Molecular analysis of genetic mutations among cross-resistant second-line injectable drugs reveals a new resistant mutation in *Mycobacterium tuberculosis*

Lesibana Malinga a,c,⁎, Jeannette Brand a, Steve Olorunjub, Anton Stoltzc, Martie van der Walt a

a South African Medical Research Council, TB Research Platform, Pretoria, South Africa
b South African Medical Research Council, Biostatistics Unit, Pretoria, South Africa
c South African Medical Research Council, TB Research Platform, Pretoria, South Africa

1. Introduction

Drug resistant tuberculosis (TB) is a global threat and a major public health problem in several countries (WHO, 2014). World Health Organization (WHO) estimate that 480 000 new cases of multidrug resistant (MDR)-TB and among them cases with extensively drug resistant (XDR)-TB were reported at 9.0% worldwide (WHO, 2014). MDR-TB is defined as concurrent resistance to isoniazid and rifampicin, while XDR-TB is MDR-TB plus resistance to one of the injectables plus resistance to quinolones. Both MDR and XDR-TB are difficult to treat and require the use of less effective second-line injectable drugs (SLIDs) which are often associated with major side effects (Jain and Dixit, 2008). Appropriate use of SLIDs of aminoglycosides (Maus et al., 2005a) is critical to treatment of MDR-TB and prevention of XDR-TB cases (Georgiou et al., 2012). XDR-TB is difficult to treat than MDR-TB and require use of capreomycin (CAP) in the intensive phase (Matteelli et al., 2014). Since 2006, CAP has replaced amikacin (AMK) and kanamycin (KAN) that forms the backbone regimen treatment of XDR-TB in South Africa (Pietersen et al., 2015; Streicher et al., 2012).

CAP resistant strains can also be cross-resistant to AMK/KAN given that mutations conferring resistance are encoded by the rrs gene (Georgiou et al., 2012). Cross-resistance within SLIDs drugs has been reported back in the early 1970s and until now, it has been difficult phenomenon to overcome in treatment of XDR-TB (Tsukamura, 1969; Tsukamura and Mizuno, 1975). To date, knowledge on mechanisms causing cross-resistance of injectable drugs against *Mycobacterium tuberculosis* (M. tuberculosis) isolates has been contradictory (Maus et al., 2005a). Understanding SLIDs cross-resistance mechanisms at a molecular level should facilitate rapid detection of XDR-TB, due to limited treatment options (Reeves et al., 2013). Moreover there is a high rate of CAP resistance in South African TB population and this leads to poor treatment outcomes (Pietersen et al., 2015). Mutations causing CAP resistance should be further investigated to increase our efforts for rapid detection of XDR-TB.

The ribosomal (rrs) A1401G mutation is commonly associated with cross-resistance between KAN, AMK, and CAP (Campbell et al., 2011; Engstrom et al., 2012). Other mutations within the 16 S RNA (i.e. G1484T, C517T, A514C) have also been implicated in cases of cross and mono-resistance within the injectable drugs (Maus et al., 2005a). Jughele et al. found an association between the A1401G mutation and resistance to AMK and KAN with moderately high specificity and sensitivity (Jughele et al., 2009). However KAN resistance is often missed by detection of A1401G and eis mutations are used to distinguish low from high level KAN resistance (Zaunbrecher et al., 2009). Cross-resistance to CAP is due to A1401G mutation, while the tlyA mutations are involved in mono-resistance (Engstrom et al., 2011). However
only 70–80% of CAP resistant isolates have A1401G mutation and this suggests that there is still at least one mechanism of cross-resistance to be discovered (Campbell et al., 2011; Pietersen et al., 2015). Moreover, association of certain genetic mutations to SLIDs needs further investigation, especially in genetically diverse strains. There is a large variation of CAP minimal inhibitory concentrations (MIC) levels in Beijing strains as compared to EuroAmerican lineage (Reeves et al., 2015).

