



HAL
open science

Molecular investigation of resistance to second line injectable drugs in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis* in France

Florence Brossier, Anne Pham, Christine Bernard, Alexandra Aubry, Vincent Jarlier, Nicolas Veziris, Wladimir Sougakoff

► To cite this version:

Florence Brossier, Anne Pham, Christine Bernard, Alexandra Aubry, Vincent Jarlier, et al.. Molecular investigation of resistance to second line injectable drugs in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis* in France. *Antimicrobial Agents and Chemotherapy*, 2017, 61 (2), pp.e01299-16. 10.1128/AAC.01299-16 . hal-01513642

HAL Id: hal-01513642

<https://hal.sorbonne-universite.fr/hal-01513642>

Submitted on 25 Apr 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Full-Length Paper**

2
3 **Molecular investigation of resistance to second line injectable drugs in multidrug-**
4 **resistant clinical isolates of *Mycobacterium tuberculosis* in France**

5
6
7 Running title: Resistance to second line injectable drugs in MDR-TB

8
9
10 Florence Brossier^{1,2,#}, Anne Pham¹, Christine Bernard^{1,2}, Alexandra Aubry^{1,2}, Vincent
11 Jarlier^{1,2}, Nicolas Veziris^{1,2}, Wladimir Sougakoff^{1,2}, on behalf of the CNR-MyRMA

12
13
14 ¹Sorbonne Universités, UPMC Univ Paris 06, INSERM, U1135, Centre d'Immunologie et des
15 Maladies Infectieuses (CIMI-Paris), Team E13 (Bacteriology), Paris, France;

16 ²AP-HP, Hôpital Pitié-Salpêtrière, Centre National de Référence des Mycobactéries et de la
17 Résistance des Mycobactéries aux Antituberculeux, Bactériologie-Hygiène, Paris, France.

18
19
20
21
22 * Correspondence to: Florence Brossier, Laboratoire de Bactériologie-Hygiène, Faculté de
23 Médecine, Pitié-Salpêtrière, 91 boulevard de l'Hôpital, F-75634 Paris Cedex 13, France. Tel:
24 (+33) 1 40 77 97 46. Fax: (+33) 1 45 82 75 77.

25 e-mail: florence.brossier@aphp.fr

26 **ABSTRACT**

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

45

46

47

48

49

50

51 **INTRODUCTION**

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

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

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

76 substitution displays low-level resistance to KAN, high-level resistance to CAP, and retains
77 susceptibility to AMK; and the *rrs* G1484T substitution displays high-level resistance to all 3
78 drugs (8, 9). However, these mechanisms in *rrs* have never been formally demonstrated by
79 allelic exchange data. The most frequent mutations in strains resistant to SLID are in *rrs*
80 region 1400, mainly A1401G, which accounts for 42 to 100% of global *M. tuberculosis*
81 strains resistant to AMK, CAP and KAN (4, 5, 7, 10-12). Other mechanisms not linked to *rrs*
82 have been shown to confer cross-resistance to some of the SLID (13-15). Mutations G-37T,
83 C-14T, C-12T, and G-10A in the promoter region of the *eis* gene (encoding an
84 aminoglycoside acetyltransferase) are responsible for resistance to KAN with minimal
85 inhibitory concentrations (MICs) sometimes close to the critical concentration used for
86 routine DST, especially for C-12T (16, 17). Such mutations are found in 30 to 80% of the
87 strains resistant to KAN without mutation in *rrs* (17-19). In addition, mutations in the *tlyA*
88 gene, which encodes a 2'-*O*-methyltransferase that modifies nucleotides in 16S rRNA and
89 23S rRNA, have been suggested to confer isolated resistance to CAP in *M. tuberculosis*,
90 because the unmethylated ribosome is insensitive to the drug (20, 21).

