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4 **Profiling and identification of novel *rpoB* mutations in rifampicin-resistant**
5 ***Mycobacterium tuberculosis* clinical isolates from Pakistan**
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28 experiment and analyzed data. TM and RF supervised this work. All authors contributed to the
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Abstract

Introduction: Rifampicin (RIF) is one of the most effective anti-tuberculosis first-line drugs prescribed along with isoniazid. However, the emergence of RIF resistance *Mycobacterium tuberculosis* (MTB) isolates is a major issue towards tuberculosis (TB) control program in high MDR TB-burdened countries including Pakistan. Molecular data behind phenotypic resistance is essential for better management of RIF resistance which has been linked with mutations in *rpoB* gene. Since molecular studies on RIF resistance is limited in Pakistan, the current study was aimed to investigate the molecular data of mutations in *rpoB* gene behind phenotypic RIF resistance isolates in Pakistan.

Method: A total of 322 phenotypically RIF-resistant isolates were randomly selected from National TB Reference Laboratory, Pakistan for sequencing while 380 RIF resistance whole-genome sequencing (WGS) of Pakistani isolates (BioProject PRJEB25972), were also analyzed for *rpoB* mutations.

Result: Among the 702 RIF resistance samples, 675 (96.1%) isolates harbored mutations in *rpoB* in which 663 (94.4%) were detected within the Rifampicin Resistance Determining Region (RRDR) also known as a mutation hot spot region, including three novel. Among these mutations, 657 (97.3%) were substitutions including 603 (89.3%) single nucleotide polymorphism, 49 (7.25%) double and five (0.8%) triple. About 94.4% of Phenotypic RIF resistance strains, exhibited mutations in RRDR, which were also detectable by GeneXpert.

Conclusion: Mutations in the RRDR region of *rpoB* is a major mechanism of RIF resistance in MTB circulating isolates in Pakistan. Molecular detection of drug resistance is a faster and better approach than phenotypic drug susceptibility testing to reduce the time for transmission of RIF

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4 resistance strains in population. Such insights will inform the deployment of anti-TB drug
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7 regimens and disease control tools and strategies in high burden settings, such as Pakistan.
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10 **Keywords:** *rpoB*; rifampicin-resistant; *Mycobacterium tuberculosis*; mutations; anti-TB drug.
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Introduction

Tuberculosis is among the top ten leading causes of death worldwide and, according to WHO estimates, ten million people developed TB in 2017 globally. The emergence of first-line drug-resistant tuberculosis (TB) [1,2], especially multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB, poses formidable challenges to controlling TB in high burden countries (WHO 2020, <https://www.who.int/publications/i/item/9789240013131>). In 2017, 558000 of global TB cases were resistant to rifampicin (RIF) [3]. RIF is one of the most effective anti-TB drugs and resistance to it serves as a valuable surrogate marker for diagnosis of multidrug-resistant tuberculosis (MDR-TB) [4]. This resistance is primarily developed through mutations in *rpoB* of *Mycobacterium tuberculosis* (MTB). In general, 96.1% of RIF resistance worldwide is associated with *rpoB* mutations, and >90% of these mutations are located in the 81 bp RIF resistance determining the region (RRDR) of *rpoB* [5,6].

Various culture-based and molecular methods are used for drug susceptibility testing in MTB. Phenotypic drug susceptibility testing (DST) is still considered the gold standard. Culture-based methods are time-consuming and deemed to be a significant obstacle for prompt diagnosis of MDR TB, its management and control [7]. RIF DST is considered to be highly reliable and accurate. However, some clinically relevant, low-level RIF resistance linked to specific *rpoB* mutations, can be easily missed by growth-based methods [8]. This problem, however, appears to have been solved with the development of molecular techniques [9,10]. In 2009, WHO, for the first time, recommended the use of a genotypic method including Genotype MTBDRplus (Hain Lifescience, Nehren, Germany) for rapid detection of drug-resistant TB [9] while also recommending a cartridge-based nucleic acid amplification test called Xpert[®] MTB/RIF (Cepheid, Sunnyvale, CA, USA) for simultaneous detection of *Mycobacterium*

