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Running title: Resistance to second line injectable drugs in MDR-TB

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ABSTRACT

The second line injectable drugs (SLID, i.e. amikacin, kanamycin, capreomycin) are key drugs for the treatment of multidrug-resistant tuberculosis. Mutations in *rrs* region 1400, *tlyA* and *eis* promoter are associated with resistance to SLID, to capreomycin and to kanamycin respectively. In this study, the sequencing data of SLID resistance-associated genes were compared to the results of phenotypic drug susceptibility testing by the proportion method for the SLID in 206 multidrug-resistant clinical isolates of *Mycobacterium tuberculosis* collected in France. Among the 153 isolates susceptible to the 3 SLID, 145 showed no mutation, 1 harbored T1404C plus G1473A mutations in *rrs* and 7 had an *eis* promoter mutation. Among the 53 strains resistant to at least 1 of the SLID, mutations in *rrs* accounted for resistance to amikacin, capreomycin and kanamycin for 81%, 75% and 44% isolates, respectively, while mutations in *eis* promoter were detected in 44% of the isolates resistant to kanamycin. By contrast, no mutations in *tlyA* were observed in the isolates resistant to capreomycin. The discrepancies observed between the genotypic (on the primary culture) and phenotypic drug susceptibility testing were explained by i) resistance to SLID with MICs close to the critical concentration used for routine DST and not detected by phenotypic testing (n=8, 15% of SLID-resistant strains), ii) low-frequency heteroresistance not detected by sequencing of drug resistance-associated genes on the primary culture (n=8, 15% of SLID-resistant strains), and iii) to other resistance mechanisms not yet characterized (n=7, 13% of SLID-resistant strains).

INTRODUCTION

The emergence of multidrug-resistant tuberculosis (MDR-TB; 580,000 cases worldwide), which is resistant to at least rifampin (RIF) and isoniazid (INH), and, more recently, extensively drug-resistant tuberculosis (XDR-TB; 55,000 cases worldwide), which is resistant to any fluoroquinolone and at least one of three second line injectable drugs (SLID, i.e., amikacin (AMK), kanamycin (KAN), or capreomycin (CAP)), is widely considered to be a serious threat to global health (1). Treatment of MDR-TB is based on the association of fluoroquinolones and SLID (2). As a consequence of inadequate use of second-line treatments, XDR-TB, with an overall successful treatment outcome of only 50%, has progressed (3). The lack of ability to perform Drug Susceptibility Testing (DST) is partly responsible of the misuse of antituberculous drugs in several countries (1).

Rapid detection of drug resistance is essential to designing appropriate treatment regimens, preventing treatment failure, and reducing the spread of drug-resistant isolates. Since conventional phenotypic methods are cumbersome and take weeks to months to obtain drug resistance profile, molecular assays for the detection of mutations that confer resistance have been increasingly used, even in areas where DST capacities are very limited or not available, and have the potential to shorten the time to detection of resistance to one working day (4-6). The molecular tests for diagnosing resistance to antituberculous drugs are based on the detection of mutations affecting the function and/or expression of chromosome-encoded targets.

The SLID bind to the 16S rRNA in the 30S ribosomal subunit and inhibit protein synthesis (7). Cross-resistance to second line injectable drugs (AMK, KAN and CAP) is known to be caused by mutations at positions 1401, 1402 and 1484 in the *rrs* gene encoding 16S rRNA with the following expression patterns: *rrs* substitution A1401G displays CAP resistance with disparities in resistance levels, and high-level resistance to AMK and KAN; the *rrs* C1402T

