Characterization of mutations conferring extensive drug resistance to 
*M. tuberculosis* isolates in Pakistan

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**Running title:** Mutations in XDR MTB strains from Pakistan

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ABSTRACT

Increasing incidence of extensively drug-resistant (XDR) *M. tuberculosis* (MTB) in high tuberculosis burden countries further highlights the need for improved rapid diagnostic assays. An increasing incidence XDR-TB in Pakistan has been reported but drug resistance associated mutations in these strains have not been evaluated previously. We sequenced the 'hot spot' regions of *rpoB*, *katG*, *inhA*, *ahpC*, *gyrA*, *gyrB*, and *rrs* genes in 50 XDR-TB strains. It was observed that 2% of rifampicin, 6% of isoniazid, 24% of fluoroquinolone and 32% of aminoglycoside/capreomycin resistance in XDR-TB strains would be undetected if only these common 'hot spot' regions were tested. The frequency of resistance conferring mutation was found to be comparable between all XDR *M. tuberculosis* strain families present including, Central Asian Strain, Beijing, East African Indian genogroups and Unique isolates. Additional genetic loci need to be tested for detection of mutations conferring fluoroquinolone, aminoglycoside and capreomycin resistance in order to improve molecular based diagnosis of regional XDR-TB strains.

**Keywords:** XDR TB, Pakistan, tuberculosis, fluoroquinolone, aminoglycoside, Central Asian Strain, genogroups, mycobacterium
INTRODUCTION

Extensively drug resistant tuberculosis (XDR-TB) has emerged as a major public health problem worldwide (22). Multi-drug resistant (MDR) *Mycobacterium tuberculosis* (MTB) strains are resistant to at least the first line anti-tuberculous agents, rifampicin (RIF) and isoniazid (35). XDR-TB are resistant to at least one fluoroquinolone (FQ) and at least one of the three second line injectable agents; amikacin (AMK), capreomycin (CAP) or kanamycin (KAN), in addition to MDR (15). According to WHO estimates of 2008, MDR TB rates vary between 1.6 – 22.3 % in high TB burden countries including Pakistan (52). Community based MDR-TB cases are reported to be 4-5% of all TB cases in Pakistan (16). Global occurrence of XDR-TB among all MDR-TB cases is reportedly 6.6% while in Asia, including Bangladesh and Indonesia, it has been reported as 1.5% (43). Recent data from Pakistan reports that the number of XDR-TB cases has increased from eleven or 1.5% of 728 MDR isolates in 2006 to fifty three or 4.5% of 1181 MDR isolates in 2009 (27). Therefore, there is an urgent need for rapid and reliable tools to diagnose MDR and XDR TB to aid appropriate treatment and management of drug resistant TB particularly in this high burden setting.

Molecular genotyping studies of mycobacterial strains have illustrated variations in the phylogeography of strains worldwide (19, 20). In addition, it has been shown that the drug resistance patterns of different *M. tuberculosis* genogroup families or clades vary worldwide. Beijing strains are the most predominant genogroup globally but their resistance patterns vary regionally (25). Beijing strains are found to be associated with drug resistance in studies from Asia, Europe and Africa (7, 30, 41). T3 and Central Asian
(CAS) family strains have been shown to be associated with MDR (2). Studies from South Africa have reported higher occurrence of F15/LAM4/KZN, known as KZN strains, amongst XDR TB (21). In Pakistan, Central Asian Strain (CAS) (43) family isolates are most prevalent followed by Beijing strains (49). However, Beijing strains were found to be associated with MDR (49). CAS strains were found to be the most prevalent (42.1%) amongst XDR-TB isolates followed by Beijing (8.8%) and T (7%) family strains (27).

