

Molecular Characterization and Spoligotyping Analysis of Multi Drug Resistant *Mycobacterium tuberculosis* from a High TB Endemic Area of Pakistan

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Abstract

Background: Multidrug resistant tuberculosis caused by *Mycobacterium tuberculosis* is an infection that is resistant to rifampicin and isoniazid. Management of Multidrug resistant tuberculosis is a serious challenge worldwide.

Objectives: To investigate hotspot mutations in *rpoB*, *katG* and *inhA* genes and its possible co-relation with predominant genotypes in Khyber Pakhtunkhwa, Pakistan.

Study design, settings and duration: This cross sectional study was conducted after approval from research and ethics committee of Provincial TB Control Program, Khyber Pakhtunkhwa in March 2015.

Materials and Methods: A total of 166 clinical isolates were analysed which were collected from programmatic management of drug-resistant tuberculosis units. All samples were characterized by phenotypic drug susceptibility test, genotypic drug resistant test (line probe assay) and spoligotyping analysis using "TB-SPRINT" micro bead assay.

Results: Out of the total 166 samples, 97 strains were resistant to rifampicin (RIF) and 106 strains were resistant to isoniazid (INH). Most common mutation in *rpoB* was S531L in 75 (77%) isolates followed by D516V in 10 (10%) and H526Y in 6 (6%) samples respectively. A rare mutation in *rpoB* gene at codon 522 and deletion of codon 518 was also reported. In 106 INH resistant strains, 97(91%) were associated with mutation in *katG* gene while resistance in 9 (8.4%) strain was due to mutation in the *inhA* promoter region. Spoligotyping analysis revealed 55 distinct types of different patterns. Spoligotyping patterns of 146 samples matched with 15 different lineage of *M.tuberculosis* in which 101 (60%) were identified as the predominant CAS1-Delhi lineage. The pattern of 20 strains (12%) did not matched to any other pattern in the SITVIT database and were named orphan KP.

Conclusion: Molecular characterization of *M.tuberculosis* is very helpful in the early identification of MDR-TB. As CAS1-Delhi is the predominant type in this region, its association with drug resistance, treatment failure and patient demographic profiles should be investigated.

Key words: Spoligotyping, *katG*, *rpoB*, line probe assay, TB-SPRINT.

Introduction

The emergence of drug resistance in *Mycobacterium tuberculosis* (*M.tuberculosis*) is a serious global threat. Pakistan contributes about 65% of tuberculosis (TB) burden in the Eastern Mediterranean region and ranks 5th among the countries with high burden of TB. Prevalence of the TB is estimated as 342 cases in one million population or 670,000 cases per year.¹ Rapid identification of drug resistance in *M.tuberculosis* is a hope to effectively treat and control this deadly

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disease. Tuberculosis (TB) is treated with first line drugs including streptomycin (STR), isoniazid (INH), rifampicin (RIF), ethambutol (ETB) and pyrazinamide (PZA). Two of these drugs INH and RIF kill more than 99% of the susceptible mycobacterial population.² Rapid genotypical assays for drug susceptibility testing (DST) is gaining more popularity for its rapid results as the phenotypical DST takes 4-8 weeks.³ These assays target specific mutations responsible for drug resistance in *M. tuberculosis* like resistant to RIF is conferred by mutation in *rpoB* gene that codes for β -subunit of RNA polymerase.⁴ More than 90% of mutation occur in specific region of this gene called rifampicin resistance determining region (RRDR) that is only 81 base pair long but contribute to more than 95% resistance to rifampicin.^{4,5} The predominant mutations reported in different molecular studies are Ser531Leu, His526Asp, His526Tyr and Asp516.

Resistance to INH is conferred by mutation in *katG*, *inhA*, *ndh*, *kasA* and *ahpC-oxyR* gene. However, 50 to 95% of INH resistance is associated with mutation in *katG* gene. The most common reported mutation is Ser315Thr substitution. Similarly, 15 to 30% of INH resistance is due to mutation in *inhA* promoter region at upstream position -15. The most common mutation in the *inhA* promoter is C8T substitution.⁶ GenoType® MTBDR plus (Hain Life-Science, Nehren, Germany) is one of the two line probe assay (LPA) that detects common mutations in *rpoB*, *katG* and *inhA* promoter region. Mutation in the RRDR region of *rpoB* is detected using 8 wild type immobilized probes along with 4 other probes that detect most common substitution at codon 516, 526 and 531 as in Figure-1.⁷ Each probe binds to its corresponding complementary codon. In the same way one wild type and two mutated probes are used to determine mutation at codon 315 of *katG* while two wild type probes and four mutation probes are used to detection mutation at -16, -15 and -8 upstream region of *inhA*.⁸

Spoligotyping stands for spacer oligonucleotide typing that is a technique used for simultaneously detection and typing of

M.tuberculosis. It is a PCR based technique that amplify direct repeat locus (DR) along with the neighboring spacer in the *Mycobacterium* genome.⁹ TB-SPRINT is commercial assay used for determining DNA polymorphism at the DR locus and simultaneously detects mutation in RRDR region of *rpoB* gene.¹⁰

The aim of the present study is to investigate hotspot mutations in *rpoB*, *katG* and *inhA* genes and its possible co-relation with predominant genotypes in the region.

