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1 **First genetic characterization of multi-drug resistant *Mycobacterium tuberculosis***
2 **isolates from Algeria**

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40 **ABSTRACT (199 words)**

41 *Objectives:* to characterize the genotypes of multidrug resistant (MDR) *Mycobacterium tuberculosis*
42 (MTB) isolated in Algeria, where a low MDR-MTB incidence rate is observed.

43 *Methods:* 10 MDR isolates and one resistant to isoniazid were investigated by PCR-Sanger
44 sequencing for 10 loci involved in resistance. Amplicon-based NGS of 15 loci was additionally
45 performed on isolates harboring novel mutations.

46 *Results:* Sanger and amplicon-NGS sequencing provided the same results concordant with GenoType
47 kits. Mutations known to be associated with resistance were described for most isolates: *rpoB* S531L
48 in 7/10 rifampicin-R MTB isolates, *katG* S315T in 9/11 isoniazid-R and promoter *inhA* c-15t in 3/11
49 of them, *embB* M306V or M306I in 2/2 ethambutol-R, *rpsL* K43R in 4/8 or *rrs* a514c associated with
50 *gidB* L16R in streptomycin-R, *gyrA* A90V in the ofloxacin-R pre-XDR isolate. New and rare
51 mutations were also described in *rpoB* (deletion 512-513-514), *katG* (S315R, M126I/ R496L), *gidB*
52 (V124G, E92A, V139A, G37V) and *gyrA* (P8A). MIRU-VNTR profiles were similar for 3 isolates
53 (lineage Cameroon) indicating a possible clonal diffusion with patients not epidemiologically related.
54 *Conclusions:* Resistant MTB isolates in Algeria harbor resistance genotypes similar as in other
55 countries but some rare patterns may result from selection and transmission processes inherent to the
56 country.

57

58 **Keywords:** *Mycobacterium tuberculosis*; multidrug-resistant; Algeria, mutation; NGS-amplicon.

59

60 1. Introduction

61 Tuberculosis (TB) remains one of the major infectious diseases with about 10 million new cases
62 causing 1.6 million deaths worldwide. The emergence of multidrug-resistance (MDR) TB, resistance
63 to rifampicin (RIF) and to isoniazid (INH), is a major problem in industrialized and developing
64 countries [1] and in the global fight against TB [2]. Algeria is classified by WHO into the group of
65 countries having moderate TB incidence (between 53 and 88 cases per 100 000 inhabitants in 2017).
66 The number of notified TB cases was 23 224 with a low rate of MDR (1.4 %) in contrast to many
67 other countries. The MDR-MTB strains from Algeria have not been studied or compared yet with
68 regard to strains isolated in Africa [3] and in Middle East [4].

69 In MDR-TB strains, mutations were classically described within a 81pb region (codons 507-533) of
70 the *rpoB* gene encoding the beta subunit of RNA polymerase. This was observed in more than 95%
71 of RIF-resistant strains [5] and serves as a surrogate marker for MDR-TB since 90% of RIF resistant
72 isolates exhibit also resistance to INH [6]. Mutations associated with INH resistance are observed in
73 *katG* encoding a catalase-peroxidase enzyme [7], in the promoter region of the *mabA* (FabG1)-*inhA*
74 operon [8] causing overexpression of *inhA* and less frequently in the *inhA* coding sequence [9]. The
75 *katG* S315T mutation is found in 50-95% of INH-resistant clinical isolates [11] and confer high level
76 resistance, whereas mutations at *inhA* promoter result in a low level resistance only [10].

77 Although considered as an old antituberculous drug and thus rarely studied, streptomycin has been
78 an alternative first-line antituberculous drug increasingly used for treating MDR-TB in Algeria. Its
79 resistance is conferred by mutations in *rrs* (in the 530 loop and the 912 region) encoding the small
80 ribosomal subunit 16S rRNA, or in *rpsL* (codons 43 and 88 mainly) encoding the ribosomal protein
81 S12 [12]. More recently, mutations in *gidB* encoding a conserved 7-methylguanosine
82 methyltransferase specific of the 16S rRNA were shown to confer low-level STR resistance in MTB
83 [13][14].

