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

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ABSTRACT

Mutations causing mono and cross-resistance among amikacin, kanamycin and capreomycin of second-line injectable drugs (SLIDs) namely are not well understood. We investigated 124 isolates of *Mycobacterium tuberculosis* for mutations within *rrs*, *eis*, *tlyA* and efflux pump (Rv1258c and Rv0194) genes involved in resistance towards SLIDs. The distribution of mutations across these genes were significantly different in strains with mono-resistance or cross-resistance. A new mutation G878A was found in *rrs* gene, among strains with capreomycin mono-resistant, or in strains with cross-resistance of capreomycin, kanamycin and amikacin. This mutation was associated with the Euro-American X3 lineage ($P < 0.0001$). Mutations in the two efflux genes Rv1258c and Rv0194 were confined to strains with only capreomycin/amikacin/kanamycin cross-resistance. We further investigated the minimum inhibitory concentration of capreomycin on isolates with new G878A mutation ranging from 8 μg/mL to 64 μg/mL. Inclusion of G878A on new molecular assays could increase the sensitivity of capreomycin resistance detection.

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 (Georghiou 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 (Georghiou 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). Jugheli et al. found an association between the A1401G mutation and resistance to AMK and KAN with moderately high specificity and sensitivity (Jugheli 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

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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 MTBDR*sl*) which decrease their sensitivity (Engstrom et al., 2012; Georghiou 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 aminoglycosides 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*, *tlyA* 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 MTBDR*sl* was run on all specimens as previously explained according to the manufacturer (Hain lifescience, Germany) (Hillemann et al., 2009). Discordant isolates were amplified into seven genes by PCR using primers of *rrs* (500, 900, and 1400), *eis*, *tlyA*, 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 H_2O (dH_2O), 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 (Ocimum 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
List of primers used for sequencing of genes.

Primer	Sequence	Target size	Reference
rrs 500	Forward:5′gatgacggccttcgggttgt3′ Reverse: 5′tctagtctgcccgtatcgcc3′	123 bp	(Honore and Cole, 1994)
rrs 900	Forward:5′gtagtccacgccgtaaacgg3′ Reverse: 5′ aggccacaagggaacgccta3′	222 bp	(Honore and Cole, 1994)
rrs 1400	Forward: 5′gtccgagtgttgcctcagg3′ Reverse: 5′gtcaactcggaggaaggtgg	516 bp	(Campbell et al., 2011)
eis	Forward:5′ gcgtaacgtcacggcgaaattc3′ Reverse: 5′gtcagctcatgcaaggtg3′	567 bp	(Campbell et al., 2011)
tlyA	Forward:5′atgtcggataggccagctg3′ Reverse:5′acttttttctacgcgcgccgtgc3′	555 bp	(Campbell et al., 2011)
Rv0194	Forward: 5′gcgacctacttgctgatgta3′ Reverse: 5′cgctggaactccagtgataa3′	700 bp	This study
Rv1258c	Forward:5′cggcattcctgatcctgtt3′ Reverse: 5′cgtgtggtcggtgaagtatt3′	700 bp	This study

Table 2
Distribution of resistance patterns among pre-XDR-TB and XDR-TB.

Resistance pattern	Pre-XDR-TB No. (%)	XDR-TB No. (%)	P*	Total No. (%)
AMK/KAN/CAP	18 (38)	29 (62)	0.02	47 (38)
AMK/KAN	2 (20)	8 (80)	0.001	10 (8)
AMK/CAP	6 (60)	4 (40)	0.37	10 (8)
KAN/CAP	3 (75)	1 (25)	0.15	4 (3)
AMK	2 (100)	0 (0)	n/a	2 (2)
KAN	13 (65)	7 (35)	0.004	20 (16)
CAP	23 (74)	8 (26)	0.000	31 (25)

AMK = Amikacin; KAN = Kanamycin, CAP = Capreomycin.
n/a = non-applicable.
* Pearson's chi-square.