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4 *tuberculosis complex* (MTBC) and RIF resistance [10]. Both these commercial assays can detect
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6 mutations located within the 81 bp hotspot region of the *rpoB* and are currently being used on a
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8 global scale [9,10].
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11 Beyond the common RRDR mutations, other significant mutations in rifampicin-resistant (RR)
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13 strains have been reported in *rpoB* [6,10–12] from 1% to 5% of total RR strains. According to
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15 the WHO report 2020 [13], half a million people developed RIF resistant TB of which 78% had
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17 MDR-TB. The three countries, India (27%), China (14%) and the Russian Federation (8%) share
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19 the largest of the global burden of 27%, 14%, and 8% respectively. Globally MDR/RR-TB was
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21 present in 3.3% of new cases and 17.7% of previously treated cases. The highest proportions was
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23 detected in previously treated cases (>50%), were in former Soviet Union countries.
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28 Pakistan is among the five highest-burden countries for RR cases, having an estimated 15000
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30 cases every year at the rate of 4.2% and 16% in new and previously treated cases, respectively
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32 [3]. Only a few small-scale studies, however, have been conducted in the country for the
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34 characterization of *rpoB* mutations among local MTB strains [14–16].
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38 Phenotypic DST is considered to be the gold standard method to infer resistance. However, some
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40 studies have reported that liquid culture systems (e.g. the MGIT platform) often fail to detect
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42 low-levels of RR-TB [17,18], including those involving H445D/L/R mutations [17,19], which
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44 have been linked to treatment failure or relapse [20–22].
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48 The aim of this study is the molecular characterization of phenotypically RR clinical isolates to
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50 determine the frequency of known as well as novel mutations within and outside the RRDR
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52 region of *rpoB*. This information will be useful in selecting and developing appropriate rapid
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54 diagnostic tools to aid in the management of MDR TB.
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4 **Materials and methods**

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7 **Ethical Approval**

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10 The current study was ethically approved by the Quaid-e-Azam University Ethical Committee
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15 **Setting**

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18 The National TB Reference Laboratory, Islamabad, Pakistan (NRL), provides DST services to
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20 50% of the country's population including the largest province (Punjab) and three regions
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22 (Islamabad Capital Region, Azad Kashmir and Gilgit Baltistan). Phenotypic DST is performed
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24 on MTB culture isolates from TB patients diagnosed as RR on GeneXpert with unknown RIF
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26 status.
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31 **Sampling**

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34 The study was conducted from January 2014 to October 2016. All MTB isolates that reported
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36 RR on phenotypic DST were identified first, and 322 (13.3%) of these were selected using
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38 sample random method for *rpoB* sequencing. The whole-genome sequencing (WGS) results of
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40 380 isolates from a previous study [23] under BioProject PRJEB25972 were also available and
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42 were resistant to RIF, which were included for analysing the *rpoB* gene mutation.
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48 **Processing of specimens for Mycobacterial culture and DST**

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51 Clinical specimens were decontaminated using the NALC-NaOH method and were inoculated on
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53 Middlebrook 7H9 Broth (Bactect MGIT 960 tube) and Lowenstein-Jensen (LJ) Media [24].
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55 Rapid BD MGIT™ TBc Identification testing was performed on positive cultures for
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57 confirmation of Mycobacterium tuberculosis complex (MTBC). Drug susceptibility testing was
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4 performed on MTBC isolates using the proportion method on LJ media for RIF at 40µg/ml, 0.2
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6 µg/ml for Isoniazid (IHN), 2.0 µg /ml for ofloxacin (OFX), 30.0 µg /ml for kanamycin(KM)
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8 [25]. Based on the DST results, MTB isolates were classified into four groups as follows: RIF
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10 mono (only RR), MDR (Resistant to both RIf and INH, Pre-XDR (MDR strain also resistant to
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12 Fluoroquinolone (FQ) or Second-line injectable (SLI) and XDR (MDR strains also resistant to
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14 FQ and SLI).
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18 19 20 **MIC determination**