substitution displays low-level resistance to KAN, high-level resistance to CAP, and retains susceptibility to AMK; and the *rrs* G1484T substitution displays high-level resistance to all 3 drugs (8, 9). However, these mechanisms in *rrs* have never been formally demonstrated by allelic exchange data. The most frequent mutations in strains resistant to SLID are in *rrs* region 1400, mainly A1401G, which accounts for 42 to 100% of global *M. tuberculosis* strains resistant to AMK, CAP and KAN (4, 5, 7, 10-12). Other mechanisms not linked to *rrs* have been shown to confer cross-resistance to some of the SLID (13-15). Mutations G-37T, C-14T, C-12T, and G-10A in the promoter region of the *eis* gene (encoding an aminoglycoside acetyltransferase) are responsible for resistance to KAN with minimal inhibitory concentrations (MICs) sometimes close to the critical concentration used for routine DST, especially for C-12T (16, 17). Such mutations are found in 30 to 80% of the strains resistant to KAN without mutation in *rrs* (17-19). In addition, mutations in the *tlyA* gene, which encodes a 2'-*O*-methyltransferase that modifies nucleotides in 16S rRNA and 23S rRNA, have been suggested to confer isolated resistance to CAP in *M. tuberculosis*, because the unmethylated ribosome is insensitive to the drug (20, 21).

The goals of the present study were to compare the sequencing data of SLID resistance-associated genes (*rrs* region 1400, *eis* promoter, *tlyA*) to the results of phenotypic DST by the proportion method for the SLID in 206 multidrug-resistant clinical isolates of *M. tuberculosis* collected in France, and to analyze the discrepancies between genotypic and phenotypic DST.

MATERIALS AND METHODS

Two-hundred and six MDR *M. tuberculosis* clinical isolates collected in 2010-2014 at the French Reference Center for Mycobacteria (NRC MyRMA) and randomly selected were included: 153 AMK/CAP/KAN susceptible isolates, and 53 resistant to at least 1 of the 3

SLID (23 R-AMK/CAP/KAN, 22 monoR-KAN, 3 R-AMK/KAN, 2 R-CAP/KAN, 3 monoR-CAP) including 29 XDR. Fifty-nine strains used for this study were isolated from patients enrolled in a previous study evaluating the performance of the MTBDR*sl* v2.0 assay (22). A table with MIRU-VNTR results and the names of the corresponding genotypes is provided as supplemental data.

In vitro DST for SLID was performed on Löwenstein-Jensen medium following the proportions method, using concentrations of 20 mg/liter for amikacin, 40 mg/liter for capreomycin, and 30 mg/liter for kanamycin (23). It has to be noted here that the AMK critical concentration used in this study was lower than the value endorsed by the WHO in the 2014 guidelines (30 mg/liter) (24). Resistance to SLID was defined as a proportion of resistant mutants ≥1% (23). The DST was repeated and MICs were determined on Middlebrook 7H10 plates (24) containing KAN at 0.625, 1.25, 2.5, 5, 10, 20, 40 mg/liter, AMK and CAP at 0.5, 1, 2, 4, 8, 16, 32 mg/liter for all the strains with unexpected combinations of resistances and mutations (and not explained by a low percentage of resistant mutants not detected by sequencing of drug resistance-associated genes on the primary culture but detected by sequencing from tubes with antibiotics). The MIC was defined as the lowest concentration of drug resulting in growth of ≤1% of the initial inoculum after 4 weeks of incubation at 37°C (9). The reference strain *M. tuberculosis* H37Rv (ATCC 27294) sensitive to all the drugs tested in our experiment was included as a control strain. The critical concentrations on 7H10 for KAN, AMK, CAP were 5.0, 4.0 and 4.0 mg/liter, respectively (24).

The SLID resistance-associated genes (*rrs* region 1400, *eis* promoter and *tlyA*) were amplified and sequenced for the 206 MDR strains as previously reported (4), using the oligonucleotide primers pairs previously described (4, 6). For the 15 strains with resistance to at least 1 of the 3 SLID not explained by mutations in resistance-associated genes on the primary culture

(culture obtained directly from the patient's sample cultivated without antibiotics), the resistance-associated genes were also sequenced from the strains that grew on tubes containing the antibiotics.