Materials and Methods

This cross sectional study involved 166 pulmonary samples that were positive after smear microscopy on ziehl neelsen (ZN) technique. All the received samples were digested and decontaminated using standard N-acetyl-L-cysteine sodium hydroxide (NALC-NaOH) method¹¹ in a biosafety level 3 facility (BSLIII) at Provincial TB Reference Laboratory, Peshawar. Decontaminated samples were inoculated on Lowenstein Jensen medium (LJ) and mycobacterium growth indicator tube (MGIT). Positive growth in the tubes was confirmed by Tbc ID device (Ref: 245159, Becton, Dickinson). All confirmed mycobacterial isolates were processed for both phenotypical DST and molecular resistance assay. Drug susceptibility test (DST) was carried out using BD BACTEC MGIT 960 SIRE kit (Ref: 245123, Becton, Dickinson) in which final drug concentration was 1ug/ml for RIF and 0.1 ug/ml for INH drug. Line probe assay (LPA) was performed for the detection of molecular resistance using GenoType® MTBDR plus assay (Ref: 30496, Hain Lifescience, Germany). Mutations were confirmed by sequencing respective genes using primers previously described by Talenti et al.¹² Briefly, 1 mL of each positive isolate was centrifuged at 1000 gx for 15 minutes. Supernatant was discarded and 100 μ L DNAs free molecular grade water was added to re-suspend the pellets. Suspension was then

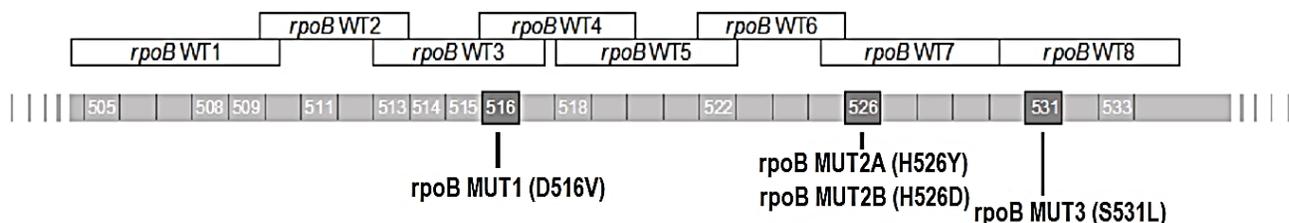


Figure 1: *rpoB* gene and common mutation sites⁸ (*rpoB* MUT1, *rpoB* MUT2A, *rpoB* MUT2B and *rpoB* MUT3)

heat inactivated followed by sonication for 15 minutes and final centrifugation at 13000 gx for 5 minutes. PCR mixture was constituted by mixing 5 µL of DNA templates and 45 µL of multiplex PCR reagent provided with the assay. PCR reaction was programmed as per manufacturer instruction. Finally 20 µL of the amplified product (Figure-2) was then hybridized to the DNA strips immobilized with specific probes. Results were interpreted as per manufacturer instructions while sensitivity and specificity was performed using Medcalc software (<https://www.medcalc.org>). Spoligotyping study was done at institute de Génétique et Microbiologie, Université Paris-Sud using TB-SPRINT assay running on microbead-based multiplexed systems (Luminex 200) and result were interpreted at SITVIT database.¹³

The study was conducted after approval from research and ethics committee of Provincial TB Control Program, Khyber Pakhtunkhwa (TB/KP /R&D-25-15) in March 2015.



Figure 2: Results of PCR product after gel electrophoresis. (157 bp *rpoB* gene and 209bp *katG* gene)

Results

A total of 166 mycobacterium isolates were tested for both DST and LPA assay. DST identified

Table 1: Mutation analysis of *rpoB* gene with corresponding probes. A total of eight wild type and three mutational probes are used that binds to complementary sequences on mycobacterial DNA. Absence of any probes after hybridization corresponds to a confirm mutation.

Codon Analyzed	Probe Name	Frequency of Missing Probe n (%)	Mutation Confirmed	Probe Used for Mutations	Frequency Mutation n (%)
505-509	<i>rpoB</i> WT1	0			
510-513	<i>rpoB</i> WT2	1 (1)	L511P		1 (1)
510-517	<i>rpoB</i> WT2/WT3	0			
513-519	<i>rpoB</i> WT3/WT4	13 (13.4)	D516V D516V	MUT1	10 (10)
516-522	<i>rpoB</i> WT4/WT5	1 (1)	Deletion:518		1 (1)
518-525	<i>rpoB</i> WT5/WT6	1 (1)	S522L		
526-529	<i>rpoB</i> WT7	6 (6.3)	H526Y	MUT2A/ MUT2B	6 (6.3)
530-533	<i>rpoB</i> WT8	75 (77.3%)	S531L	MUT3	75 (77.3)

97 (57 %) isolates resistant to RIF and 129 (77%) isolates resistant to INH. LPA identified all 97 (100%) rifampicin resistant mutant and 106 (82%) INH mutant and could not detect 23 (18%) isolates that were phenotypically resistant to INH. Resistance in these strains suggests mutation in other genes that confer resistance to INH but not target in LPA.