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23 Minimum inhibitory concentration (MIC) was determined for newly reported mutations as well
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25 as some already well-characterized mutations for comparison using the Bactec MGIT 960 system
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27 and LJ media, as described in previous studies [26–29]. The drug concentrations tested for RIF
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29 included 0.125, 0.25, 0.5, 1, 2, 4, and 8 µg /mL for MGIT and 5, 10, 20, 40, 80, and 160 µg
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31 /mL for LJ media.
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35 36 **PCR amplification and sequencing of *rpoB***

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39 DNA for polymerase chain reaction (PCR) was extracted by heating bacterial colonies at 90°C
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41 for 20min in 100 microliters of water followed by sonication using ELMA E-30H Sonicator
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43 (Elma Schmidbauer GmbH) for 15 minutes. PCR was performed using 25 µL of Qiagen
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45 HotStarTaq Master Mix containing 2.5 units of HotStarTaq DNA Polymerase I x PCR Buffer
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47 (Contains 1.5 mM MgCl₂) and 200 µM of each dNTP, 2 µL of each forward and reverse 10 µM
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49 primer, 8 µL of DNA and 5 µL of water with a total volume of 50 microliter in a Gene Amp PCR
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51 systems 9700 (Applied Biosystem). PCR cycling conditions began with 15 minutes hot start,
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53 followed by 45 cycles at 94°C for 45 seconds, annealing and extension for 1 min 30 sec at 72°C,
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55 and a final amplification at 72°C for 10 min. Primers *rpoB*geneSA (5' -
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4 GGTTCGCCGCGCTGGCGCGAAT-3') and rpoBgeneRB (5' -
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6 GACCTCCTCGATGACGCCGCTTTCT-3') were used for amplification of a 1764 bp region of
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8 the *rpoB* [30].
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11 Sequencing was performed using the following primers: forward 5'-
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13 GGGAGCGGATGACCACCCA-3', and reverse 5'-GCGGTACGGCGTYTTCGATGAAC-
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15 3'(21) on 3130 Genetic Analyser by Applied Biosystems using the BigDye™ Terminator v3.1
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17 Cycle Sequencing Kit at University College, London. WGS was performed on the Illumina
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19 platform at Supranational TB Reference Laboratory (SRL) in Milan, Italy, in another study [23].
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22 23 24 **Sequencing Analysis**

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26 For strains on which *rpoB* sequencing was performed, analysis of the chromatogram generated
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28 by the sequencer was conducted by Molecular Evolutionary Genetics Analysis Version 7
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30 (MEGA 7) software and compared to H37Rv *rpoB* sequence Rv0667. PhyResSE web tool was
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32 used for *rpoB* polymorphism and phylogenetic lineage analysis [31]. PhyResSE is a reliable and
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34 simple viewer for MTB WGS DNA reads. The server combines the most reliable methods from
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36 FastQC, SAMtools, QualiMap, BWA, and others with validated samples. Single and paired-end
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38 data from Next Generation Sequencing of *Mycobacterium tuberculosis* could be analyzed with
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40 validated approaches. Mutations were characterized using both MTBC numbering system and
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42 the *E. coli* numbering system [32].
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47 48 **Quality Control**

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50 NRL participates annually in proficiency testing conducted by the WHO collaborating centre and
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52 Supra National Reference Laboratory, ITM in Antwerp, Belgium. For internal quality control,
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54 susceptible strain H37Rv and two known rifampicin-resistant strains selected from the DST
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56 proficiency testing panel were included with each batch for DST.
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Results

During the study period (January 2014 to October 2016), 2421 strains were reported as phenotypically resistant to RIF including 3% as mono RIF resistant, 45% MDR, 46% pre-XDR and 6% XDR (Table 1), *rpoB* gene sequencing was performed on 322(13.3%) isolates and already available WGS data was analysed for an additional 380(15.6%) isolates [23]. Among the 702(29%) phenotypically resistant isolates for which *rpoB* sequencing data was analysed, mutations in *rpoB* were identified in 675 isolates (96.1%), and 27 (3.8%) were reported as wild type.