84 Other mutations were reported to be associated or conferring resistance to other antituberculous
85 drugs. Briefly resistance to ethambutol (EMB) was associated with mutations (mostly codon 306) in

86 *embB* encoding arabinosyltransferases involved in the biosynthesis of arabinogalactan and
87 lipoarabinomannan [15][16]. Pyrazinamide (PZA) resistance, which is difficult to assess
88 phenotypically [17], was associated with mutations in *pncA* encoding pyrazinamidase (PZAse) that
89 activates PZA into the active pyrazinoic acid [18] . Lastly , fluoroquinolone resistance is conferred
90 by mutations in the quinolone resistance–determining region (QRDR) of the DNA gyrase *gyrA* (most
91 often) and *gyrB* genes [19].

92 Our objectives were to genetically characterize some MDR-TB isolates from Algeria, by
93 epidemiological typing, using MIRU-VNTR genotyping to distinguish clones and lineages, and
94 searching for mutations associated with resistance. For this, we followed different technical
95 approaches that could be done directly on crude DNA, such as LiPA kits, PCR –Sanger sequencing
96 of gene regions known to confer resistance and amplicon-based NGS approach [5][20]. Our results
97 showed that classical mutations were found, with concordant results with all the techniques, in most
98 strains and lineages. However, rare and new mutations were also detected showing the specificity of
99 the MDR selection and transmission in this country.

100

101 2. **Materials and methods**

102 2.1. *MTB* complex clinical strains

103 Ten MDR *MTB* clinical isolates were studied. They were from 10 patients (from 21 to 88 years old)
104 diagnosed with pulmonary TB from December 2014 to December 2015 at the National Reference
105 Laboratory for Tuberculosis and Mycobacteria at the Pasteur Institute of Algeria. An additional
106 clinical isolate from another patient was included in the study and was resistant to INH and STR but
107 susceptible to RIF.

108 *MTB* Isolates were grown on Löwenstein-Jensen (LJ) media at 37°C and drug susceptibility testing
109 was carried out at Pasteur Institute Algiers, on LJ media by the conventional proportional method
110 [21] with the reference strain *MTB* H37Rv as quality control.

111 2.2 *DNA* extraction

112 DNA was extracted from MTB cultures on LJ, by suspending a loop of colonies in 400µl of TE buffer
113 (10mM Tris/HCL (pH 8.0), 1mM EDTA). Samples were vortexed, heated at 95°C for 120 min and
114 suspensions were centrifuged at 12 000 rpm for 5 min. DNA extracts were shown to be culture
115 negative. DNA was then stored at -20°C and brought to the French NRC laboratory where it was
116 used crude for all the molecular experiments.

117 2.3. Genotype MTBDRplus and Genotype MTBDRsl

118 Isolates were submitted to the detection of mutations conferring INH and RIF resistance by the line
119 probe assay kit GenoType®MTBDRplus VER 2.0 and those conferring resistance to
120 fluoroquinolone and injectable drugs by the kit GenoType® MTBDRsl VER 2.0 (Hain Lifescience
121 - Bruker, Nehren, Germany) according to the manufacturer's protocol.

122 2.4. PCR amplification and Sanger sequence analysis

123 Pairs of specific primers were designed to amplify 10 loci (*rpoB*, *katG*, *inhA*, *promoter inhA*, *rpsL*,
124 *rrs*, *gidB*, *embB*, *pncA*, *gyrA*) associated with resistance to anti-tuberculosis drugs. Primer sequences
125 are provided in the supplement Table. For *rpoB*, *embB*, *rrs* and *gyrA*, only the regions including
126 mutations known to confer resistance were sequenced. For *rpsL*, *inhA*, *pncA*, *gidB*, the entire genes
127 were sequenced to detect resistance associated mutations throughout the gene.

128 Briefly, a 25µl PCR reaction mixture was prepared containing 1µl of each primer at 10 pmol/µl, 12µl
129 of Go Taq ® Green (Promega, WI, USA), 2µl of sample DNA and 8, 5µl of sterile distilled water.