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

95

96

97 **MATERIALS AND METHODS**

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

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

106 *In vitro* DST for SLID was performed on Löwenstein-Jensen medium following the
107 proportions method, using concentrations of 20 mg/liter for amikacin, 40 mg/liter for
108 capreomycin, and 30 mg/liter for kanamycin (23). It has to be noted here that the AMK
109 critical concentration used in this study was lower than the value endorsed by the WHO in the
110 2014 guidelines (30 mg/liter) (24). Resistance to SLID was defined as a proportion of
111 resistant mutants $\geq 1\%$ (23). The DST was repeated and MICs were determined on
112 Middlebrook 7H10 plates (24) containing KAN at 0.625, 1.25, 2.5, 5, 10, 20, 40 mg/liter,
113 AMK and CAP at 0.5, 1, 2, 4, 8, 16, 32 mg/liter for all the strains with unexpected
114 combinations of resistances and mutations (and not explained by a low percentage of resistant
115 mutants not detected by sequencing of drug resistance-associated genes on the primary culture
116 but detected by sequencing from tubes with antibiotics). The MIC was defined as the lowest
117 concentration of drug resulting in growth of $\leq 1\%$ of the initial inoculum after 4 weeks of
118 incubation at 37°C (9). The reference strain *M. tuberculosis* H37Rv (ATCC 27294) sensitive
119 to all the drugs tested in our experiment was included as a control strain. The critical
120 concentrations on 7H10 for KAN, AMK, CAP were 5.0, 4.0 and 4.0 mg/liter, respectively
121 (24).

122 The SLID resistance-associated genes (*rrs* region 1400, *eis* promoter and *tlyA*) were amplified
123 and sequenced for the 206 MDR strains as previously reported (4), using the oligonucleotide
124 primers pairs previously described (4, 6). For the 15 strains with resistance to at least 1 of the
125 3 SLID not explained by mutations in resistance-associated genes on the primary culture

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

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

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

138

139

140 **RESULTS**

141 A significant proportion of the strains included in the present study were isolated from
142 patients born in the former Soviet Union: 37% (n=76) among the 206 MDR clinical isolates,
143 85% (n=45) among the 53 MDR isolates resistant to at least AMK, KAN or CAP, and 95%
144 (n=21) among the 22 MDR isolates mono-resistant to KAN. The phylogenetic diversity has
145 been evaluated by determining the MIRU-VNTR codes of the strains (supplemental Table).
146 The main clades corresponded to Beijing (44 S-SLID and 35 R-SLID), LAM (27 S-SLID and
147 11 R-SLID), Haarlem (11 S-SLID and 4 R-SLID) and non typeable strains generally linked to
148 the T strains family (35 S-SLID and 1 R-SLID). The remaining strains belonged to the S
149 (n=8), Ghana (n=6), Delhi-CAS (n=5), EAI (n=4), Ural (n=6), Cameroon (n=3), Uganda II
150 (n=2), NEW-1 (n=1), Bovis (n=1), Africanum (n=1), X (n=1) and TUR (n=1) families.

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

169 Three strains were resistant to AMK and KAN, but susceptible to CAP (Table 1). One
170 R-AMK/KAN strain (no. 24) showed a *rrs* A1401G mutation known to be associated with
171 AMK, KAN and CAP resistance. This strain was confirmed to be susceptible to CAP (MIC =
172 1 mg/liter) (Table 1). The second R-AMK/KAN strain (isolate no. 25) showed only a C-14T
173 mutation in the *eis* promoter. When the colonies growing on tube containing AMK (with 1%
174 of resistant mutants) were sequenced, this strain exhibited no other mutation than the C-14T
175 mutation in the *eis* promoter. The MIC of AMK for this isolate was 4 mg/liter, i.e. identical to

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

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

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

192 Among the 22 monoR-KAN isolates, 17 displayed a mutation in the *eis* promoter from
193 the primary cultures (G-10A: n=8, C-12T: n=1, C-14T: n=2, G-37T: n=6) and no mutation in
194 *rrs* and *tlyA* (Table 1). For the 5 remaining monoR-KAN strains, we found no mutation in *rrs*,
195 *eis* promoter and *tlyA* on the primary sequencing, but all had a percentage of resistant mutants
196 varying between 1 and 10%. When the sequencing was done from the tubes with antibiotics,
197 the 5 strains showed an *eis* promoter mutation on tubes with KAN (C-14T in 3 strains and G-
198 37T in 2 strains) (Table 1).