Among 675 isolates with mutations in *rpoB*, substitutions of nucleotides were identified in 657 (97.3%), one having both substitution and deletion and 17 (2.7%) with either insertion or deletion. A total of 63 different types of polymorphism were identified including single nucleotide polymorphism (SNP) in 603 isolates (89.3%), double nucleotide polymorphism (DNP) in 49 isolates (7.25%) and triple nucleotide polymorphisms (TNP) in five (0.74%) isolates as shown in Table 2. Mutations were detected outside RRDR in 12 isolates which include mutation at Ile491Phe in 6 isolates, Ile491Met in one isolate, Ile491Leu in one isolate, Val170Phe in three isolates and a change in Tyr474His .

In the *rpoB*, the highest number of amino acid changes were found at position Ser450 (64.1%) followed by Asp435 (13.3%) and His445 (12.7%). The most frequent amino acid change noted was Ser450Leu (60.3%). This amino acid change was seen at a frequency of 72.2 % in XDR 61.3% in pre-XDR compared to 58.7% in MDR and isolates. Amino acid changes observed at frequencies of four times or more are shown in Table 3, while amino acid changes seen with lesser frequencies are listed in Table 3s.

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4 In 12 isolates (1.8%), three different types of mutations were detected outside the 81 base pair
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6 hotspot region including Ile491Phe in six isolates, Ile491Met in 1 isolate, Ile491Leu in 1 isolate,
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8 Val170Phe in three isolates and a change in Tyr474His as shown in Table 3 and 3s. Mutation
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10 was located within Rifampicin resistant determining region (RRDR) in 663 (94.4%) isolates,
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12 including three new mutations identified as shown in Figure. In the RRDR region in one isolate,
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14 the mutation detected was due to the insertion of arginine (R) at position 432 of *rpoB* (insertion
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16 codon CGC1294). In another isolate, deletion of GAA codon at position 1308-10 resulted in
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18 substitution Gln436His and deletion of amino acid asparagine (N) at 437. The mutation was
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20 detected in another isolate due to deletion of CACCAGCCAGCTG/C at 1281-1294 resulting in
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22 deletion of 4 amino acid threonine (T), serine (S), glutamine (Q) and leucine (L).
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28 Phenotypic genotypic results, MICs, Genotypes, DST Pattern and GenBank accession numbers
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30 of newly reported mutations are summarized in Table 4. In our study, we were able to
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32 characterize 344 isolates into phylogenetic lineages. The most common lineage found in our
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34 research was Delhi/CAS in 257 cases (74.7%) followed by Euro American Super Linage in
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36 45(13.1%), Beijing in 20 (5.8%), EAI in 16 (4.7%), Haarlem in 2 isolates while Cameroon in
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38 one, TUR and Ural was identified in one isolates. Predominant lineage Delhi/CAS was found in
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40 79.2% of MDR, 73.3% of pre-XDR, 61.9% in XDR and 55.6% of rifampicin mono resistant
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42 isolates.
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47 48 49 **Discussion**