The R-KAN and S-KAN isolates were compared for mutations using Fisher's exact test. The MDR isolates susceptible to AMK/CAP/KAN and MDR resistant to at least 1 of the 3 SLID were also compared for country of birth of patients using Fisher's exact test. *P* values were two-tailed, and *P* values ≤0.05 were considered significant.

The nucleotide sequences determined for the mutant genes included in the present report were deposited into the GenBank database under the following accession numbers: GU323404, GU323405, KU160149, and KU160150 for the *rrs* mutants A1401G, G1484T, C1402T and T1404C+G1473A, respectively; KU160151-KU160154 for the *eis* promoter G-10A, C-12T, C-14T, and G-37T.

RESULTS

A significant proportion of the strains included in the present study were isolated from patients born in the former Soviet Union: 37% (n=76) among the 206 MDR clinical isolates, 85% (n=45) among the 53 MDR isolates resistant to at least AMK, KAN or CAP, and 95% (n=21) among the 22 MDR isolates mono-resistant to KAN. The phylogenetic diversity has been evaluated by determining the MIRU-VNTR codes of the strains (supplemental Table). The main clades corresponded to Beijing (44 S-SLID and 35 R-SLID), LAM (27 S-SLID and 11 R-SLID), Haarlem (11 S-SLID and 4 R-SLID) and non typeable strains generally linked to the T strains family (35 S-SLID and 1 R-SLID). The remaining strains belonged to the S (n=8), Ghana (n=6), Delhi-CAS (n=5), EAI (n=4), Ural (n=6), Cameroon (n=3), Uganda II (n=2), NEW-1 (n=1), Bovis (n=1), Africanum (n=1), X (n=1) and TUR (n=1) families.

In total, 53 isolates were resistant to at least one of the three SLID. Among the 23 R-AMK/CAP/KAN isolates, 20 isolates showed a A1401G mutation (including one with an additional C-14T mutation in the *eis* promoter), 1 isolate a C1402T mutation in *rrs*, and 2 isolates a C-14T mutation in the *eis* promoter (Table 1). For the three latter strains, resistance to AMK (1 strain) and AMK and CAP (2 strains) was not explained by mutations in drug resistance-associated genes when the sequencing was done from the primary culture (tubes without antibiotics). The R-AMK/CAP/KAN strain with the mutation C1402T in *rrs* (isolate no. 21), which accounts for resistance to KAN and CAP but not to AMK, showed no other mutation than *rrs* C1402T when the sequencing was done from the tubes containing AMK on which 10% of resistant mutants grew. The corresponding AMK MIC value was 2 mg/liter (Table 1). When tested from tubes containing AMK or CAP, one of the 2 R-AMK/CAP/KAN strains (isolate no. 22) displaying only mutation C-14T in the *eis* promoter, which accounts for KAN resistance only, finally showed a *rrs* A1401G mutation in addition to the *eis* C-14T promoter mutation when the sequencing was done from the 2% of colonies growing on AMK or CAP-containing tubes. For the remaining strain (isolate no. 23) which showed 100% of resistant mutant on AMK or CAP and had MIC values of 16 mg/liter for both drugs, no other mutation than *eis* C-14T was detected in *rrs* or *tlyA* after sequencing of the resistance-associated genes from tubes containing the corresponding antibiotics (Table 1).

Three strains were resistant to AMK and KAN, but susceptible to CAP (Table 1). One R-AMK/KAN strain (no. 24) showed a *rrs* A1401G mutation known to be associated with AMK, KAN and CAP resistance. This strain was confirmed to be susceptible to CAP (MIC = 1 mg/liter) (Table 1). The second R-AMK/KAN strain (isolate no. 25) showed only a C-14T mutation in the *eis* promoter. When the colonies growing on tube containing AMK (with 1% of resistant mutants) were sequenced, this strain exhibited no other mutation than the C-14T mutation in the *eis* promoter. The MIC of AMK for this isolate was 4 mg/liter, i.e. identical to

the AMK breakpoint value (Table 1). The third R-AMK/KAN strain (no. 26) had no mutation detectable from the primary culture yielding 3% of resistant mutants on AMK and KAN. When the colonies growing on tubes containing AMK and KAN were tested, a *rrs* A1401G mutation, but no mutation in the *eis* promoter and *tlyA*, was detected.