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

201 mutation in *rrs*, and for 7 isolates with *eis* promoter mutations (G-10A: n=3, C-12T: n=1, C-
202 14T: n=1, G-37T: n=2) (Table 1). Isolate no. 54 (*rrs* T1404C plus G1473A) was confirmed by
203 MIC determination to be susceptible to the 3 drugs (MICs of KAN, AMK and CAP = 1.25, 1
204 and 1 mg/liter, respectively). For the 7 other strains (isolates no. 55-61), for which the
205 phenotype of drug susceptibility was not explained by the mutations found from the primary
206 cultures, they were confirmed to be susceptible to KAN with MIC values ranging from 2.5 to
207 5 mg/liter, except for isolate no. 60 which displayed a KAN MIC value of 10 mg/liter (Table
208 1).

209

210

211 **DISCUSSION**

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

217 In our study, mutations in *rrs* region 1400 accounted for resistance to AMK for 81%
218 (21/26) (considering that *rrs* C1402T is not associated with AMK-R), to CAP for 75%
219 (21/28), and to KAN for 44% (22/50) (Table 1). These figures are in accordance with those
220 previously published by other groups, which reported mutations in *rrs* region 1400, mainly
221 A1401G, accounting for resistance to SLID for respectively: 56% to 100% for AMK, 51% to
222 96% for CAP and 44% to 84% for KAN (4, 5, 7, 8, 10-12, 14, 26, 27). We observed in our
223 study that *eis* promoter mutations were present in 22/50 (44%) of the R-KAN strains on the
224 primary cultures. Among the detected mutations, G-10A, C-14T and G-37T were more
225 frequent (8/50 (16%), 6/50 (12%) and 7/50 (14%), respectively), while C-12T was rare (1/50

226 (2%). It is noticeable that a significant number of S-KAN strains (7/156 (4.5%) also showed
227 mutations in the *eis* promoter (although these mutations are more common in R-KAN strains;
228 $P = 0.0004$) (see Table 1). Finally, no mutation in *tlyA* was observed in any of our isolates, so
229 *tlyA* does not seem to be implicated in CAP resistance in our MDR clinical isolates. Mutations
230 in the *tlyA* gene associated with CAP resistance were reported to be rare in the surveyed
231 literature (found in ~0-3% of resistant strains) and their implication in resistance not
232 undoubtedly established (4, 7, 8, 10, 11, 14, 26-31).

233 Overall, among the 206 strains, discrepancies between genotypic and phenotypic DST
234 were observed in 23 (11%) strains. On one hand, sequencing performed on primary culture
235 did not show mutations in *rrs* region 1400, *tlyA* or *eis* promoter that could account for
236 resistance in 15/53 strains resistant to at least 1 of the 3 SLID. On the other, mutations in the
237 *eis* promoter were found in 7/153 strains susceptible to KAN, and 1 strain showing a *rrs*
238 1401G mutation associated to AMK, KAN but also CAP resistance was found to be
239 susceptible to CAP in phenotypic DST (Table 1). Three hypotheses, that are discussed below,
240 can be made to explain the discrepancies between the genotypic results (performed on the
241 primary culture) and the phenotypic DST results: 1) a low percentage of resistant mutants
242 precluding the detection by sequencing of resistance-associated genes on the primary culture,
243 2) resistance to SLID with MICs close to the critical concentration used for routine DST and
244 not detected by phenotypic testing, and 3) other resistance mechanisms not yet characterized.

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

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

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

276 is therefore tempting to suggest that mutations in the *eis* promoter can confer MICs close to
277 the critical concentration used, an hypothesis that would account for the significant proportion
278 of discrepant S-KAN results observed in our study: 27% (3/11) for G-10A, 50% (1/2) for C-
279 12T, 14% (1/7) for C-14T, and 25% (2/8) for C-37T. In France, AMK is the main SLID for
280 MDR TB treatment and KAN is not used. However, in countries where KAN is the preferred
281 SLID (as in Former Soviet Union) and *eis* promoter mutations dominate, these limits of
282 phenotypic testing should be taken into account in the therapeutic strategy (35). It has to be
283 pointed out here that the question of whether these isolates with *eis* promoter mutations and
284 phenotypic susceptibility to KAN should be reported as resistant to KAN should be addressed
285 by determining the impact of such mutations on patient outcome. In a recent study, Van Deun
286 et al showed that rifampin susceptible strains displaying *rpoB* mutations are associated with
287 poorer outcome than *rpoB* wild-type strains (36). In view of such results, we think that any
288 strain displaying a mutation in the *eis* promoter should be not considered as susceptible until
289 clinical studies analyze patient outcome.