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52 In this study, we investigated mutations occurring in the *rpoB* among phenotypic resistant 702
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54 MTB isolates from Pakistan. We reported mutations in RRDR region in 663 (94.4%) isolates
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56 including three novel mutations, while outside the RRDR region were detected in 12 isolates
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58 (1.8%), whereas no mutation was identified in 27 (3.8%) of the phenotypically resistant isolates.
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4 Mutations detected in *rpoB* were substitutions in 657 (97.3%), deletions, and insertions in 18
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6 (2.7%) isolates. Our data is consistent with the previously reported studies in RRDR and outside
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8 RRDR [5,30,33]. In a previous study [34], five isolates did not harbor any mutation in the RRDR
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10 of RpoB region. In Swaziland 30% of RR cases, harbored mutations I outside the RRDR [35]. In
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12 Uganda, 18% (8/45), in China five mutations were detected outside the RRDR region [15,
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14 16]. Similarly, In Syria, one mutation was detected outside the RRDR region [37].
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22 Pakistan is among the five highest-burden countries for RR cases, the most frequent mutations
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24 reported in RRDR regions were Ser450 (64.1%), Asp435 (13.3%) and His445 (12.7%). Earlier
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26 studies performed in Pakistan, were on small sample sizes, have also reported similar frequencies
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28 of changes at amino acid position 450 (52 and 56.4%), amino acid 435 (4.84% and 15%) and
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30 amino acid 445 (7 and 9.68%) [15,16]. Similar frequencies of mutations were reported by other
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32 countries in Asia, e.g., Ser450 mutation rates were reported in India (59%), China (60%) Nepal
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34 (58.7), and in Russia (66.6%). Similarly, Asp435 were reported in the range of 5.3% to 15.6%
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36 and His445 from 15.3 to 29.2 % in these countries [12,38–41].
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42 Except for the three new types of mutations we have identified in this study, other mutations
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44 have been previously reported and associated with drug resistance in different studies which are
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46 available TB dream database [42] and in a review study [11] which is part of the WHO
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48 guideline, “The use of next-generation sequencing technologies for the detection of
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50 mutations associated with drug resistance in *Mycobacterium tuberculosis* complex: technical
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52 guide”. We characterized three novel mutations, two out of these mutations (deletion of four
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54 amino acids TSQL from 427-430 and an insertion of amino acid R at position 432) were reported
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56 RIF-resistant by Xpert as well as a phenotypic method using Liquid MGIT 960 and Solid
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4 Lowenstein Jensen media. MIC of these newly reported mutation was above critical
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6 concentration on both MGIT and LJ media and were classified as resistant.
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9 However, one novel mutation due to the substitution of amino acid Gln436His and deletion of
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11 Asn437 was resistant to Xpert and phenotypic DST on LJ but susceptible to Liquid MGIT 960.
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13 MIC was determined for this strain and it was 0.5 µg/ml on MGIT 960 which is below the
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15 critical concentration of 1 µg/ml and will be reported as susceptible. However, MIC on LJ
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17 media was 80 µg/ml which was above the critical concentration of 40 µg/ml and was classified
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19 as resistant. Previous studies have also reported mutations which were resistant on solid media
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24 DST while susceptible on MGIT DST [7,43]. Laboratories performing DST on MGIT only are
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26 likely to misdiagnose such cases, leading to treatment failure and proliferation of resistant
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28 strains. However, it has been reported previously that deletion of Asn437 of the M. tuberculosis
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30 RpoB causes rifampicin resistance in recombinant M. smegmatis [44].
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33 These newly-identified mutations are very important as drug susceptibility testing is moving
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35 from phenotypic to genotypic method especially whole-genome sequencing. Once this report is
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37 published and data of these mutations is made available to those databases which identify and
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39 classify resistance causing mutations. These will be updated and once they will be identified
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41 elsewhere will get classified as resistant conferring mutations. This data will also be helpful as a
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43 lot of new diagnostics tools are in the process of development as well as getting upgraded. The
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45 sequences of the newly identified mutations have been submitted to GenBank and are
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47 available under accession numbers shown in Table 4. All mutations identified outside the RRDR
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49 region have been previously reported in different studies [11,33,42,45]. The frequency of such
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51 mutations reported in our study correlates to studies conducted in different regions [46] including
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53 Pakistan [47]. However, under-reporting of these mutations cannot be disregarded as Xpert
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4 testing services are decentralized and RR cases are only systematically referred for DST. It is,
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6 therefore, important that samples from patients reported as rifampicin sensitive on Xpert but with
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8 poor response to treatment should be referred for phenotypic DST or sequencing to avoid
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10 misdiagnosis of resistant strains, otherwise ineffective treatment will result in proliferation and
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12 transmission of these strains. In Swaziland, 30% of RR cases were detected due to mutation
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14 outside RRDR [35].
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19 In our work, no mutation was detected in 27 (3.8%) of the phenotypically RIF-resistant isolates
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21 (Table 1). These isolates may carry other mechanisms of resistance, such as modified efflux
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23 pumps [48,49] but the possibility of laboratory error cannot be excluded with certainty. In
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25 conclusion, our study has provided valuable information on mutations associated with resistance
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27 to rifampicin in clinical isolates from Pakistan. We were able to characterize and report the
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29 frequency of different mutations inside as well as outside RRDR in our population including
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31 three novel mutations. These findings described will help compare and evaluate the new
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33 diagnostic tools for diagnosis of MDR TB.
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37 38 **Conclusion**