Two strains were resistant to CAP and KAN but displayed susceptibility to AMK (Table 1). One of these 2 strains (isolate no. 27), with 100% of resistant mutant on CAP, harbored an *eis* promoter mutation G-37T and no mutation in *rrs* region 1400 and *tlyA* when tested from the tubes with or without CAP. The CAP MIC value for this isolate was 16 mg/ml (Table 1). For the other R-CAP/KAN strain (isolate no. 28), with no mutation on primary culture and 3% of resistant mutants on CAP and KAN, a *rrs* A1401G mutation was finally detected on CAP and KAN tubes.

Three strains were monoR to CAP. They had no mutation in *rrs* region 1400, *tlyA* and *eis* promoter on primary culture (Table 1). Two displayed 100% of resistant mutants and 1 only 2% of resistant mutants on the CAP-containing tubes. The elevated MIC values for isolates no. 29 and 30 were concordant with the DST results (Table 1). For the 3 strains, we detected no mutation on primary cultures, nor from the colonies growing on the CAP tubes.

Among the 22 monoR-KAN isolates, 17 displayed a mutation in the *eis* promoter from the primary cultures (G-10A: n=8, C-12T: n=1, C-14T: n=2, G-37T: n=6) and no mutation in *rrs* and *tlyA* (Table 1). For the 5 remaining monoR-KAN strains, we found no mutation in *rrs*, *eis* promoter and *tlyA* on the primary sequencing, but all had a percentage of resistant mutants varying between 1 and 10%. When the sequencing was done from the tubes with antibiotics, the 5 strains showed an *eis* promoter mutation on tubes with KAN (C-14T in 3 strains and G-37T in 2 strains) (Table 1).

We included in our study 153 isolates susceptible to AMK/KAN/CAP. No mutation was detected in these isolates, except for one isolate with a T1404C plus G1473A double

mutation in *rrs*, and for 7 isolates with *eis* promoter mutations (G-10A: n=3, C-12T: n=1, C-14T: n=1, G-37T: n=2) (Table 1). Isolate no. 54 (*rrs* T1404C plus G1473A) was confirmed by MIC determination to be susceptible to the 3 drugs (MICs of KAN, AMK and CAP = 1.25, 1 and 1 mg/liter, respectively). For the 7 other strains (isolates no. 55-61), for which the phenotype of drug susceptibility was not explained by the mutations found from the primary cultures, they were confirmed to be susceptible to KAN with MIC values ranging from 2.5 to 5 mg/liter, except for isolate no. 60 which displayed a KAN MIC value of 10 mg/liter (Table 1).

DISCUSSION

When MDR TB is detected, the main therapeutic issue that must be addressed is to determine the susceptibility of the strain to second line drugs, particularly fluoroquinolones and SLID (AMK, KAN and CAP). Since *in vitro* testing is particularly cumbersome, difficult to interpret for second line drugs and takes weeks to months to obtain drug resistance profile, rapid detection of resistance to these drugs by molecular methods is of major interest.