Phenotypically resistant isolates that lack genetic mutations in known regions lead to discordance in molecular assays (e.g. GenoType MTBDRsS) which decrease their sensitivity (Engstrom et al., 2012; Georgiou et al., 2012). Currently available molecular assays rely on few mutations to accurately detect SLIDs resistance, especially in the case of CAP drug. It has been shown that mutations within *M. tuberculosis* transporter proteins lead to cross-resistance due to efflux pump mechanisms (Engstrom et al., 2011; Jugheli et al., 2009).

The G133C of Rv1258c efflux pump caused cross-resistance to aminoglycosides (Reeves et al., 2013). Moreover, both Rv1258c and eis genes are upregulated by whiB7 (Rv3197A) which might contribute to cross-resistance of aminoglycosides (Reeves et al., 2013). Novel mutations located in whiB7 lead to aminoglycoside cross-resistance in *M. tuberculosis* (Reeves et al., 2013). A combination of mutations in different regions is important to accurately predict SLIDs cross-resistance within pre-XDR and XDR-TB cases (Georghiou et al., 2012). We investigated the association of phenotypic resistance cases of SLIDs with mutations within *rrs*, *eis*, *thyA* and efflux pumps (Rv1258c and Rv0194) genes. We further determined the MIC resistance to CAP caused by the detected G878A mutation.

2. Materials and methods

2.1. Bacterial strains

One hundred and twenty four culture isolates received from 2008 to 2012 and stored at the Medical Research Council TB laboratory in Pretoria, South Africa were used. Isolates were selected based on pre-XDR and XDR-TB criteria. The laboratory is a former Supranational TB Reference Laboratory and has been previously involved in WHO proficiency testing schemes until 2013 (Mativandlela et al., 2013). Repeat testing was done on all pre-XDR and XDR-TB isolates. The strains were previously tested for AMK (1 μg/mL), KAN (5 μg/mL), CAP (2.5 μg/mL) and ofloxacin (2 μg/mL) drugs using MGIT 960 system. The isolates were classified as susceptible or resistant based on DST performed earlier using standardized and quality assured methods.

2.2. Minimal inhibitory concentration determination

To determine the minimal inhibitory concentration (MIC 64–0.125 μg/mL) levels of AMK, KAN and CAP resistant isolates of *M. tuberculosis* strains based on conventional DST, a microplate alamarBlue assay (MABA) was performed as described previously (Collins and Franzblau, 1997). Briefly, the cultures were grown to mid-log phase on 7H9 OADC. Once an OD of 0.6 was reached, 100 μL of the culture was added to a solution of 98 μL of 7H9 OADC and 2 μL of CAP, AMK and KAN drug (Sigma Aldrich). Alamar Blue reagent (Thermo Fischer, US) and 10% v/v Tween 80 of 25 μL each were added to the wells of the microplate and further incubated for 24 hours. After one day of incubation, resistance was detected by change of a blue to pink color. The MIC was recorded as the well without color change at the lowest concentration. The H37Rv (ATCC 27294) was used as negative control and was susceptible to all drugs tested.

2.3. DNA extraction, amplification and sequencing

Crude DNA was isolated from MGIT cultures by boiling method. Briefly, 1000 μL of culture was transferred to a 1.5 mL Eppendorf tube and centrifuged at 8000 × g and supernatant discarded. The pellet was re-suspended in 100 μL of deionised water, heat killed (20 minutes), sonicated (15 minutes) and supernatant transferred to a new tube and stored at −20 °C until further processing. Genotype MTBDRsS was run on all specimens as previously explained according to the manufacturer (Hain life science, Germany) (Hillemann et al., 2009). Discordant isolates were amplified into seven genes by PCR using primers of *rrs* (500, 900, and 1400), *eis*, *thyA*, Rv0194 and Rv1258c genes synthesized by integrated DNA technologies (*Table 1*). The 25 μL of the cocktail reaction was made up of 11.5 μL of Hot Start mix (Kapa Biosystems, Cape Town, South Africa), 1 μL each of sense and antisense primer, 7.5 μL distilled H2O (dH2O), and 2 μL of DNA. The amplification protocol was performed at 95 °C (15 minutes), followed by 30 cycles of 95 °C (30 seconds), 60 °C (30 seconds), 72 °C (30 seconds), with a final step at 72 °C for 5 minutes. The PCR products were purified using purification kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol, to remove unincorporated primers and nucleotides. Direct sequencing of the genes was performed at Central Analytical Facility, Stellenbosch, South Africa. These sequences were subjected to multiple sequence alignment with H37Rv genome (GenBank accession number NC_000962) using BioEdit software version 7.2 (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