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41 The application of sequencing technologies in TB endemic regions will assist clinical
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43 management and personalised anti-TB drug approaches, as well as disease control through
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45 surveillance activities. The management of MDR-TB strains is essential for the control of TB. In
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47 our study, we found that the mutations underlying MDR-TB are established variants. Known and
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49 putative drug resistance markers in established loci are detectable using low-cost sequencing-
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51 based approaches (e.g. amplicon-based), suitable for a low resource setting. More than 94%
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53 strains with resistance to rifampicin exhibited mutations in RRDR, detectable by Xpert and the
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55 line probe assay. Although these current molecular assays are applicable for the early detection
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4 of rifampicin resistance in the region. However, a significant subset of mutations not detected by
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6 these rapid methods was identified, and these pose a risk for the further development of drug
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8 resistance. Failure to consider the possibility of drug-resistant TB can result in inadequate
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10 drug regimens, amplification of drug resistance, and additional disease transmission. Large-scale
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12 molecular approaches including whole genome sequencing and analysis will assist with
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14 understanding the epidemiology and risk groups or factors underpinning the transmission of TB,
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16 as well as provide insights into drug resistance and compensatory mechanisms. Such insights
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18 will inform the deployment of anti-TB drug regimens and disease control tools and strategies in
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20 high burden settings, such as Pakistan.
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27 **Acknowledgements**

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29 We will like to thank all colleagues at National TB reference, Laboratory and University College
30
31 London for their help in technical assistance for DST and sequencing.
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36 **Conflict of interest statement**

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38 None of the authors has any conflict of interest with the material in this manuscript.
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37 **Figure legends**