Conventional drug susceptibility testing (DST) is highly sensitive and specific but takes around 6-9 weeks. Molecular based assays for drug resistance detection may represent an alternative to conventional DST (28, 51). Molecular tests target common mutations in ‘variable regions’ of drug resistance genes. Resistance to RIF is well characterized and more than 95% of strains have mutations in a 81bp hot spot region of the \textit{rpoB} gene (40, 50). INH resistance can occur due to mutations in several genes such as \textit{katG}, \textit{inhA}, \textit{kasA}, \textit{oxyR} and \textit{ahpC} (9, 33, 56). However 70-80% of INH resistance is associated with mutations in codon 315 of \textit{katG} (9, 56). FQ resistance mutations in 320 and 375 bp hypervariable regions of \textit{gyrA} and \textit{gyrB} genes encoding DNA gyrase are thought to account for resistance in 50-90% of the phenotypic FQ resistant isolates (14, 24, 48). Resistance to aminoglycosides (amikacin and kanamycin) and capreomycin is thought to be associated with mutations in 16S rRNA gene (\textit{rrs}) particularly at positions 1401, 1402, and 1484, leading to AMK, KAN and CAP resistance (5, 32).

The frequency of specific resistance conferring gene mutations has been found to differ between \textit{M. tuberculosis} genogroups. In MDR strains of the Beijing genotype, the most common mutation for RIF resistance is codon 531 of \textit{rpoB} gene (23, 37, 46). Data
from Pakistan shows the mutation at codon 526 of rpoB gene is to be the second most common mutation amongst CAS MDR TB isolates after mutation at codon 531 (6).

Currently the World Health Organization has endorsed the use of line probe assays (INNO-LiPA Rif and MTBDRplus) in addition to conventional culture susceptibility method, for rapid detection of first line drug resistance (36, 53). The MTBDRsl line probe assay is for rapid detection of second line drug resistance (11). Together, these tests target rpoB, katG, inhA and gyrA hot spot region mutations. As prevalent MTB strain types vary geographically as does their frequency and pattern of drug resistance mutation (36, 37), local data is essential to the development of appropriate molecular based assays. No data on mutations of XDR TB strains is currently available from Pakistan and this is the first study where first and second line drug resistance conferring mutations in XDR-TB strains have been characterized.

MATERIALS AND METHODS

Mycobacterium tuberculosis isolates

M. tuberculosis strains were obtained from the strain bank at Aga Khan University Clinical Microbiology laboratory, Pakistan. Fifty XDR-TB isolates which had been previously spoligotyped were selected randomly for the study (27). These strains were from the period 2004 – 2009 and included 26 CAS isolates, 4 Beijing and T family strains each, 2 East African Indian (EAI) and U family strains each, one Pak-3 and one X family strain and 10 unique strains.

Culture and antibiotic susceptibility testing
Susceptibility testing was performed using the agar proportion method on enriched Middle brook 7H10 medium (BBL) at the following cut-off concentrations; rifampicin (RIF) 1 µg/ml, isoniazid 0.2µg/ml, streptomycin (S) 10µg/ml and ethambutol (EMB) 5 µg/ml. Pyrazinamide (PZA) sensitivity was carried out using the BACTEC 7H12 medium pH 6·0 at 100 µg/mL (BACTEC™ PZA test medium, Becton Dickinson USA). MDR TB strains were further tested with ciprofloxacin (CIP) 2 µg/mL, ethionamide (ETH) 25 µg/mL, capreomycin (CAP) 10 µg/mL, amikacin (AMK) 6 µg/mL and kanamycin (KAN) 6 µg/mL on Middlebrook 7H11 agar. The sensitivity data in this study reports breakpoints and MICs were not determined. The susceptibility of XDR strains included in this study was confirmed by repeating the susceptibility testing by same methodology. Reference strain *M. tuberculosis* H37Rv (ATCC 27294) was used as a control with each batch of susceptibility testing (54).