2.4. Spoligotyping

The PCR products were amplified using primers purchased from manufacturer (Ocmium Biosciences, India) and the procedure was performed as previously described (Kamerbeek et al., 1997). After amplification, hybridization was performed on denatured DNA using a 43 spacer membrane. The direct repeat (DR) region was amplified by PCR with primers derived from the DR sequences. The amplified PCR product was hybridized to a set of 43 immobilized oligonucleotides on the membrane. The products were detected by chemiluminescence (Amersham Biosciences) and by exposure to X-ray film (HyperfilmECL, Amersham). The spoligotypes were reported by using a binary code as previously described (Cowan et al., 2002).

2.5. Statistical analysis

The genetic data for each strain was entered in a Microsoft Office Access 2013 program. The genetic, genotype, and DST data sets were further analyzed on Epi Info (version 3.5.1, 2008) and STATA 13.0 softwares. Fishers exact and chi square test were used to measure the level of association.

2.6. Ethics approval

The permission to use the strains was sought from University of Pretoria, Faculty of Health Sciences Research Ethics Committee (206/2012).

### Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rrs</em> 500</td>
<td>123 bp (Honore and Cole, 1994)</td>
</tr>
<tr>
<td><em>rrs</em> 900</td>
<td>222 bp (Honore and Cole, 1994)</td>
</tr>
<tr>
<td><em>eis</em> 1400</td>
<td>516 bp (Campbell et al., 2011)</td>
</tr>
<tr>
<td><em>thyA</em></td>
<td>567 bp (Campbell et al., 2011)</td>
</tr>
<tr>
<td>Rv0194</td>
<td>555 bp (Campbell et al., 2011)</td>
</tr>
<tr>
<td>Rv1258c</td>
<td>700 bp This study</td>
</tr>
</tbody>
</table>

List of primers used for sequencing of genes.
3.2. Molecular analysis of cross-resistance

Distribution of mutations among cross and mono-resistant cases.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>AMK/KAN/CAP</th>
<th>AMK/KAN</th>
<th>AMK/CAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=10</td>
<td>n=10</td>
<td>n=4</td>
</tr>
<tr>
<td>rrs1400</td>
<td>23</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>rrs900</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>rrs500</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>eis</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>tlyA</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Rx0194</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Rv1258c</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total (%)</td>
<td>33 (45.8%)</td>
<td>7 (9.7%)</td>
<td>5 (6.9%)</td>
</tr>
</tbody>
</table>

**AMK** = Amikacin; **KAN** = Kanamycin; **CAP** = Capreomycin; **nP** = non-applicable.

3.3. Molecular analysis of mono-resistance

We had a total of 53/124 (43%) isolates with mono-resistant profiles to CAP, KAN and AMK drugs (Tables 2 and 3). Most were resistant to CAP, 31/53 (58%), 21/53 (37%) with KAN resistance while only two isolates were AMK mono-resistant. The KAN/CAP and AMK/KAN to 70% and KAN/CAP to 75%. Mutations in the AMK mono-resistant isolates (Table 3).

3.4. The association of G878A mutation and genotype

Spoligotyping revealed high genotypic diversity within 124 isolates. The most prevalent families were of EuroAmerican lineage, S 44 (35%), X2 5 (4%), X3 31 (25%), T 16 (13%) and LAM 9 (7%), while Beijing 8 (7%), Haarlem 7 (6%) and East Africa Indian 4 (3%) genotypes. The A1401G mutation was found in 1 (4%) Beijing, 2 (9%) East African Indian, 7 (30%) S, 7 (30%) T1, 1 (4%) X2, 5 (22%) X3 distributed among all genotypes. The new mutation of G878A was found in 16/31 of the EuroAmerican X3 genotype compared to 5/88 in the rest of the genotypes (95% confidence interval: 5.98–58.94; P < 0.0001) (Table 4).