290 Concerning the *rrs* C1402T mutation found in 1 R-AMK/CAP/KAN isolate in our study
291 (isolate no. 21), it has to be highlighted that if this mutation is rather associated with
292 resistance to only CAP and KAN, it has been reported that the MIC of AMK associated to this
293 mutation can be close to the critical concentration of the drug (8). Accordingly, the strain
294 displayed a MIC of 2 mg/liter, a value close to the breakpoint value (4 mg/liter). Similarly,
295 isolate no. 25 which showed a low percentage of mutants resistant to AMK on primary
296 cultures (1%) displayed a MIC of 4 mg/liter for the drug (Table 1). Thus, AMK resistance in
297 these 2 isolates can be regarded as borderline and one cannot exclude that the 2 strains would
298 have been ranked as S-AMK if a higher AMK concentration had been used on primary DST
299 (30 mg/liter according to the WHO 2014 guidelines (24). One has to note that the WHO
300 provides no evidence based on which the recommended critical concentrations have been set,

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

303 The accuracy of the molecular tests in predicting susceptibility for AMK and CAP seems to
304 be also limited by as-yet uncharacterized resistance mechanisms (third hypothesis).
305 Considering the results reported here, 3 R-AMK strains (isolates no. 21, 23 and 25) had no
306 AMK resistance-associated gene mutations from the tubes containing AMK. If strains no. 21
307 and 25 display borderline resistance to AMK (R-AMK on DST with MIC values of 2 and 4
308 mg/liter, respectively), isolate no. 23 was confirmed to be undoubtedly resistant to AMK with
309 a MIC of 16 mg/liter, which strongly suggests that an unknown mechanism of resistance is
310 present in this strain. Similarly, 5 R-CAP strains (strains n°23, 27, 29-31) had no CAP
311 resistance-associated gene mutations on sequencing analysis performed from the tubes
312 containing CAP. It is worth to note here that genes such as *whiB7* and *rrl* have previously
313 been implicated in resistance to KAN and CAP in *M. tuberculosis* (20, 38, 39). Since very
314 little is currently known about the contribution of such genes on SLID resistance, one cannot
315 exclude that they may contribute to CAP-R in isolates no. 23, 27, 29 30 and 31 (10, 18, 38,
316 40).

317 One S-AMK/CAP/KAN strain (isolate no. 54) showed a double mutation T1404C plus
318 G1473A in *rrs*. If the *rrs* G1473A mutation has never been reported, *rrs* T1404C was
319 described by Walker et al in 53 strains including 51 susceptible to the 3 SLID, 1 R-KAN and
320 1 R-AMK (41). Therefore this mutation doesn't seem to be implicated in AMK, CAP or KAN
321 resistance, as confirmed by the low MICs found for our isolate (Table 1).

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

326 Our study has some limitations, in particular because we used a collection of strains
327 representative of clinical isolates received at the French Reference Center for Mycobacteria.
328 In this set of strains, among the MDR isolates, those with resistance to at least one SLID, and
329 particularly monoR-KAN, are associated with patients born in the Former Soviet Union ($P =$
330 0.0007 and $P = 0.007$, respectively). This association between monoR-KAN and Former
331 Soviet Union-born patients is not surprising since KAN is the preferred SLID in this country.
332 Therefore, our results may be biased by the country or origin of the patients. We have also to
333 mention that an epidemiological link was detected for 3 clusters of 2 strains each (6 strains)
334 among the 53 isolates resistant to at least 1 of the 3 SLID and 7 clusters of S-
335 AMK/KAN/CAM isolates (16 strains) by taking into account (i) the phylogenetic lineages of
336 clinical isolates based on the Mycobacterial Interspersed Repetitive Unit Variable Number
337 Tandem Repeat (MIRU-VNTR) 24-loci (40), (ii) the strain characteristics (resistance
338 phenotype and genotype) and (iii) patient characteristics data (country of birth, city where the
339 diagnosis was made, family ties) (Table 1).

340

341

342 **CONCLUSION**

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

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

353

354

355

356 **ACKNOWLEDGEMENTS**

357 This work was supported by grants from the Ministère de la Recherche (grant UPRES EA
358 1541) and from the Institut National de la Santé et de la Recherche Médicale (INSERM) and
359 the Université Pierre et Marie Curie (UPMC).