130 Amplification consisted in an initial denaturation at 94°C for 5 min followed by 35 cycles of
131 denaturation at 94°C for 30s, annealing temperatures varies from 50° at 60°C for 30 s and extension
132 at 72°C for 30 s, followed by a final extension at 72°C for 10 min. PCR products sizes were verified
133 by electrophoresis in 1% agarose gel and further purified by using NucleoFast® 96 PCR Macherey-
134 Nagel. Sequencing of both strands of the PCR product was performed using the Big Dye™
135 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Inc, Foster City, CA, USA)
136 and using a 3313xL sequencing instrument according to the protocol supplied by the manufacturer.

137 Sequences were analyzed using BioEdit sequence alignment software. The obtained sequences were
138 aligned in a multiple sequence alignments with reference sequences of *rpoB*, *katG*, *inhA*, promoter
139 *inhA*, *embB*, *pncA*, *rrs*, *rpsL*, *gid* and *gyrA* genes in MTB H37Rv (GenBank accession number
140 NC_000962.3) to identify mutations. All mutations found were compared against those included in
141 the drug resistance Mutation Database [22] (<https://tbdreamdb.ki.se/Info/>).

142

143 2.5. Amplicon-based Next Generation sequencing (NGS)

144 Fifteen genes involved in drug resistance (*rpoB*, *katG*, *inhA* and its promoter, *embB*, *pncA*, *rrs*, *rpsL*,
145 *gidB*, *gyrA*, *gyrB*, *rplC*, *tlyA*, *ethA*, *ethR*) were sequenced following the amplicon strategy using 454
146 technologies for two clinical MDR isolates which showed unknown mutations after Sanger
147 sequencing. Primers used are listed in the supplement Table. A barcode sequence was added to each
148 primer to distinguish different samples during the analysis.

149 Each amplicon was amplified and purified two times with Taq DNA polymerase High Fidelity
150 (Invitrogen, Waltham, MA, USA) and Agencourt AMPure XP magnetic beads (Brea, CA, USA),
151 according to the amplicon library preparation manual of GS Junior (Roche diagnostics, Indianapolis,
152 IN, USA). Purified libraries were pooled in an equimolar mix measured previously with Quant-iT
153 Picogreen dsDNA assay kit (Life technologies, Eugene, OR, USA). The size distribution of amplicons
154 was determined on an Agilent 2100 Bioanalyzer with D1000 DNA Chips (Agilent Technologies,
155 Santa Clara, USA). Amplicon pool were amplified with GS Junior Lib-A and sequencing using GS
156 Junior titanium sequencing kit in 454-GS Junior instrument. Sequence analysis was performed by the
157 software GS Junior Sequencer v3.0, GS Run Browser v3.0 and GS Amplicon Variant Analyzer v3.0.

158

159 2.6 MIRU–VNTR

160 The isolates from the eleven patients were compared using 24-locus mycobacterial interspersed
161 repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) typing, as previously described [22].

162

163 3. Results

164 The results of the genetic characterization of the 11 MTB isolates are detailed in the Table 1 for
165 loci associated with rifampicin (*rpoB*) and isoniazid (*katG*, *inhA*, promoter *inhA*) resistance and in
166 Table 2 for other loci associated with streptomycin (*rpsL*, *rrs*, *gidB*), ethambutol (*embB*),
167 pyrazinamide (*pncA*), fluoroquinolone (*gyrA*), kanamycin and amikacin (*rrs*) resistance.

168 3.1 mutations defining multidrug resistance as RIF plus INH resistance

169 Among the 10 MDR-TB isolates, a single nucleotide mutation resulting in Ser531Leu substitution
170 (numbering system in *E. coli*, the corresponding position is 450 in the numbering system of MTB)
171 was the most prevalent and observed in 7 (70%) isolates. The second prevalent codon was 526
172 (numbering system in *E. coli*, the corresponding position is 445 in the numbering system of MTB),
173 with mutation found in 2/10 (20%) isolates leading to the H526D and H526Q substitutions.
174 Surprisingly, a 9pb deletion from 1290 to 1298 nucleotides (nt) corresponding to the codons 512, 513
175 and 514 (numbering system in *E. coli*, the corresponding positions are 431, 432 and 433 in the
176 numbering system of MTB) (GenBank accession no. PRJEB24182 or ERP105991) was described in
177 one MDR-TB isolate. The remaining MDR-TB isolate had a mutation leading to D516V (numbering
178 system in *E. coli*, the corresponding position is 435 in the numbering system of MTB).