**DNA extraction and amplification**

DNA was extracted by cetyltrimethylammoniumbromide (CTAB) method (29). XDR strains were further subjected to PCR amplification and DNA sequencing in specific ‘hot spot’ target regions of the following genes; *rpoB* for RIF region 1694-2189 bp (38). For INH, *katG* region 872-1568 bp (3), *inhA* region -168-79 bp and *ahpC* region -182-100 bp (37); for FQ, *gyrA* region 78-397 bp and *gyrB* region 1330-1742 bp (18). For AMK, CAP and KAN the *rrs* region 1202-1565 bp was sequenced using specific primers (18). PCR products were purified using QIA quick Qiagen PCR purification kit and subjected to DNA sequencing in order to determine the genetic sequence in the ‘hot spot’ regions of the genes under investigation.
DNA Sequence Analysis

DNA sequences were analyzed after DNA sequencing by BLAST using multiple sequence alignments (www.ncbi.nlm.nih.gov/BLAST). Mutations were detected in respective gene by comparing with *Mycobacterium tuberculosis* H37Rv (wild type, reference laboratory strain).

RESULTS

Demographic information of XDR-TB strains

All strains were from pulmonary specimens. Forty six per cent of isolates were from the province of Sindh, 40% from Punjab, 12 % from Khyber Pakhtunkhwa Province and 2% from Baluchistan. Twenty eight isolates were from male and 22 from female patients. The average age of the male and female patients from which isolates were obtained was 37.28 years and 33.09 years respectively with a median age of 33 years in males and 34.5 years in females.

Antimicrobial susceptibility pattern of XDR TB strains

The drug susceptibility patterns of XDR TB strains and cut-off values for first and second line anti-tuberculous agents are summarized in Table 1. All isolates were resistant to RIF, INH, PZA amongst first line, and CIP, AMK and KAN amongst second line drugs. Twenty isolates were susceptible to S and only one isolate was susceptible to EMB while
30 isolates were resistant to all first line drugs including EMB and PZA. Twenty two isolates were sensitive to CAP, 28 were sensitive to ETH, while 8 XDR isolates were resistant to all the second line anti-tuberculous agents tested.

Detection of mutations in \textit{rpoB} gene for Rifampicin resistance

To investigate the mutations associated with rifampicin resistance we examined the 495 bp region including the 81bp hyper-variable RIF resistance detection region (RRDR) of \textit{rpoB} gene in each XDR isolate. Mutations were detected in 49 of 50 isolates, affecting six amino acid codons of \textit{rpoB}; 531 (68%), 516 (24%), 526 (4%) and 513 (2%), Table 2. Forty four isolates (88%) had a single mutation and five (10%) had two mutations each. Two strains had a mutation outside of the RRDR at codon 575 along with one at codon 516.

Detection of mutations in \textit{katG}, \textit{inhA} and \textit{ahpC} genes leading to INH resistance

Mutations leading to INH resistance were first investigated in the hot spot region of \textit{katG}. Forty one (82%) isolates had mutations at codon 315 (Table 2). Mutations were either S315T (76%) or S315N (6%). One strain had a change at codon 328. No mutations were detected in eight (16%) isolates. The 8 strains without mutations in \textit{katG} were further subjected to sequencing in the \textit{inhA} and \textit{ahpC} promoter regions. Two strains had a transition at the \textit{inhA} -15 promoter site. Three of the six without either \textit{katG} or \textit{inhA} mutations had a transition at \textit{ahpC} -88. None had dual mutations in \textit{inhA} and \textit{ahpC} genes. We could not detect any mutations responsible for INH for 3 strains based on \textit{katG}, \textit{inhA} and \textit{ahpC}. 

8
Detection of mutations in gyrA and gyrB gene for Fluoroquinolone resistance

Sequencing of the gyrA gene revealed that 11/50 (22%) isolates had a mutation at codon 90 while 26/50 (52%) had variable mutations at codon 94, Table 3. Forty seven strains had AGC to ACC polymorphism at codon 95 and 11 of these had no other mutation. No gyrA mutation was detected in one isolate. As the codon 95 mutation is reportedly not associated with drug resistance, all 11 isolates and the one without a mutation were further tested for gyrB gene mutations. Only 1 of the 12 tested displayed a mutation at codon 471 of the gyrB gene.