360 Members of the CNR-MyRMA (French National Reference Center for Mycobacteria) are as
361 follows: Emmanuelle Cambau, Emilie Lafeuille, Faiza Mougari, Laurent Raskine, Florence
362 Reibel, Jérôme Robert.

363

364

365

366

367

368

369

370

371

372

373

374

375

376 **REFERENCES**

- 377 1. **World Health Organization.** 2016. Global tuberculosis report 2016.
378 WHO/HTM/TB/2016.13. World Health Organization, Geneva, Switzerland.
- 379 2. **World Health Organization.** 2011. Guidelines for the programmatic management of
380 drug-resistant tuberculosis: 2011 update. WHO HTM/TB/2011.6. World Health
381 Organization, Geneva, Switzerland.
- 382 3. **Kliiman K, Altraja A.** 2009. Predictors of poor treatment outcome in multi- and
383 extensively drug-resistant pulmonary TB. *Eur Respir J* **33**:1085-1094.
- 384 4. **Brossier F, Veziris N, Aubry A, Jarlier V, Sougakoff W.** 2010. Detection by
385 MTBDRsI test of complex resistance mechanisms to second-line drugs and
386 ethambutol in multidrug-resistant strains of *Mycobacterium tuberculosis*. *J Clin*
387 *Microbiol* **48**:1683-1689.
- 388 5. **Feng Y, Liu S, Wang Q, Wang L, Tang S, Wang J, Lu W.** 2013. Rapid diagnosis of
389 drug resistance to fluoroquinolones, amikacin, capreomycin, kanamycin and
390 ethambutol using genotype MTBDRsI assay: a meta-analysis. *PLoS One* **8**(2):e55292.
- 391 6. **Huang WL, Chi TL, Wu MH, Jou R.** 2011. Performance assessment of the
392 GenoType MTBDRsI test and DNA sequencing for detection of second-line and
393 ethambutol drug resistance among patients infected with multidrug-resistant
394 *Mycobacterium tuberculosis*. *J Clin Microbiol* **49**:2502-2508.
- 395 7. **Georghiou SB, Magana M, Garfein RS, Catanzaro DG, Catanzaro A, Rodwell**
396 **TC.** 2012. Evaluation of genetic mutations associated with *Mycobacterium*
397 *tuberculosis* resistance to amikacin, kanamycin and capreomycin: a systematic review.
398 *PLoS One* **7**(3):e33275.

- 399 8. **Maus CE, Plikaytis BB, Shinnick TM.** 2005. Molecular analysis of cross-resistance
400 to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*.
401 Antimicrob Agents Chemother **49**:3192-3197.
- 402 9. **Reeves AZ, Campbell PJ, Willby MJ, Posey JE.** 2015. Disparities in capreomycin
403 resistance levels associated with the *rrs* A1401G mutation in clinical isolates of
404 *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **59**:444-449.
- 405 10. **Du Q, Dai G, Long Q, Yu X, Dong L, Huang H, Xie J.** 2013. *Mycobacterium*
406 *tuberculosis rrs* A1401G mutation correlates with high-level resistance to kanamycin,
407 amikacin, and capreomycin in clinical isolates from mainland China. Diagn Microbiol
408 Infect Dis **77**:138-142.
- 409 11. **Feuerriegel S, Cox HS, Zarkua N, Karimovich HA, Braker K, Rüsç-Gerdes S,**
410 **Niemann S.** 2009. Sequence analyses of just four genes to detect extensively drug-
411 resistant *Mycobacterium tuberculosis* strains in multidrug-resistant tuberculosis
412 patients undergoing treatment. Antimicrob Agents Chemother **53**:3353-3356.
- 413 12. **Theron G, Peter J, Richardson M, Barnard M, Donegan S, Warren R, Steingart**
414 **KR, Dheda K.** 2014. The diagnostic accuracy of the GenoType® MTBDRsl assay for
415 the detection of resistance to second-line anti-tuberculosis drugs. Cochrane Database
416 Syst Rev **10**:CD010705. Review.
- 417 13. **Alangaden GJ, Kreiswirth BN, Aouad A, Khetarpal M, Igno FR, Moghazeh SL,**
418 **Manavathu EK, Lerner SA.** 1998. Mechanism of resistance to amikacin and
419 kanamycin in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **42**:1295-
420 1297.
- 421 14. **Jugheli L, Bzkalava N, de Rijk P, Fissette K, Portaels F, Rigouts L.** 2009. High
422 level of cross-resistance between kanamycin, amikacin, and capreomycin among