In our study, mutations in *rrs* region 1400 accounted for resistance to AMK for 81% (21/26) (considering that *rrs* C1402T is not associated with AMK-R), to CAP for 75% (21/28), and to KAN for 44% (22/50) (Table 1). These figures are in accordance with those previously published by other groups, which reported mutations in *rrs* region 1400, mainly A1401G, accounting for resistance to SLID for respectively: 56% to 100% for AMK, 51% to 96% for CAP and 44% to 84% for KAN (4, 5, 7, 8, 10-12, 14, 26, 27). We observed in our study that *eis* promoter mutations were present in 22/50 (44%) of the R-KAN strains on the primary cultures. Among the detected mutations, G-10A, C-14T and G-37T were more frequent (8/50 (16%), 6/50 (12%) and 7/50 (14%), respectively), while C-12T was rare (1/50

(2%)). It is noticeable that a significant number of S-KAN strains (7/156 (4.5%) also showed mutations in the *eis* promoter (although these mutations are more common in R-KAN strains; $P = 0.0004$) (see Table 1). Finally, no mutation in *tlyA* was observed in any of our isolates, so *tlyA* does not seem to be implicated in CAP resistance in our MDR clinical isolates. Mutations in the *tlyA* gene associated with CAP resistance were reported to be rare in the surveyed literature (found in ~0-3% of resistant strains) and their implication in resistance not undoubtedly established (4, 7, 8, 10, 11, 14, 26-31).

Overall, among the 206 strains, discrepancies between genotypic and phenotypic DST were observed in 23 (11%) strains. On one hand, sequencing performed on primary culture did not show mutations in *rrs* region 1400, *tlyA* or *eis* promoter that could account for resistance in 15/53 strains resistant to at least 1 of the 3 SLID. On the other, mutations in the *eis* promoter were found in 7/153 strains susceptible to KAN, and 1 strain showing a *rrs* 1401G mutation associated to AMK, KAN but also CAP resistance was found to be susceptible to CAP in phenotypic DST (Table 1). Three hypotheses, that are discussed below, can be made to explain the discrepancies between the genotypic results (performed on the primary culture) and the phenotypic DST results: 1) a low percentage of resistant mutants precluding the detection by sequencing of resistance-associated genes on the primary culture, 2) resistance to SLID with MICs close to the critical concentration used for routine DST and not detected by phenotypic testing, and 3) other resistance mechanisms not yet characterized.

In the frame of the first hypothesis, a low percentage of resistant mutants can preclude the detection by sequencing of resistance-associated genes on the primary culture. Indeed, the molecular tests are less efficient than conventional culture-based DST in finding resistance in samples with heteroresistant bacteria, i.e. a minority of resistant variants in a susceptible main population. A previous study showed that 1% resistant bacteria in a mixture of susceptible and resistant *M. tuberculosis* was not detected by line probe assay or Sanger sequencing,

while it is generally detected by using phenotypic DST (32). The same study showed that a proportion of more than 10% resistant bacteria was required for detection of resistance by Sanger sequencing (32). Therefore, we resequenced *rrs* 1400, *tlyA* and *eis* promoter regions from the colonies growing on the tubes containing the SLID when an unexplained resistance was noted on the primary culture. The complementary sequencing allowed the identification of mutations responsible for drug resistance for 2/5 (40%) AMK resistant strains without mutation on the primary culture (isolates no. 22, 26), 2/7 (29%) for CAP (isolates no. 22, 28) and 7/7 (100%) for KAN (isolates no. 26, 28, 49-53). Thus, 15% (8/53) of resistant strains displayed heterogeneous resistance to SLID, a situation in which the diagnostic performance of genotypic testing was poor because minority population was present in only a few percent. It can be noted here that the isolates 26 and 28 should be cross-resistant to the 3 drugs because of the A1401G mutation. Such random-susceptible results (CAP for isolate no. 26, and AMK for isolate no. 28) are due to the fact that the resistant population occurs at a low percentage of the total population.