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40 Figure 1: Depiction of novel mutations detected in the RpoB (hotspot region RRDR) of *M.*
41 *tuberculosis* strains using MTB numbering system. Mutation site has been shown with blue
42 arrows. H37Rv was used as a reference for comparison.
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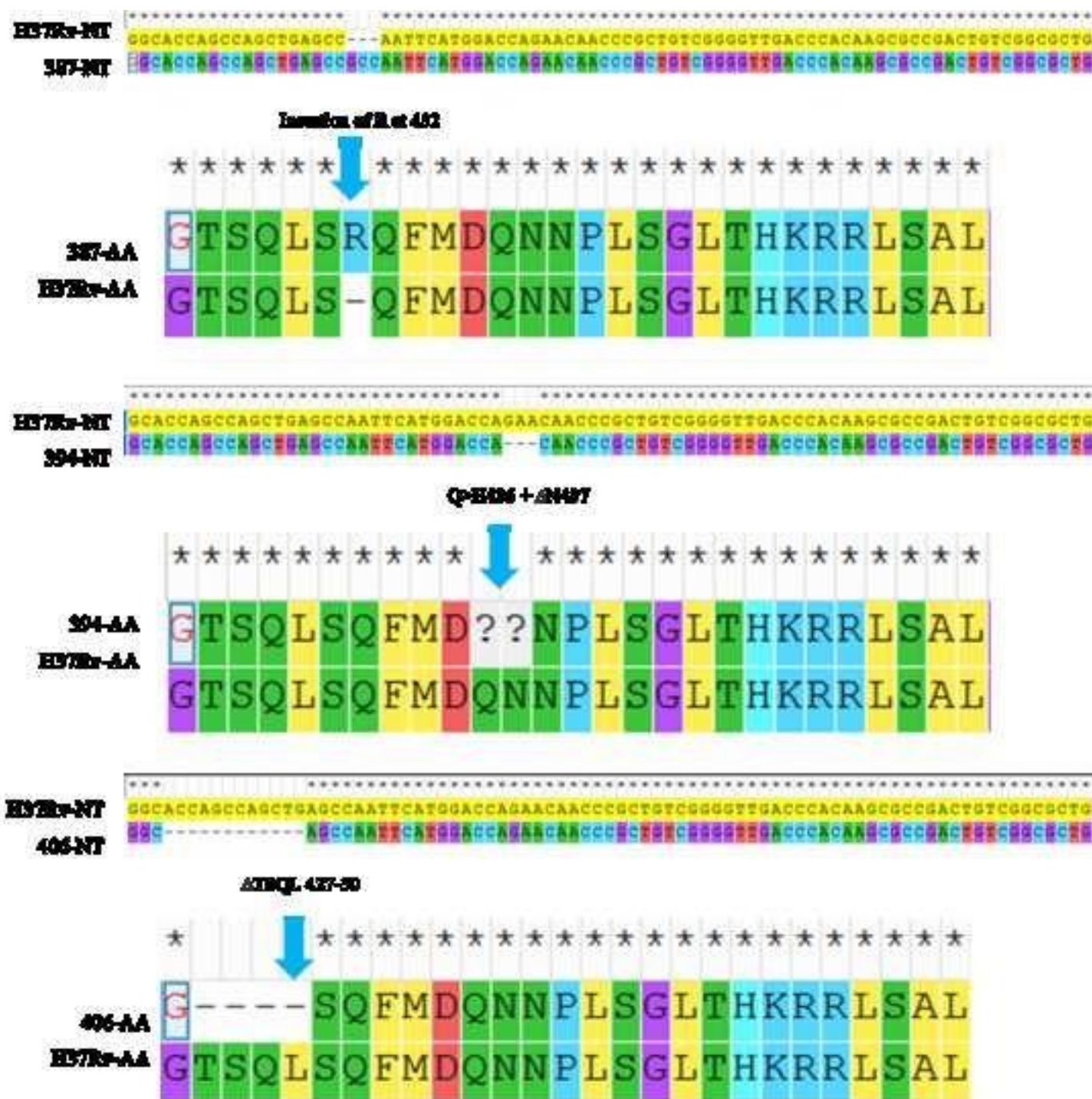


Table 1:- Total drug resistance and RIF resistance isolates with sequencing data

Phenotypic DST profiles	¹ Total resistant isolates (2421)		² Total resistant isolates with sequencing data (702)		³ Isolates with <i>rpoB</i> mutation (675)	
	*N	%	N	%	N	%
Mono RIF resistant	65	3%	28	4%	25	4%
MDR	1094	45%	305	43%	291	43%
PRE-XDR	1119	46%	331	47%	323	48%
XDR	143	6%	38	5%	36	5%

*N: Number.

¹Total resistant isolates recorded at National TB reference laboratory, Pakistan (NRL).²Resistant isolates whose sequence data is available.³RIF resistant isolates with *rpoB* mutations.

Table 2: Different types of mutations detected in *rpoB* of RIF resistant isolates.