Detection of mutations in rrs genes for AMK resistance

We next analyzed the 1400 region of rrs gene to investigate resistance conferring mutations for AMK, KAN and CAP. Mutations were detected in 35/50 (70%) strains, Table 3. Thirty-three of the strains had A to G transition at nucleotide (nt) 1401, while one had a mutation at nt 1344 and another at nt 1484.

Relationship between MTB genogroups and the resistance conferring mutations in XDR-MTB isolates

The distribution of mutations according to genogroups of the MTB clinical isolates showed the most common single rpoB mutation to be S531L, Table 4. Most strains had a single mutation but 3 CAS and 2 unique genotype strains revealed 2 mutations each. Of the katG gene mutations codon 315 was the most common mutation (Table 4). Of the 8 strains without katG mutations; 4 were CAS family, 2 were Unique strains, while one
was an EAI and another a Beijing family isolate. Of these, only the EAI and Unique
group isolates showed an \textit{inhA} -15 region mutation. The 3 strains with mutations in the
\textit{ahpC} region belonged to CAS, Beijing and Unique genogroups while, the 3 strains for
which INH resistance could not be detected belonged to the CAS family.

\textit{GyrA} gene mutations at codon 90 and 94 were present amongst all XDR genogroups
tested. The 12 strains where \textit{gyrA} gene resistance was not detected belonged to CAS,
EAI, T and Unique genogroups (Table 4). Analysis of \textit{gyrB} gene mutations identified a
codon 271 mutation in one Unique isolate but none in the CAS, EAI and T genogroups.

All XDR isolates showed a predominant \textit{rrs} gene mutation at nt 1401. One CAS isolate
had additional mutations at nt1344/nt1358 while one Unique isolate had a mutation at
nt1484. The 15 XDR strains where no \textit{rrs} mutation were detected comprised of, CAS,
EAI, Beijing, T, U and Unique genogroups (Table 4).

\textbf{DISCUSSION}

Culture and DST remain the most sensitive and reliable method of detection of \textit{M. tuberculosis} drug resistance. However, phenotypic testing is time consuming and requires
specialized laboratory facilities. Therefore there is a need for rapid molecular based
methods for detection of drug resistance. This study illustrates that using molecular based
testing for antimicrobial susceptibility testing of XDR MTB isolates using only the
primary mutational hot spot regions of \textit{rpoB}, \textit{katG}, \textit{gyrA} and \textit{rrs} genes, a proportion of
phenotypically resistant XDR strains particularly those with fluoroquinolone (24%), amikacin/kanamycin and capreomycin resistance (32%) would remain undetected. The sensitivity patterns of the strains indicate limited treatment options available for these XDR isolates, with 8 of 50 strains resistant to all first and second line anti-tuberculous agents tested.

Rifampicin resistance due to common mutation at codons 531, 516 and 526 of the \( rpoB \) gene is consistent with data from Pakistan and other countries (4, 6, 12, 47). We observed a high frequency of mutations at codon 516 of \( rpoB \) gene in XDR strains especially in CAS spoligotypes. This differs from reports of low frequency of codon 516 mutations amongst XDR-TB isolates from India and China (4, 47). Also, it has previously been reported that the codon 526 mutation of \( rpoB \) is associated with MDR TB resistance in CAS isolates (6).