- 423 *Mycobacterium tuberculosis* isolates from Georgia and a close relation with mutations
424 in the *rrs* gene. Antimicrob Agents Chemother **53**: 5064-5068.
- 425 15. **Krüüner A, Jureen P, Levina K, Ghebremichael S, Hoffner S.** 2003. Discordant
426 resistance to kanamycin and amikacin in drug-resistant *Mycobacterium tuberculosis*.
427 Antimicrob Agents Chemother **47**:2971-2973.
- 428 16. Pholwat S, Stroup S, Heysell S, Ogarkov O, Zhdanova S, Ramakrishnan G, Houpt E.
429 2016. Eis C-14G and C-15G mutations do not confer kanamycin resistance in *M.*
430 *tuberculosis*. Antimicrob Agents Chemother doi:10.1128/AAC.01775-16:ahead of
431 print.
- 432 17. **Zaunbrecher MA, Sikes RD Jr, Metchock B, Shinnick TM, Posey JE.** 2009.
433 Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis*
434 confers kanamycin resistance in *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S
435 A **106**:20004-20009.
- 436 18. **Chakravorty S, Lee JS, Cho EJ, Roh SS, Smith LE, Lee J, Kim CT, Via LE, Cho**
437 **SN, Barry CE 3rd, Alland D.** 2015. Genotypic susceptibility testing of
438 *Mycobacterium tuberculosis* isolates for amikacin and kanamycin resistance by use of
439 a rapid sloppy molecular beacon-based assay identifies more cases of low level drug
440 resistance than phenotypic Lowenstein-Jensen testing. J Clin Microbiol **53**:43-51.
- 441 19. **Gikalo MB, Nosova EY, Krylova LY, Moroz AM.** 2012. The role of *eis* mutations
442 in the development of kanamycin resistance in *Mycobacterium tuberculosis* isolates
443 from the Moscow region. J Antimicrob Chemother **67**:2107-2109.
- 444 20. **Johansen SK, Maus CE, Plikaytis BB, Douthwaite S.** 2006. Capreomycin binds
445 across the ribosomal subunit interface using *tlyA*-encoded 2'-O-methylations in 16S
446 and 23S rRNAs. Mol Cell **23**:173-182.

- 447 21. **Maus CE, Plikaytis BB, Shinnick TM.** 2005. Mutation of *tlyA* confers capreomycin
448 resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **49**:571-577.
- 449 22. **Brossier F, Guindo D, Pham A, Reibel F, Sougakoff W, Veziris N, Aubry A.** 2016.
450 Performance of the new version (v2.0) of the GenoType MTBDRsl test for detection
451 of resistance to second-line drugs in multidrug-resistant *Mycobacterium tuberculosis*
452 complex strains. *J Clin Microbiol* **54**:1573-1580.
- 453 23. **World Health Organization.** 2008. Policy guidance on drug-susceptibility testing
454 (DST) of second-line antituberculosis drugs. WHO/HTM/TB/2008.392. Geneva,
455 Switzerland.
- 456 24. **World Health Organization.** 2014. Companion handbook to the WHO guidelines for
457 the programmatic management of drug-resistant tuberculosis.
458 WHO/HTM/TB/2014.11. World Health Organization, Geneva, Switzerland.
- 459 25. **Juréen P, Angeby K, Sturegård E, Chryssanthou E, Giske CG, Werngren J,**
460 **Nordvall M, Johansson A, Kahlmeter G, Hoffner S, Schön T.** 2010. Wild-type
461 MIC distributions for aminoglycoside and cyclic polypeptide antibiotics used for
462 treatment of *Mycobacterium tuberculosis* infections. *J Clin Microbiol.* **48**:1853-1858.
- 463 26. **Engström A, Perskvist N, Werngren J, Hoffner SE, Juréen P.** 2011. Comparison
464 of clinical isolates and in vitro selected mutants reveals that *tlyA* is not a sensitive
465 genetic marker for capreomycin resistance in *Mycobacterium tuberculosis*. *J*
466 *Antimicrob Chemother* **66**:1247-1254.
- 467 27. **Via LE, Cho SN, Hwang S, Bang H, Park SK, Kang HS, Jeon D, Min SY, Oh T,**
468 **Kim Y, Kim YM, Rajan V, Wong SY, Shamputa IC, Carroll M, Goldfeder L, Lee**
469 **SA, Holland SM, Eum S, Lee H, Barry CE, III.** 2010. Polymorphisms associated
470 with resistance and cross-resistance to aminoglycosides and capreomycin in

