentage of mutations at each codon. Maximum frequency of mutations detected at codon 518, 20% mutations at codon 526 found in examined isolates.

3.2 Nucleotide sequence accession numbers:

The new alleles found in this study have been submitted in DDBJ (DNA data bank of Japan) under accession no: AB711167, AB711168, AB711169, AB711170, AB711171, AB711172, AB711173, AB711174, AB711175, AB711176, AB711177 and AB711178.

4 Discussion

The major point of concern is that individuals involved with MDR-MTB strains are complicated to treat compared to those individuals infected with drug-susceptible strains. In this study thirteen different mutations in the rpoB gene were detected from Karachi, Pakistan, which is an important addition in investigation of global drug resistance rpoB gene mutations. Rifampin is a crucial drug in tuberculosis treatment. It is rarely found as mono-resistance. It is also considered a marker for multiple drug resistance (MDR) strains [15]. Rifampin resistance in M. tuberculosis is associated with mutations in the rpoB gene; it has been demonstrated in a genetic study that more than 95% resistance is due to mutations in rpoB gene [16].

TABLE
MUTATIONS IN *RPOB* GENE CONFERRING RESISTANT TO RIFAMPIN IN MDR-MTB PAKISTANI ISOLATES

*Codon No.	Mutated bases	Type of Mutations	Change in Amino acid
509	AGC to AGA	Substitution	S to R
511	CTG to TTG	Silent	L no change
513	CAA to CAG	Silent	Q no change
515	ATG to ATC	Substitution	M to I
516	GAC to TAC	Substitution	D to Y
518	AAT to ATC	Substitution	N to I
519	AAC to GAC	Substitution	N to D
521	CTG to ATG	Substitution	L to M
521	CTG to CTC	Silent	L no change
522	TCG to TCC	Silent	S no change
526	CAC to CGC	Substitution	H to R
526	CAC to CAT	Silent	H no change
526	CAC to CTC	Substitution	H to L

* *E.coli* codon numbering system is used.

Highly conservative rpoB genes play an essential role in physiology of the M. tuberculosis. The rpoB not only encode β-subunit of polymerase, it is also engaged to other genes rpoA, rpoC and rpoD genes. These genes produce proteins involved in gene transcription [5].

Our depicted mutations in the rifampin resistance-determining regio*n* (RRDR) 507 to 533 rpoB gene are comparable to those reported from other parts of global world, which reflect a global pattern of rifampin resistance rpoB gene mutations [16]. In our sequencing analysis a novel pattern of mutation at codon 518 Asparagines (N) to Isoleucine (I) detected most frequently in examined strains, and a comparative data of different amino acids gave an understanding for further analysis of probable effects of the mutation. It has an association to previously published data [15, 17]. Mutations at codon 509 Serine (S) to Arginine (R), at codon 519 Asparagine (N) to Aspartate (D) and at codon 521 Leucine (L) to Methionine (M) were not reported earlier from Pakistan. Some similar mutations on other codons have been reported from this geographical region [18, 19]. We also detected some silent mutations at codon 511, 513 and 522. Furthermore, we detected the variable mutations at 526 of rpoB gene and variability at codon 526 reported by various researchers around the world including Pakistan [19]. Mutations in our study showed an association to previously reported data [2, 4, 6, 7, 10, 20].

In this investigation 60% for all examined isolates demonstrated mutations at codon 518 and 20% at codon 526 that might present the most important mutations in isolates from Karachi (Figure 2). Some similar mutations were reported earlier from Pakistani isolates at codon 515, 516 and 526, while at codon 511, 513 and 522 we observed a new pattern of substitution mutations from same geographical area [18, 19]. Our analysis also identified no mutations detected in four of our examined strains, although these were resistant to rifampin. Same observations have been reported by other groups and suggested that mutation outside the hyper variable region might cause resistance against tuberculosis [8]. There is also another possibility that in such resistant strains, changes occurred in genes whose products may participate in antibiotic permeation or metabolism [13]. A characteristics finding of our study is the high frequency of double mutations occurring at two separate codons in 7 isolates, whereas in sample number 2, three substitutions mutation were de-

International Journal of Scientific & Engineering Research Volume 4, Issue 11, November-2013
ISSN 2229-5518

tected on different codons, although a mutation at codon 526 in the same strains was a silent mutation (Figure 3). To understand the genetic features, the CLUSTALW2 tool was used in our study. The multiple alignments by CLUSTALW2 among our examined strains and H37Rv was made to understand the pattern of substitution mutations at different codons in various examined strains, which give a clear understanding about the change in mutated codon bases as compared to H37Rv (Figure 3).