Conversely, in case of resistance to SLID with MIC values close to the critical concentration used for routine DST, phenotypic DST can miss resistant strains detected by genotypic DST (second hypothesis). In particular, low-level KAN resistance caused by *eis* promoter mutations (detectable by the MTBDR*sl* v2.0 test endorsed by the WHO) may be missed by phenotypic tests alone (17, 18, 28, 31, 33, 34). One study has shown that the phenotypic DST on Löwenstein-Jensen medium does not adequately detect moderate- to low-level KAN resistance, and that the MGIT or MycoTB method should be preferred for testing phenotypic resistance to KAN (18). This could explain why we missed in our study the phenotypic diagnosis of KAN resistance for 7 strains harboring an *eis* promoter mutation (isolates no. 55 to 61) (Table 1). Interestingly, among the 7 isolates, 6 (no. 55-59 and 61) were confirmed to display MIC values close to the KAN breakpoint value (5 mg/liter) (Table 1). It

is therefore tempting to suggest that mutations in the *eis* promoter can confer MICs close to the critical concentration used, an hypothesis that would account for the significant proportion of discrepant S-KAN results observed in our study: 27% (3/11) for G-10A, 50% (1/2) for C-12T, 14% (1/7) for C-14T, and 25% (2/8) for C-37T. In France, AMK is the main SLID for MDR TB treatment and KAN is not used. However, in countries where KAN is the preferred SLID (as in Former Soviet Union) and *eis* promoter mutations dominate, these limits of phenotypic testing should be taken into account in the therapeutic strategy (35). It has to be pointed out here that the question of whether these isolates with *eis* promoter mutations and phenotypic susceptibility to KAN should be reported as resistant to KAN should be addressed by determining the impact of such mutations on patient outcome. In a recent study, Van Deun et al showed that rifampin susceptible strains displaying *rpoB* mutations are associated with poorer outcome than *rpoB* wild-type strains (36). In view of such results, we think that any strain displaying a mutation in the *eis* promoter should be not considered as susceptible until clinical studies analyze patient outcome.

Concerning the *rrs* C1402T mutation found in 1 R-AMK/CAP/KAN isolate in our study (isolate no. 21), it has to be highlighted that if this mutation is rather associated with resistance to only CAP and KAN, it has been reported that the MIC of AMK associated to this mutation can be close to the critical concentration of the drug (8). Accordingly, the strain displayed a MIC of 2 mg/liter, a value close to the breakpoint value (4 mg/liter). Similarly, isolate no. 25 which showed a low percentage of mutants resistant to AMK on primary cultures (1%) displayed a MIC of 4 mg/liter for the drug (Table 1). Thus, AMK resistance in these 2 isolates can be regarded as borderline and one cannot exclude that the 2 strains would have been ranked as S-AMK if a higher AMK concentration had been used on primary DST (30 mg/liter according to the WHO 2014 guidelines (24). One has to note that the WHO provides no evidence based on which the recommended critical concentrations have been set,

a point that has to be considered when explaining the discrepancies between genotype and phenotype, as previously suggested (9, 37).

The accuracy of the molecular tests in predicting susceptibility for AMK and CAP seems to be also limited by as-yet uncharacterized resistance mechanisms (third hypothesis). Considering the results reported here, 3 R-AMK strains (isolates no. 21, 23 and 25) had no AMK resistance-associated gene mutations from the tubes containing AMK. If strains no. 21 and 25 display borderline resistance to AMK (R-AMK on DST with MIC values of 2 and 4 mg/liter, respectively), isolate no. 23 was confirmed to be undoubtedly resistant to AMK with a MIC of 16 mg/liter, which strongly suggests that an unknown mechanism of resistance is present in this strain. Similarly, 5 R-CAP strains (strains n°23, 27, 29-31) had no CAP resistance-associated gene mutations on sequencing analysis performed from the tubes containing CAP. It is worth to note here that genes such as *whiB7* and *rrl* have previously been implicated in resistance to KAN and CAP in *M. tuberculosis* (20, 38, 39). Since very little is currently known about the contribution of such genes on SLID resistance, one cannot exclude that they may contribute to CAP-R in isolates no. 23, 27, 29 30 and 31 (10, 18, 38, 40).