Mutation/polymorphism type		Clinical isolates with mutations (#n)	*Polymorphism types detected (n)
Indels	Deletions	8	6
	Insertions	9	2
Substitution	Single Nucleotide Polymorphism	603	27
	Double Nucleotide Polymorphism	49	32
	Triple Nucleotide Polymorphism	5	4
	Deletion + substitution	1	1
Indels + Substitution			
Total		675	72

*Different types at different locations of *rpoB*. N; number

Table 3: Frequency of different mutations among various RIF resistant isolates

Codon Change	#A. A Change (RpoB) <i>MTB</i> Numbering System	A.A Change (RpoB) <i>E. coli</i> Numbering System	RIF-mono resistant (n=25)	<i>MDR</i> n=291	<i>PRE-XDR</i> n=323	<i>XDR</i> n=36	*Total n= 675 (%)
TCG→TTG	Ser450Leu	Ser531Leu	12	171	198	26	407(60.3)
GAC→GTC	Asp435Val	Asp516Val	0	20	25	2	47(7.0)
TCG→TGG	Ser450Trp	Ser531Trp	1	9	10	3	23(3.4)
CTG→CCG	Leu452Pro	Leu533Pro	2	7	9	1	19(2.8)
GAC→TAC	Asp435Tyr	Asp516Tyr	0	5	10	1	16(2.4)
CAC→TAC	His445Tyr	His526Tyr	2	3	10	0	15(2.2)
CAC→GAC	His445Asp	His526Asp	2	7	3	0	12(1.8)
CAC→CTC	His445Leu	His526Leu	0	8	4	0	12(1.8)
CAC→AAC	His445Asn	His526Asn	0	4	4	0	8(1.2)
ATC→TTC	Ile491Phe	Ile572Phe	0	2	4	0	6(0.9)
Insertion of TTC →1300	Phe433ins	Phe514ins	1	3	2	0	6(0.9)
CAC→CGC	His445Arg	His526Arg	0	4	1	0	5(0.7)
CAC→TGC	His445Cys	His526Cys	0	1	4	0	5(0.7)
CTG→CCG	Leu430Pro	Leu511Pro	0	2	2	1	5(0.7)
CAA→CTA	Gln432Leu	Gln513Leu	0	2	1	1	4(0.6)
CAA→AAA	Gln432Lys	Gln513Lys	0	1	3	0	4(0.6)
CTG→CGG,	Leu430Arg ,	Leu511Arg ,	0	1	3	0	4(0.6)
GAC→TAC	Asp435Tyr	Asp516Tyr					
ATG→ATA	Met434Ile ,	Met515Ile ,	0	3	1	0	4(0.6)
,	Asp435Tyr	Asp516Tyr					
GAC→TAC							
ATG→GTG,	Met434Val ,	Met515Val ,	2	0	2	0	4(0.6)
CAC→AAC	His445Asn	His526Asn					

*Frequency of each mutation among total RIF resistance mutations.

#AA: Amino Acid

Table 4: Novel mutations detected in **RpoB** among **RIF resistant** *MTB* isolates

#Codon change	Amino acid change MTB Numbering	Amino acid change E.Coli Numbering	GX Res ult	LJ MIC in µg /ml	MGIT MIC in µg /ml	Type of mutation	Genotype	*Res- Type	GeneBank accession numbers
Ins of cgc at 1294	Insertion of Arg at 432	R513ins (inframe)	R	>80	2	Insertion	Delhi/CAS	MDR	MT411895
Caccag ccagctg /C from 1279- 1290	deletion of aa TSQL at 427-30	Del T508- S509- Q510- L511 (inframe)	R	>80	4	Deletion	Euro- American Super Linage	RIF- R	MT411896
CAGA AC/CA C at 1308- 10	Gln436His + Asn437del	Q517H+ Asn518del	R	80	0.5	Substitution + deletion	Delhi/CAS	PRE- XDR	MT411897

*Type of resistance. MDR; Multidrug resistance, XDR; Extensively drug resistance, #Codon change in *rpoB* gene.
GX; GeneXpert, LJ; Lowenstein Jenson media