With a significant global reservoir of *M. tuberculosis* disease and thread of multiple drug resistance (MDR), there is a great need for novel research into this old age disease. Our results improve the understanding of rifampin resistance at a molecular level by providing data about mutations happening at different codons of *rpoB* gene among the MDR clinical isolates from Karachi, Pakistan. This subsequent study reports new mutations responsible for rifampin (RIF) resistance in Pakistani isolates.

Comparative multiple alignment of MDR-MTB

```
                                                                526         522
   07-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGAGGGTCAACCCCGA 60
   12-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGCGGGTCAACCCCGA 60
   01-rpoB-gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGCGGGTCAACCCCGA 60
   13-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   10-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   11-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCGGA 60
   14-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   15-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   20-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   rpoB-gene-H37Rv       CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   22-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   23-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   21-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
19-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   18-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   17-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   16-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   05-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   08-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   06-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   04-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   02-rpoB-gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTAGGTCAACCCCGA 60
   03-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
   09-rpoB-Gene-MTB      CTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGA 60
                         ********************************************  ********* **
                       521  519 518    516 515 513     511 509
   07-rpoB-Gene-MTB      CAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   12-rpoB-Gene-MTB      CAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   01-rpoB-gene-MTB      CAGCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   13-rpoB-Gene-MTB      CAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   10-rpoB-Gene-MTB      CAGCGGGTTGTTCTGGTCCATGAATTGGCTCAACTGGCTGGTGCCGAAGAACTCCTTGAT 120
   11-rpoB-Gene-MTB      CAGCGGGTTGTTCTGGTCCATGAACTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   14-rpoB-Gene-MTB      CAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   15-rpoB-Gene-MTB      CAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   20-rpoB-Gene-MTB      CAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   rpoB-gene-H37Rv       CAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   22-rpoB-Gene-MTB      CAGCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   23-rpoB-Gene-MTB      CAGCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   21-rpoB-Gene-MTB      CAGCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   19-rpoB-Gene-MTB      CAGCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   18-rpoB-Gene-MTB      CAGCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   17-rpoB-Gene-MTB      CAGCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   16-rpoB-Gene-MTB      CAGCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   05-rpoB-Gene-MTB      GAGCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   08-rpoB-Gene-MTB      CATCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   06-rpoB-Gene-MTB      CAGCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   04-rpoB-Gene-MTB      CAGCGGGTCGATCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   02-rpoB-gene-MTB      CAGCGGGTTGATCTGGTCCATGAATTGGCTCAGCTGTCTGGTGCCGAAGAACTCCTTGAT 120
   03-rpoB-Gene-MTB      CAGCGGGTTGATCTGGTACATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
   09-rpoB-Gene-MTB      CAGCGGGTTGTTCTGGTAGATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACTCCTTGAT 120
                          ** ***** * ******  ***** ******* *** ***********************
```

Fig. 3. Multiple alignments of mutated (negative strand) sequences with wild type H37Rv clarify the mutations in our examined MDR-MTB strains.

International Journal of Scientific & Engineering Research Volume 4, Issue 11, November-2013
ISSN 2229-5518

References

[1] World Health Organization. Global Tuberculosis Control. A short update to the 2009 report. 2009.

[2] Zeinab HH, Gomaa FAM, Shehata MMK. Effect of low dose of gamma radiation on multidrug resistant *Mycobacterium tuberculosis*. J Am Sci 2010; 6: 774-780.

[3] Khan MM, Ali S, Kamal M. The Base "G" in TB86 Primer Frequently Used in the Amplification of *katG* Isoniazid Resistance Gene of *Mycobacterium tuberculosis* is an Insertion. J Basic & Appl Sci 2012; 8: 181-182.