3.5. The G878A mutation on CAP MIC with different genotypes

To determine the phenotypic impact of G878A mutation on CAP drug-resistance MIC levels of eight samples were established (Table 5). Five isolates displayed MICs of moderate to high (8–64 μg/mL) resistance to CAP drugs belonging to EuroAmerican X3 genotype. Three isolates had their MIC at 32 μg/mL, two were of S genotype. One isolation belonging to Beijing genotype had a moderate MIC at 8 μg/mL. The AMK/KAN cross-resistant isolates with C14T mutations showed MIC levels of 4–16 μg/mL for both drugs. However, five isolates had their MIC at 32 μg/mL and thus were independent of genotype.

4. Discussion

The study reports an association of rrs, eis, tlyA, Rv1258c and Rx0194 mutations with SLID cross and mono-resistant cases within XDR and pre-XDR-TB. The mutations were distributed across all cases but those in Rv1258c and Rx0194 were confined to AMK/KAN/CAP and AMK/CAP cross-resistant cases respectively.
belonged to the EuroAmerican family, which could explain our lower high genotypic diversity among cross-resistant isolates, most of them strains (Said et al., 2012; Sirgel et al., 2012), but we found the mutation have been found among AMK/CAP and KAN/CAP resistant isolates (Zimenkov et al., 2013). Others have con-
cross-resistance, despite it been shown to be present in KAN susceptible 
tion has a low MIC level of KAN at 5 μg/mL (Gikalo et al., 2012). We results confirm this mutation’s presence in KAN resistant isolates. Strains harbouring this mutation are however still susceptible to CAP (Zimenkov et al., 2013). The eis C14T and C12T mutations are considered as borderline or low-level to KAN drug (Gikalo et al., 2012). Mutations within eis gene increases the virulence of M. tuberculosis that are thought to be responsible for preservation of bacterial fitness, and could be the reason for the extensive transmission of drug-resistant TB strains in Russia (Casali et al., 2014). Absence of AMK/CAP cross-resistance in strains harbouring mutations in the eis gene has been recently demonstrated (Casali et al., 2014; Gikalo et al., 2012). Our study also confirms that none of the eis mutations found in AMK/CAP cross-resistant isolates.

The Y177H mutation within Rv1258c found in one XDR-TB isolate with AMK/CAN cross-resistance. The Rv1258c is a stable gene region (Ainsa et al., 1998) and appearance of a mutation could lead to higher efflux pump activity, as suspected among isolates with this cross-resistance (Albert et al., 2010; Reeves et al., 2013). Interestingly two mutations within Rv0194 of R83G and G170V were detected in pre-XDR-TB and XDR-TB isolates respectively. Similar to other investigators we found mutations within Rv0194 in an XDR-TB strain that lacked known mutations (Ilina et al., 2013; Liu et al., 2013). The eis C14T and C12T mutations are considered as borderline or low-level to KAN drug (Gikalo et al., 2012). Mutations within eis gene increases the virulence of M. tuberculosis that are thought to be responsible for preservation of bacterial fitness, and could be the reason for the extensive transmission of drug-resistant TB strains in Russia (Casali et al., 2014). Absence of AMK/CAP cross-resistance in strains harbouring mutations in the eis gene has been recently demonstrated (Casali et al., 2014; Gikalo et al., 2012). Our study also confirms that none of the eis mutations found in AMK/CAP cross-resistant isolates.