- 471 *Mycobacterium tuberculosis* isolates from South Korean patients with drug-resistant
472 tuberculosis. J Clin Microbiol **48**:402-411.
- 473 28. **Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, Hooks**
474 **DP, Cowan LS, Plikaytis BB, Posey JE.** 2011. Molecular detection of mutations
475 associated with first- and second-line drug resistance compared with conventional drug
476 susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother
477 **55**:2032-2041.
- 478 29. **Leung KL, Yip CW, Yeung YL, Wong KL, Chan WY, Chan MY, Kam KM.**
479 2010. Usefulness of resistant gene markers for predicting treatment outcome on
480 second-line anti-tuberculosis drugs. J Appl Microbiol **109**:2087-2094.
- 481 30. **Perdigão J, Macedo R, Malaquias A, Ferreira A, Brum L, Portugal I.** 2010.
482 Genetic analysis of extensively drug-resistant *Mycobacterium tuberculosis* strains in
483 Lisbon, Portugal. J Antimicrob Chemother **65**:224-227.
- 484 31. **Sirgel FA, Tait M, Warren RM, Streicher EM, Bottger EC, van Helden PD, Gey**
485 **van Pittius NC, Coetzee G, Hoosain EY, Chabula-Nxiweni M, Hayes C, Victor**
486 **TC, Trollip A.** 2012. Mutations in the *rrs* A1401G gene and phenotypic resistance to
487 amikacin and capreomycin in *Mycobacterium tuberculosis*. Microb Drug Resist
488 **18**:193-197.
- 489 32. **Folkvardsen DB, Svensson E, Thomsen VØ, Rasmussen EM, Bang D, Werngren**
490 **J, Hoffner S, Hillemann D, Rigouts L.** 2013. Can molecular methods detect 1%
491 isoniazid resistance in *Mycobacterium tuberculosis*? J Clin Microbiol **51**:1596-1599.
- 492 33. **Hoshide M, Qian L, Rodrigues C, Warren R, Victor T, Evasco HB 2nd, Tupasi T,**
493 **Crudu V, Douglas JT.** 2014. Geographical differences associated with single-
494 nucleotide polymorphisms (SNPs) in nine gene targets among resistant clinical isolates
495 of *Mycobacterium tuberculosis*. J Clin Microbiol **52**:1322-1329.

- 496 34. **World Health Organization.** 2016. The use of molecular line probe assays for the
497 detection of resistance to second-line anti-tuberculosis drugs. Policy guidance.
498 WHO/HTM/TB/2016.07. World Health Organization, Geneva, Switzerland.
- 499 35. **Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya**
500 **I, Corander J, Bryant J, Parkhill J, Nejentsev S, Horstmann RD, Brown T,**
501 **Drobniewski F.** 2014. Evolution and transmission of drug-resistant tuberculosis in a
502 Russian population. *Nat Genet* **46**:279-286.
- 503 36. **Van Deun A, Aung KJ, Bola V, Lebeke R, Hossain MA, de Rijk WB, Rigouts L,**
504 **Gumusboga A, Torrea G, de Jong BC.** 2013. Rifampin drug resistance tests for
505 tuberculosis: challenging the gold standard. *J Clin Microbiol* **51**:2633-2640.
- 506 37. **Yu X, Wang G, Chen S, Wei G, Shang Y, Dong L, Schön T, Moradigaravand D,**
507 **Parkhill J, Peacock SJ, Köser CU, Hairong H.** 2016. *Mycobacterium tuberculosis*
508 wild-type and non-wild-type MIC distributions for the novel fluoroquinolone
509 antofloxacin compared with ofloxacin, levofloxacin, and moxifloxacin. *Antimicrob*
510 *Agents Chemother*, **60**:5232-5237.
- 511 38. **Reeves AZ, Campbell PJ, Sultana R, Malik S, Murray M, Plikaytis BB, Shinnick**
512 **TM, Posey JE.** 2013. Aminoglycoside cross-resistance in *Mycobacterium tuberculosis*
513 due to mutations in the 5' untranslated region of *whiB7*. *Antimicrob Agents Chemother*
514 **57**:1857-1865.
- 515 39. **Köser CU, Bryant JM, Parkhill J, Peacock SJ.** 2013. Consequences of *whiB7*
516 (*Rv3197A*) mutations in Beijing genotype isolates of the *Mycobacterium tuberculosis*
517 complex. *Antimicrob Agents Chemother* **57**:3461.
- 518 40. **Kaur S, Rana V, Singh P, Trivedi G, Anand S, Kaur A, Gupta P, Jain A, Sharma**
519 **C.** 2016. Novel mutations conferring resistance to kanamycin in *Mycobacterium*
520 *tuberculosis* clinical isolates from Northern India. *Tuberculosis (Edinb)* **96**:96-101.