[4] Paolo M, Federica P, Penati V, Canducci F, Migliori GB, Cirillo DM. Use of genotype MTBDR assay for molecular detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical strain in Italy. J Clin Microbiol 2006; 44: 2485-2491.

[5] Kunin CM. Antimicrobial activity of rifabutin. Clin Infect Dis 1996; 22: 3-14.

[6] Negi SS, Singh U, Gupta S, Khare S, Rai A, Lal S. Characterization or *rpoB* gene for detection of rifampin drug resistance by SSCP and sequence analysis. J Med Microbiol 2009; 27: 226-30.

[7] Hsing-Yu H, Chung-Yu C, Lin-li C, Shui-Feng C, Ya-Hui, C, Yi-Jing C. *Mycobacterium tuberculosis* in Taiwan. J Med Microbiol 2003; 52: 239-245.

[8] Valim ARM, Rossetti MLR, Ribeiro MO, Zaha A. Mutation in *rpoB* gene of multidrug resistant *Mycobacterium tuberculosis*. J Clin Microbiol 2000; 38: 3119-3122.

[9] Telenti A, Honore N, Bernasconi C, March J, Ortega A, Takiff HE, Cole ST. Genotyping assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. J Clin Microbiol 1997; 35: 719-723.

[10] Telenti A, Imboden P, Marchesi F, Schmidheini T, BodmerT. Direct automated detection rifampin-resistant *Mycobacterium tuberculosis* by polymerase chain reaction and single-strand confirmation polymorphism analyses. Antimicrob Agent Chemother 1993; 37: 2054-2058

[11] Raunak S, Joshi RN, Joshi K, Poudel BH, Shrestha BG. Analysis of *katG* ser315Thr Mutation in Multidrug Resistant *Mycobacterium tuberculosis* and SLC11A1 Polymorphism in Multidrug Resistance Tuberculosis in central Development Region of Nepal using PCR-RFLP Technique: A Pilot Study. Nepal J Biotechnol 2011; 1: 14-21.

[12] Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*. Tuber Lung Dis 1998; 79: 3-29.

[13] Kapur V, Li LL, Iordanescu S, Hamrick M R, Wanger A, Kreiswirth BN, Musser JM. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*) encoding RNA Polymerase Beta subunit in rifampicin-resistant *Mycobacterium tuberculosis* strain from New York City and Taxas. J Clin Microbiol 1994; 32: 1095-1098.

[14] Yue J, Shi W, Xi J, Li Y, Zeng E, Wang H. Mutations in the ***rpoB*** gene of Multidrug-Resistant ***Mycobacterium tuberculosis*** isolates from China. J Clin Microbiol 2003; 42: 2209-2212.

[15] Kim BJ, Kim SY, Park BH, Lyu MA, Park IK, Ba GH, Kim SJ, Cha CY, Kook YH. Mutations in *rpoB* gene of *Mycobacterium tuberculosis* that interfere with PCR single strand conformation polymorphism analysis for rifampin susceptibility testing. J Clin Microbiol 1997; 35: 492-494.

[16] Glynn JR, Whiteley J, Bifani PJ, Kremer K, Van Soolingen D. Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. Emerg Infect Dis 2002; 8: 843-9.

[17] Chen L, Gan X, Li N, Wang J, Li K, Zhang H. *rpoB* gene mutation profile in rifampicin-resistant *Mycobacterium tuberculosis* clinical isolates from Guizhou, one of the highest incidence rate regions in China. J Antimicrob Chemother 2010; 65: 1299-1301.

[18] Asho A, Hasan R, Jabeen K, Jabeen N, Qadeer E, Hasan Z. Characterization of Mutations Conferring Extensive Drug Resistance to *Mycobacterium tuberculosis* Isolates in Pakistan. Antimicrob Agent Chemother 2011; 55**:** 5654–5659.

[19] Asho A, Hasan Z, Moatter T, Tanveer M, Hasan R. *M.tuberculosis* Central Asian Strain 1 MDR isolates have more mutations in *rpoB* and *katG* genes compared with other genotypes. Scand J Infect Dis 2009; 41: 37-44.

[20] Mani C, Selvakumar N, Narayanan S, Narayanan PR. Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* Clinical Isolates from India. J Clin Microbiol 200; 39: 2987-2990.