The eis C14T gene is associated with AMK/KAN cross-resistance (Campbell et al., 2011; Rodwell et al., 2014) and including it in molecular assays increases the sensitivity for SLIDs resistance, as was shown in a new version of the Genotype MTBDRsl (v2.0) (Tagliani et al., 2015). The eis C14T mutation is regarded as a very good marker for KAN resistance and causes very high levels of KAN resistance (MICs 16 to 32 μg/mL) (Gikalo et al., 2012). Our CAP MIC data ranges between 8–64 μg/mL with no mutations within eis resistant isolates (Zimenkov et al., 2013). The eis C14T and C12T mutations are considered as borderline or low-level to KAN drug (Gikalo et al., 2012). Mutations within eis gene increases the virulence of M. tuberculosis that are thought to be responsible for preservation of bacterial fitness, and could be the reason for the extensive transmission of drug-resistant TB strains in Russia (Casali et al., 2014). Absence of AMK/CAP cross-resistance in strains harbouring mutations in the eis gene has been recently demonstrated (Casali et al., 2014; Gikalo et al., 2012). Our study also confirms that none of the eis mutations found in AMK/CAP cross-resistant isolates.

The V177H mutation within Rv1258c found in one XDR-TB isolate with AMK/CAN cross-resistance. The Rv1258c is a stable gene region (Ainsa et al., 1998) and appearance of a mutation could lead to higher efflux pump activity, as suspected among isolates with this cross-resistance (Albert et al., 2010; Reeves et al., 2013). Interestingly two mutations within Rv0194 of R83G and G170V were detected in pre-XDR-TB and XDR-TB isolates respectively. Similar to other investigators we found mutations within Rv0194 in an XDR-TB strain that lacked known mutations (Ilina et al., 2013; Liu et al., 2013). The eis C14T mutation is contradictory in KAN resistance and has been found in isolates with an additional eis of C14T mutation (Jnawali et al., 2013). The rrsl 500 mutations are usually found in isolates that are resistant to KAN but at a low frequency (Jugheli et al., 2009; Maus et al., 2005a). The G878A mutation has not yet been reported in KAN/CAP cross-resistance. The KAN/CAP cross-resistant isolates usually lack known mutations however changes within whiB7 and Rv3728 have been reported (Casali et al., 2012; Casali et al., 2014; Du et al., 2013).

Capreomycin mono-resistance is associated with mutations in rrs and tlyA genes (Georghiou et al., 2012; Maus et al., 2005b) but tlyA mutations are regarded as weak markers due the diversity of mutations and their appearance in susceptible isolates as well (Engstrom et al., 2011; Engstrom et al., 2012; Sowajasatakul et al., 2014). We detected the rrsl G878A mutation in 21/124 (17%) resistant isolates and of those 9/21 (43%) were resistant to CAP. The rrsl G878A mutation has been previously been detected and its function not well understood (Daum et al., 2012). Our CAP MIC data ranges between 8–64 μg/mL on isolates harbouring this mutation, while there might be an association with EuroAmerican X3 lineage. We were able to show association of G878A with EuroAmerican X3 lineage ($P < 0.0001$). The G878A mutation may be a novel mutation for CAP resistance and an intrinsic resistant marker for X3 genotype. Most recently, Reeves et al. also mentioned that a CAP MIC of 8 μg/mL with no mutations within rrsl gene could be due to an unidentified mechanism (Reeves et al., 2015).

Discordance of CAP resistance between molecular and phenotypic methods is common due to unreliable critical concentrations and lack
of consistent molecular markers (Reeves et al., 2015). This limitation of CAP phenotypic testing highlights the importance of molecular diagno-
sis of CAP resistance. We used the WHO recommended critical concentra-
tions of 2.5 μg/mL in MGIT 960, similar discordant results were found by
others (Kam et al., 2010; Rodwell et al., 2014). A higher critical con-
centration of 10 μg/mL could clearly distinguish between resistant and
susceptible CAP isolates (Fitzwater et al., 2013; Trollip et al., 2014).
In conclusion, our study shows an association of certain genetic
markers with different SLIDs cross-resistant patterns. The G878A muta-
tion predominantly found in strains of the EuroAmerican X3 family is a
new mechanism of resistance to CAP. The inclusion of this mutation in
diagnostic assays may increase the sensitivity for SLIDs resistance. In-
formation on predominant genotypes and mutations in regions can furth-
ernore be used when developing region-specific assays. Most of the SLIDs
cross-resistance was associated with XDR-TB and this highlights the
need to increase the sensitivity of diagnostic assays. A combination of muta-
tions is required to improve the detection of SLIDs cross-resistance.

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