- 521 41. **Walker TM, Kohl TA, Omar SV, Hedge J, Del Ojo Elias C, Bradley P, Iqbal Z,**
522 **Feuerriegel S, Niehaus KE, Wilson DJ, Clifton DA, Kapatai G, Ip CL, Bowden R,**
523 **Drobniewski FA, Allix-Beguec C, Gaudin C, Parkhill J, Diel R, Supply P, Crook**
524 **DW, Smith EG, Walker AS, Ismail N, Niemann S, Peto TE, Modernizing Medical**
525 **Microbiology Informatics G.** 2015. Whole-genome sequencing for prediction of
526 *Mycobacterium tuberculosis* drug susceptibility and resistance: a retrospective cohort
527 study. *Lancet Infect Dis* **15**:1193-1202.
- 528 42. **Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsç-Gerdes S, Willery E,**
529 **Savine E, de Haas P, van Deutekom H, Roring S, Bifani P, Kurepina N,**
530 **Kreiswirth B, Sola C, Rastogi N, Vatin V, Gutierrez MC, Fauville M, Niemann S,**
531 **Skuce R, Kremer K, Locht C, van Soolingen D.** 2006. Proposal for standardization
532 of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat
533 typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* **44**:4498-4510.
- 534

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			<i>rrs</i> region 1400 ^d	<i>eis</i> pro	<i>tlyA</i>		
R	R	R	23 (13)	1-19 20 21 22-23	A1401G (19) A1401G (1) C1402T (1) wt (2)	wt C-14T wt C-14T	wt wt wt wt	R-AMK (10%) (MIC 2) R-AMK(2%)-CAP(2%) R-AMK(100%)-CAP(100%) (MIC AMK 16, CAP 16)	[AMK] : <i>rrs</i> C1402T, <i>eis</i> pro wt, <i>tlyA</i> wt [AMK, CAP] : <i>rrs</i> A1401G, <i>eis</i> pro C-14T, <i>tlyA</i> wt [AMK, CAP] : <i>rrs</i> wt, <i>eis</i> pro C-14T, <i>tlyA</i> wt
R	S	R	3 (2)	24 25 26	A1401G (1) wt (1) wt (1)	wt C-14T wt	wt wt wt	S-CAP (MIC 1) R-AMK (1%) (MIC 4) R-AMK(3%)-KAN(3%)	[AMK] : <i>rrs</i> wt, <i>eis</i> pro C-14T, <i>tlyA</i> wt [AMK], [KAN] : <i>rrs</i> A1401G, <i>eis</i> pro wt, <i>tlyA</i> wt
S	R	R	2 (1)	27 28	wt (1) wt (1)	G-37T wt	wt wt	R-CAP (100%) (MIC 16) R-CAP(3%)-KAN(3%)	[CAP]: <i>rrs</i> wt, <i>eis</i> pro G-37T, <i>tlyA</i> wt [CAP], [KAN]: <i>rrs</i> A1401G, <i>eis</i> pro wt, <i>tlyA</i> wt

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

^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 brackets

^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).