In the current study 97 (61%) of strains showed mutation in RRDR region. Most frequent mutation in *rpoB* was S531L in 75 (77%) isolates, D516V in 10 (10%) isolates and H526Y in 6 (6%) isolates respectively. Less frequent mutations were L511P and S522L. Deletion of one codon was also observed at position 518 in an isolate associated with RIF resistance (Table-1).

Among 106 INH resistant isolates, 97(91%) were associated with *katG* mutation while 24 (22%) with promoter region of *inhA*. The predominant mutation in *katG* was S315T in 97 (100%) strains confirmed by MUT1 and MUT2 probes of LPA assay (Table-2). Mutation in *inhA* was shown in 24 (22%) isolates. The most frequent mutation was C15T in 20 (83%) isolates followed by T8C mutation in 4 (17%) isolates (Table-3). Analysis showed that *InhA* mutations was associated with mutation in *katG* in 15 strains while only 9 isolates were causing INH resistant without any association with *katG* gene.

All samples produce valid spoligotype patterns of which 146 (88%) matched to known pattern of 15 clads while pattern of 20 (12%) remained unmatched in the SITVIT database and were declared as new types. Sublineage of Common Asian Strain was the predominant genotype family in 121 (82%) samples, followed by Beijing in 9 (6%) and T1 in 7 (5%) samples. Frequency of MDR was higher among Beijing 8 (89%) while 41 (41%) strains were MDR in CAS1-Delhi family. A brief summary of drug resistance corresponding to each genotype along with MDR frequency in each genotype is given in Figure-3.

samples and positive colonies from culture growth. The assay has 95-98% sensitivity and specificity to detect RIF resistance while its sensitivity and specificity to detect INH resistance varies from 57 to 100%.¹⁴

Sensitivity of MTBDR plus assay has been evaluated in early studies ranging from 67 to 97%.^{15,16} In this study the assay showed 100% sensitivity and 98% specificity to detect RIF resistance while it was 82% sensitive and 100% specific to detect INH resistance. These findings are in accordance with Farooqi et al.¹⁵ who has reported 92.5% sensitivity and 98.2% specificity to detect RIF resistance while for INH resistance sensitivity and specificity was 76.6% and 100% respectively. In our study the most frequent mutation in *rpoB* was (77%) at codon 531, 13% at codon 516 and 6% at codon 526. These findings were concordant with the results of previous studies.¹⁷⁻¹⁹ Khan et al. has also reported 52%, 15% and 7% mutation at codon 531, 526 and 516 respectively that are agreed with the current study.²⁰ In this study we observed deletion of codon 518 that is rarely reported and not likely detected by LPA assay in previous studies from Pakistan although reported elsewhere.^{15,21} Two other rare mutations at codons 522 and 511 were also observed in the current study. In these findings serine was substituted by leucine (TCG->TTG) at codon 522 while leucine was substituted by proline (CTG->CCG) at position 511. Silent mutation at 511 has been reported in two early studies.²⁰ We are therefore reporting a missense mutation (L511P) and a substitution S522L that was never reported from Pakistan before.

Multiple probes are used in LPA to detect both high-level (mutations) and low level INH resistance in this study MTBDR plus failed to detect 17% (n=23) isolates that were resistant to INH. Resistance in these strains may be contributed to mutation of other genes that confer resistance to INH but not target in LPA assay.²²

However, the reported 84% mutation of S315T in *katG* is concordant with the result of Farooqi et al.¹⁵ who reported 66.1% mutation and Ali et al.²³ reported 63% of mutation. Similarly our finding of 8% contribution to INH resistance by mutation in *inhA* gene is consistent with other study by Farooqi et al.¹⁵ who found 11.9% mutation in *inhA*. In the current study it was shown 80 (83%) *inhA* mutation was contributed to transition of C to T at position -15 and 17.3% was due to T to C transition at position -8 in the promoter region of *inhA* gene.

Spoligotyping analysis of the current study revealed that CAS-1 Delhi is the predominant genotype 60% in Khyber Pakhtunkhwa. It was

confirmed that Beijing genotype was less dominant 5% that is consistent to other studies.^{18,24,25}

Furthermore, Beijing strains do not contribute too much to MDR-TB incidence because of its low prevalence in this region although the MDR-TB is more likely associated with Beijing strain. In this study 20 spoligo patterns did not match any of the previously published patterns in SITVIT database. Further study will reveal the fact of its diversity and its relation to new and recurrent infection.

Molecular epidemiology of *M.tuberculosis* was uncovered for the first time in Khyber Pakhtunkhwa, which is a highly TB endemic area of Pakistan. Molecular characterization of *M. tuberculosis* is very helpful in the early identification of MDR-TB.

As Common Asian Strain (CAS1 Delhi) is the predominant type in this region. It is therefore recommended to study association with drug resistance, treatment failure and patient demographic profiles should be investigated in much detail.

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Conflict of interest: None declared.

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