3. Results

3.1. Level of drug resistance and association among pre-XDR-TB and XDR-TB

Of the 124 isolates, 47 (38%) were cross-resistant to AMK/KAN/CAP drugs. Among those, 29 (62%) and 18 (38%) (62% vs 38%, p=0.02) (Table 2) isolates belonged to XDR-TB and pre-XDR-TB cases respectively. The AMK/KAN and AMK/CAP cross-resistant cases were both ten (8%) while four cases (2%) were resistant to KAN/CAP drugs. The AMK/KAN resistance was found in eight (80%) of XDR-TB strains, while AMK/CAP resistance was found in 60% of pre-XDR-TB. The KAN/CAP resistance was found in 75% of XDR-TB cases. Mono-resistant cases of AMK, KAN and CAP drugs were two (2%), 20 (16%) and 31 (25%) respectively. Most of the 23 (74%) CAP and 13 (65%) KAN isolates belonged to pre-XDR-TB. The Genotype MTBDR*sl* assay detected A1401G mutation in 22 (47%) and one (10%) cases with AMK/KAN/CAP and AMK/KAN resistance respectively. A combination of Genotype MTBDR*sl* and DNA sequencing revealed 72 mutations within *rrs*, *eis*, *tlyA*, Rv1258c, Rv0194 genes and increased sensitivity of both AMK/KAN/CAP and AMK/KAN to 70% and KAN/CAP to 75%. Mutations in KAN and CAP mono-resistant strains were detected by sequencing at a frequency of 70% and 32% respectively (Table 3).

3.2. Molecular analysis of cross-resistance

For the analysis of AMK/KAN/CAP cross-resistance, we sequenced seven genetic regions namely *rrs* (1400, 900, 500), *eis*, *tlyA*, Rv1258c and Rv0194. We detected 33 (70%) AMK/KAN/CAP cross-resistant isolates with mutations within these regions. The *rrs* A1401G was found in 23 (49%), followed by *rrs* G878A in four isolates, *eis* C14T and C12T occurred within two isolates while *tlyA* T257G change found in one isolate. In another isolate while various mutations within the 500 region were also found. One isolate (XDR) had a novel amino acid change of Y177H within Rv1258c efflux pump.

We had 24 isolates with cross-resistance to AMK/KAN and AMK/CAP drugs (Table 2), ten (42%) among both AMK/KAN and AMK/CAP drugs, and only four isolates with CAP/KAN resistance. We also sequenced seven regions namely *rrs* (1400, 900, 500), *eis*, *tlyA*, Rv1258c and Rv0194. The frequency of detected mutations were seven 7/10 (70%) for AMK/KAN, five (50%) for AMK/CAP and three (75%) for KAN/CAP. Four of the ten AMK/KAN resistant isolates had mutations of *eis* C14T while another three had *rrs* mutations (A1401G, G878A and G837T). The two of the ten isolates had T1238A and G878A that were detected in the *rrs* regions of 1400 and 900 respectively. One isolate had various mutations within *rrs* 500 region. Sequence analysis of Rv0194 efflux pump revealed G170V and R83G novel mutations in two AMK/CAP resistant isolates.

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 *rrs* (1400, 900, 500), *eis*, *tlyA*, Rv1258c and Rv0194 mutations were found in 10/31 (32%) and 14/21 (70%) respectively for CAP and KAN mono-resistant isolates but none 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 level at 32 μg/mL, two were of S genotype. One isolate 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 Rv0194 mutations with SLIDs cross and mono-resistant cases within XDR and pre-XDR-TB. The mutations were distributed across all cases but those in Rv1258c and Rv0194 were confined to AMK/KAN/CAP and AMK/CAP cross-resistant cases respectively.

Table 3
Distribution of mutations among cross and mono-resistant cases.

Mutation	AMK*/KAN*/CAP*** n=47	AMK/KAN n=10	AMK/CAP n=10	KAN/CAP n=4	CAP n=31	KAN n=20	Total#
*rrs*1400	23	1	1	0	0	1	26
*rrs*900	4	2	1	1	9	5	22
*rrs*500	1	0	1	1	0	3	6
eis	3	4	0	1	0	5	13
tlyA	1	0	0	0	1	0	2
Rv0194	0	0	2	0	0	0	2
Rv1258c	1	0	0	0	0	0	1
Total (%)	33 (45.8%)	7 (9.7%)	5 (6.9%)	3 (4.2%)	10 (13.9%)	14 (19.4%)	72

*AMK = Amikacin; **KAN = Kanamycin, ***CAP = Capreomycin, #$P < 0.000$.

Table 4
Distribution of the G878A mutation among genotypes of *Mycobacterium tuberculosis*.

Phenotypic Resistance	Strains harbouring G878A mutation	EuroAmerican Lineage			Non-EuroAmerican Lineage		Total
		X3	X2	S	Beijing	EIA	
AMK/KAN/CAP	4 (18.2%)	3			1		4
CAP/KAN	1 (4.5%)	1					1
AMK/CAPC	1 (4.5%)			1			1
AMK/CAP	1 (4.5%)	1					1
CAP	10 (50%)	9	1				10
KAN	4 (18.2%)	2		1		1	4
Total (%)	21 (100%)	16 (75%)	1 (5%)	2 (10%)	1 (5%)	1 (5%)	21 (100%)

AMK = Amikacin; KAN = Kanamycin, CAP =Capreomycin.