The most common resistance mutation for INH resistance in the XDR strains was at codon 315 of \( katG \) gene, correlating with the data reported in other studies (4, 12). We also observed one isolate with a mutation at codon 328 of \( katG \), which has been identified previously amongst XDR isolates (12, 47). Testing \( katG \) and \( inhA \) hot spot regions identified mutations in 44 of 50 isolates while another \( ahpC \) mutation were found in other 3 strains. INH resistance can also be due to changes in the \( kasA \) and \( oxyR \) genes (13) and it may be necessary to study these genes to investigate the molecular basis for isoniazid resistance in these XDR TB isolates.
The most common mutations for FQ were *gyrA* gene codons 94 and 90 with a low frequency of mutation at codon 91. This is consistent with reports of XDR strains from India, China, USA and Germany (4, 12, 18, 47). In contrast, a study from Portugal reported a higher occurrence of mutation at codon 91 of *gyrA* (42). We found that the sequence change at codon 94 exhibited the highest variability. Ninety-four percent of the XDR strains revealed mutation at codon 95 of *gyrA* gene however this mutation does not contribute towards FQ resistance and instead serves as evolutionary marker for classification of MTB strains into three Principal Genetic Group (PGG) (44). The ACC polymorphism at codon 95 confirms the PGG1 grouping of CAS and Beijing strains (26). However, we could only detect 76% of FQ resistant isolates via mutations at *gyrA* codons 91 and 94, as compared with 100% detection of FQ resistance in XDR TB isolates from a recent hospital based study in Mumbai, India (4). One XDR isolated had a novel A471V mutation in the *gyrB* gene, which may be responsible for FQ resistance. Eleven (22%) phenotypically resistant FQ isolates did not appear to have mutations in either *gyrA* or *gyrB* genes. Alternative mechanisms for FQ resistance have been attributed to drug efflux pumps and it may be possible that FQ resistance in these isolates may be attributable to those (1, 45).

Screening of the *rrs* gene to detect resistance for AMK, KAN and CAP revealed A1401G mutation to be most common and present in 66% of XDR isolates, consistent with reports from India and China (4, 34, 47). All isolates with *rrs* nt 1401 mutation were KAN and AMK resistant but 51.5% were CAP susceptible. Mutations at nt 1484 has also been reported previously to be associated with AMK, KAN and CAP resistance (4, 42). Additional point mutations in *rrs* may contribute to resistance perhaps also the
concomitant mutations at nt 1344 and 1358. The impact of rrs mutations nt 1252, 1462 and 1472 could not be determined as these were concomitant with nt 1401. No rrs gene mutations were detected in 15 (30%) XDR isolates, which included both amikacin/kanamycin and capreomycin resistant strains. Our data of XDR-TB isolates with mutations other than rrs nt 1401 and 1484 are higher than reported in XDR-TB isolates from India, where a detection rate of 98% for KAN, AMK and CAP resistance is stated (4). CAP and KAN resistance can also be due to mutations in the tlyA and eis genes respectively (39, 55). Therefore, additional genetic loci may be responsible for resistance to aminoglycosides and capreomycin in M. tuberculosis and additional sites need to be included for molecular based testing for resistance to second line drugs in order to avoid mis-identification of XDR isolates.

It is possible that the discordance between the phenotypic and genotypic resistance to FQ and AMK/KAN/CAP observed may be due to lower sensitivity of currently available genotypic as compared to phenotypic methods. It is possible that the such differences relate to alternate mutations in regions other than the currently known target regions for these drugs (17). In addition mycobacterial cultures may also comprise a mixed population of resistant and sensitive bacteria (8) and therefore DNA extracted from sample specimens may be a lower representation of the resistant genotype leading to a falsely low detection rate in the specimen. However, given the increasing levels of drug resistance in MTB strains worldwide and the imperative to rapidly detect and treat these strains, the molecular based testing needs to be emphasized. A comparative analysis of the genogroups of the XDR strains indicated that the mutations were common to all
genogroups and that there was no association between a specific mutation and the strain type.

Overall, our data highlights the need to include testing of additional genes for surveillance of drug resistance and indicates that molecular testing based on common mutational ‘hot spot’ regions of \textit{rrs} and \textit{gyrA} genes is not sufficient to detect resistance in XDR strains globally. Insufficient detection of XDR-TB strains would lead to a further increase in drug resistant TB in an environment already over-burdened by the disease.