One S-AMK/CAP/KAN strain (isolate no. 54) showed a double mutation T1404C plus G1473A in *rrs*. If the *rrs* G1473A mutation has never been reported, *rrs* T1404C was described by Walker et al in 53 strains including 51 susceptible to the 3 SLID, 1 R-KAN and 1 R-AMK (41). Therefore this mutation doesn't seem to be implicated in AMK, CAP or KAN resistance, as confirmed by the low MICs found for our isolate (Table 1).

Finally, for the unexpected susceptibility to CAP (MIC = 1 mg/liter) in 1 R-AMK/KAN isolate with a *rrs* A1401G mutation (mutation known to be associated with AMK, KAN and also CAP resistance) (isolate n°24), this discrepancy also deserves to be further investigated at the mechanistic level.

Our study has some limitations, in particular because we used a collection of strains representative of clinical isolates received at the French Reference Center for Mycobacteria. In this set of strains, among the MDR isolates, those with resistance to at least one SLID, and particularly monoR-KAN, are associated with patients born in the Former Soviet Union (P = 0.0007 and P = 0.007, respectively). This association between monoR-KAN and Former Soviet Union-born patients is not surprising since KAN is the preferred SLID in this country. Therefore, our results may be biased by the country or origin of the patients. We have also to mention that an epidemiological link was detected for 3 clusters of 2 strains each (6 strains) among the 53 isolates resistant to at least 1 of the 3 SLID and 7 clusters of S-AMK/KAN/CAM isolates (16 strains) by taking into account (i) the phylogenetic lineages of clinical isolates based on the Mycobacterial Interspersed Repetitive Unit Variable Number Tandem Repeat (MIRU-VNTR) 24-loci (40), (ii) the strain characteristics (resistance phenotype and genotype) and (iii) patient characteristics data (country of birth, city where the diagnosis was made, family ties) (Table 1).

CONCLUSION

The results presented in this study raise the questionable ability of PCR sequencing and phenotypic DST to properly classify strains as susceptible or resistant since discrepancies were observed in 23/206 (11%) strains. PCR sequencing performed on primary culture did not detect any mutation in *rrs* region 1400 (for AMK, CAP, KAN-R) and in *eis* promoter (for KAN-R) in 19% (5/26) isolates R-AMK, 25% (7/28) R-CAP and 16% (8/50) R-KAN. Phenotypic DST did not detect resistance among 8 strains with mutations conferring low-level resistance. Finally, it is of crucial importance to determine whether resistance to SLID with MICs close to the critical concentration used for routine DST and not detected by phenotypic

methods, or heterogeneous resistance not detected by genotypic methods, have an impact on treatment efficacy.

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Table 1. Genotypic and phenotypic results regarding SLID for the 206 MDR *M. tuberculosis* clinical isolates including the 53 MDR resistant to at least 1 of the 3 SLID

Aminoglycosides phenotype			No. of isolates (no. of XDR)	Isolates numbering	Sequencing of resistance-associated genes on strains on primary culture[a] (no. of isolates)			Phenotype not explained by mutation in resistance-associated genes on primary culture (% of resistant mutant in the proportion method on Löwenstein-Jensen) (MIC mg/liter on 7H10)[b]	Sequencing of resistance-associated genes on tube with antibiotics[c]
AMK	**CAP**	**KAN**			***rrs* region 1400[d]**	***eis* pro**	***tlyA***		
R	R	R	23 (13)	1-19	A1401G (19)	wt	wt		
				20	A1401G (1)	C-14T	wt		
				21	C1402T (1)	wt	wt	R-AMK (10%) (MIC 2)	[AMK] : *rrs* C1402T, *eis* pro wt, *tlyA* wt
				22-23	wt (2)	C-14T	wt	R-AMK(2%)-CAP(2%)	[AMK, CAP] : *rrs* A1401G, *eis* pro C-14T, *tlyA* wt
								R-AMK(100%)-CAP(100%) (MIC AMK 16, CAP 16)	[AMK, CAP] : *rrs* wt, *eis* pro C-14T, *tlyA* wt
R	S	R	3 (2)	24	A1401G (1)	wt	wt	S-CAP (MIC 1)	
				25	wt (1)	C-14T	wt	R-AMK (1%) (MIC 4)	[AMK] : *rrs* wt, *eis* pro C-14T, *tlyA* wt
				26	wt (1)	wt	wt	R-AMK(3%)-KAN(3%)	[AMK], [KAN] : *rrs* A1401G, *eis* pro wt, *tlyA* wt
S	R	R	2 (1)	27	wt (1)	G-37T	wt	R-CAP (100%) (MIC 16)	[CAP]: *rrs* wt, *eis* pro G-37T, *tlyA* wt
				28	wt (1)	wt	wt	R-CAP(3%)-KAN(3%)	[CAP], [KAN]: *rrs* A1401G, *eis* pro wt, *tlyA* wt