Our study is in agreement with other's on the association of the *rrs* A1401G mutation with AMK/KAN/CAP cross-resistance (Campbell et al., 2011; Engstrom et al., 2012; Jugheli et al., 2009; Maus et al., 2005a) but 20% of isolates were found to lack this mutation (Georghiou et al., 2012; Liu et al., 2013). We combined mutations within *eis*, *rrs*, *tlyA*, Rv1258c and Rv0194 to report a sensitivity of 70% in prediction of cross-resistance. Jugheli et al. found higher sensitivities by using A1401G mutation on its own, but most of these investigators' isolates belonged to Beijing genotype (Jugheli et al., 2009). This mutation was found to have a significant association with the Beijing genotype as compared to non-Beijing genotypes (Miotto et al., 2012). We found high genotypic diversity among cross-resistant isolates, most of them belonged to the EuroAmerican family, which could explain our lower sensitivity. The EuroAmerican family has significantly higher phylogenetic diversity than Beijing genotype (Casali et al., 2014). The A1401G mutation have been found among AMK/CAP and KAN/CAP resistant strains (Said et al., 2012; Sirgel et al., 2012), but we found the mutation in only one isolate with AMK/KAN resistance.

The *eis* C12T mutation was found in one isolate with AMK/KAN/CAP cross-resistance, despite it been shown to be present in KAN susceptible isolates (Zimenkov et al., 2013). Others have confirmed that this mutation has a low MIC level of KAN at 5 μg/mL (Gikalo et al., 2012; Huang et al., 2011; Tukvadze et al., 2014).

Table 5
The effect of mutations on minimal inhibitory concentrations of second-line drugs with different genotypes.

Strain	DST profile	Mutation	KAN MIC	AMK MIC	CAP MIC	Genotype
Cross-resistance						
361	AMK/KAN/CAP	C12T	8	8	16	X3
429	AMK/KAN/CAP	G878A	4	8	8	Beijing
102	AMK/KAN/CAP	C14T	16	16	64	S
S358	AMK/KAN/CAP	Y177H	16	16	32	H
Dual-resistance						
908	AMK/KAN	C14T	8	16	ND	X3
121	AMK/KAN	C14T	8	8	ND	X3
1989	AMK/KAN	C14T	16	16	ND	S
151	AMK/KAN	C14T	4	4	ND	X3
Mono-resistance						
1799	KAN	G985T	4	ND	ND	S
887	KAN	G37T	8	ND	ND	S
671	KAN	G37T	4	ND	ND	T1
211	KAN	A1219T	16	ND	ND	T1
B107	KAN	C517T	4	ND	ND	T1
591	CAP	G878A	ND	ND	32	S
569	CAP	G878A	ND	ND	32	S
49	CAP	G878A	ND	ND	32	X3
94	CAP	G878A	ND	ND	8	X3
212	CAP	G878A	ND	ND	32	X3
1040	CAP	G878A	ND	ND	32	X3
33	CAP	G878A	ND	ND	64	S
S85	CAP	T257G	ND	ND	8	S
H37Rv	Sensitive	WT	2	1	1	

AMK-Amikacin, KAN-Kanamycin, CAP-Capreomycin, MIC-Minimal Inhibitory Concentrations, ND-not done.

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 increased the sensitivity for SLIDs resistance, as was shown in a new version of the Genotype MTBDR*sl* (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 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* G37T 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 are found in AMK/CAP cross-resistant isolates.

The Y177H mutation within Rv1258c found in one XDR-TB isolate with AMK/KAN/CAP 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., 2014).

The C517T mutation is contradictory in KAN resistance and has been found in isolates with an additional *eis* of C14T mutation (Jnawali et al., 2013). The *rrs* 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; Sowajassatakul et al., 2014).

We detected the *rrs* G878A mutation in 21/124 (17%) resistant isolates and of those 9/21 (43%) were resistant to CAP. The *rrs* 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 *rrs* 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 diagnosis of CAP resistance. We used the WHO recommended critical concentrations 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 concentration 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 mutation 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. Information on predominant genotypes and mutations in regions can furthermore 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 mutations is required to improve the detection of SLIDs cross-resistance.

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