ACKNOWLEDGEMENTS

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peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. Nature

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Table 1: Anti-microbial susceptibility profile of XDR TB isolates against anti-tuberculous drugs

<table>
<thead>
<tr>
<th>Resistance to anti-tuberculous agents^ (µg/mL)</th>
<th>No. of isolates</th>
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<tbody>
<tr>
<td><strong>1st line drugs</strong></td>
<td></td>
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<tr>
<td>RMP (1), INH (0.2), PZA (100)</td>
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<tr>
<td>RMP (1), INH (0.2), PZA (100), EMB (5)</td>
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<tr>
<td>RMP (1), INH (0.2), PZA (100), EMB (10), S (10)</td>
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<tr>
<td><strong>2nd line drugs</strong></td>
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</tr>
<tr>
<td>CIP (2), AMK (6), KAN (6)</td>
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</tr>
<tr>
<td>CIP (2), AMK (6), KAN (6), CAP (10)</td>
<td>28</td>
</tr>
<tr>
<td>CIP (2), AMK (6), KAN (6), CAP (10), ETH (25)</td>
<td>8</td>
</tr>
</tbody>
</table>

^ RIF, rifampicin; INH, isoniazid; PZA, pyrazinamide; EMB, ethambutol; S, streptomycin; CIP, ciprofloxacin; AMK, amikacin; KAN, kanamycin; CAP, capreomycin; ETH, ethionamide

Drug resistance of isolates was determined as described in methodology, the values in parenthesis represent cut-offs for each drug tested.
Table 2: Resistance conferring mutations to 1st line TB drugs in 50 XDR isolates

<table>
<thead>
<tr>
<th>Gene</th>
<th>Codon/nucleotide change</th>
<th>Amino acid/nucleotide change</th>
<th>No. of isolates</th>
<th>Genotype(^#) (n)</th>
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<td>rpoB</td>
<td>TCG→TTG</td>
<td>S531L</td>
<td>33</td>
<td>CAS (16), EAI (1), Beijing (3), T (4), U (2), X (1), Pak3 (1), Unique (5)</td>
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- ‘A’ insertion at nt 1942 and ‘G’ deleted from nt 1944
- ‘G’ deleted from codon 531

<table>
<thead>
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<th>Gene</th>
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<td></td>
<td>CAA→AAA</td>
<td>Q513K</td>
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<td>Unique (1)</td>
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<tr>
<td></td>
<td>ATG→ATC, GAC→TAC</td>
<td>M515I, D516Y</td>
<td>3</td>
<td>CAS (1), Unique (2)</td>
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<td></td>
<td>GAC→TAC</td>
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<td>GAC→GTC</td>
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### katG

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<td>AGC→ACC</td>
<td>S315T</td>
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<td>CAS (18), EAI (1), Beijing (3), T (4), U (2), X (1), Pak3 (1), Unique (7)</td>
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<tr>
<td>AGC→AAC</td>
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<td>AGC→ACC, GCG→GGG</td>
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<td>CAS (1)</td>
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### inhA

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### ahpC

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<td>No mutation</td>
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<td>CAS (3), EAI (1), Unique (1)</td>
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</tr>
</tbody>
</table>

# CAS, Central Asian Strain; EAI, East African Indian; T- clade; U- clade; Unique isolates, shared types which did not match any in the SpolDB 4.0 database, Pasteur, Guadelope

* isolates sequenced for *inhA* and *ahpC* gene promoter regions respectively
Table 3: Resistance conferring mutations to 2nd line TB drugs in 50 XDR isolates

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence change</th>
<th>Amino acid change</th>
<th>No. of isolates</th>
<th>Genotype (n)</th>
</tr>
</thead>
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<td>Mutation</td>
<td>Frequency</td>
<td>Description</td>
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**gyrB**

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**rrs**

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<sup>+</sup> isolates subjected to gyrB gene sequencing
Table 4: Drug resistance gene mutations in XDR-TB isolates versus genotypes

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<th></th>
<th>rpoB codon</th>
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<tr>
<td></td>
<td>513 515 516 526 531 575 ND</td>
<td>315 328 242 379 ND</td>
<td>90 91 94 96 111 ND</td>
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<td>6 1 *1 13 *1 6</td>
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<td>1 1</td>
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<td>Beijing (4)</td>
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<td>7 1 *2 2</td>
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Total no. of strains with each mutation

|                  | 1 3 33 *3 1 41 1 | 8 11 1 26 *1 12 | 33 1 *4 15 |

* denotes concurrent mutations; ND, none detected