S	R	S	3 (3)	29-31	wt (3)	wt	wt	R-CAP (2%) (MIC 8)	[CAP] : *rrs* wt, *eis* pro wt, *tlyA* wt
								R-CAP (100%)[d] (MIC 16)	[CAP] : *rrs* wt, *eis* pro wt, *tlyA* wt
								R-CAP (100%)[d]	[CAP] : *rrs* wt, *eis* pro wt, *tlyA* wt
S	S	R	22 (10)	32-39	wt (8)[e]	G-10A	wt		
				40-45	wt (6)	G-37T	wt		
				46-47	wt (2)	C-14T	wt		
				48	wt (1)	C-12T			
				49-53	wt (5)	wt	wt	R-KAN (1%)	[KAN] : *rrs* wt, *eis* pro C-14T, *tlyA* wt
								R-KAN (2%)	[KAN] : *rrs* wt, *eis* pro G-37T, *tlyA* wt
								R-KAN (2%)	[KAN] : rrs wt, eis pro C-14T, *tlyA* wt
								R-KAN (3%)	[KAN] : *rrs* wt, *eis* pro G-37T, *tlyA* wt
								R-KAN (10%)	[KAN] : *rrs* wt, *eis* pro C-14T, *tlyA* wt
S	S	S	153	54	T1404C+G1473A (1)	wt	wt	S-SLID (MIC KAN 1.25, AMK 1, CAP 1)	
				55-57	wt (3)	G-10A	wt	S-KAN (MIC 2.5, 2.5, 5.0)	
				58-59	wt (2)	G-37T	wt	S-KAN (MIC 2.5, 5)	
				60	wt (1)	C-14T	wt	S-KAN (MIC 10)	
				61	wt (1)	C-12T	wt	S-KAN (MIC 2.5)	
				62-206	wt (145)[d]	wt	wt		

[a] primary culture = culture obtained directly from the patient's sample cultivated without antibiotics

[b] The critical concentrations on Löwenstein-Jensen for KAN, AMK, CAP were 30, 20 and 40 mg/liter, respectively (23). It has to be noted here that the AMK critical concentration used in this study was lower than the value endorsed by the WHO in the 2014 guidelines (30 mg/liter) (24). The critical concentrations on 7H10 for KAN, AMK, CAP were 5.0, 4.0 and 4.0 mg/liter, respectively (24). The MICs of H37Rv ATCC 27294 were 1 mg/liter for each drug, concordant with Juréen et al (25).

[c] antibiotics are indicated in brakets

[d] expected effect of the mutation:

rrs A1401G: resistance of high level to AMK and KAN, resistance of different levels to CAP

rrs C1402T: resistance of high level to CAP, resistance to KAN with MICs close to the critical concentration used for routine DST, and retained susceptibility to AMK

[e] Isolates with the same MIRU-VNTR 24-loci and epidemiological link: one cluster of 2 monoR-CAP isolates (n=2), two clusters of 2 monoR-KAN isolates each (n=4), and seven clusters of S-AMK/KAN/CAM isolates (n=16).