179 The results obtained by GenoType MTBDRplus were concordant with those of the Sanger sequencing
180 (Table 1).

181 Among the eleven isoniazid-resistant isolates, 9/11 (88.9%) harbored the classical *katG* S315T
182 mutation, but one isolate showed the rare *katG* S315R. The remaining isolate contained double rare
183 mutations in *katG*, M126I and R496L. Mutations in promoter *inhA* were observed in 3/11 (27.3%)
184 isolates associated with *katG* S315T. In three isolates, the mutation *inhA* A190S was observed (Table
185 1). The isolate with INH resistance but RIF susceptibility harbors a common *katG* S315T mutation

186 3.2 mutations for other first line antituberculous drugs

187 Streptomycin resistance was observed for 7/10 (70%) of MDR-TB and also for the INH-resistant
188 strain. The mutation K43R in *rpsL* was prevalent in 4/8 (50 %) isolates and co-existed with the

189 mutation V139A in *gidB* (GenBanK accession no. PRJEB24182 or ERP105991) in three isolates
190 (Table 2). Mutations in *rrs* at position 514 (numbering system of MTB , 524 in *E. coli*) was
191 observed in two of the remaining isolates and co-existed with the mutation L16R in *gidB* (Table 2).
192 In addition, a deletion of one nucleotide at position 115 in *gidB* and the mutation V124G in *gidB*
193 (GenBanK accession no. PRJEB24182 or ERP105991) were observed in STR-resistant isolates.
194 Two novel polymorphisms in *gidB* were observed G37V (GenBanK accession no. PRJEB24182 or
195 ERP105991) and E92A (GenBanK accession no. PRJEB24182 or ERP105991) in STR-susceptible
196 isolates.
197 Contrary to the high frequency of STR resistance, ethambutol resistance was observed in only two
198 isolates where *embB* mutations were observed as M306V (n=1) and M306I (1). Unexpectedly, four
199 MDR-TB determined as ethambutol-susceptible isolates harbored also *embB* mutations at other
200 positions: D328Y (3) and Y319S (1) (Table 2).

201 Since PZA resistance could not be tested phenotypically, the detection of *pncA* mutation was the
202 only way to know about the frequency of this clinically relevant resistance. *pncA* mutation were
203 found in 8/10 (80%) of the MDR–TB isolates. Mutations were various: V155A (3), T142M (2),
204 P62R (1), V139L (1) and an insertion of two nucleotides (GG) in the latter isolate (Table 2).

205 *3.3 mutations for XDR resistance*

206 Of the ten MDR-TB isolates, only one isolate was resistant to OFX with a *gyrA* A90V mutation,
207 also detected by GenoType MTBDRsl. This mutation is known to confer low level resistance to
208 fluoroquinolones. *gyrA* polymorphisms, not associated with resistance, were also observed: *gyrA*
209 S95T (7), E21Q (11), and P8A (2) (Table 2).

210 Mutations conferring resistance to amikacin, kanamycin or capreomycin in the *rrs* gene were not
211 found, although the phenotypic susceptibility could not been tested either.

212 *3.4 amplicon-based NGS versus PCR sequencing results*

213 The isolates Alg 3 and Alg 5 were studied by amplicon NGS, since this method could sequence in
214 one experiment the entire genes of the ten loci investigated by PCR –Sanger sequencing (see above)

215 plus five additional loci (*gyrB*, *ethA*, *ethR*, *tlyA*, *rplC*) (Table 3). The maximum coverage was
216 >50X and the minimum coverage was 40X. Amplicon NGS found the same mutations than in
217 Sanger sequencing.

218 3.5 *MIRU–VNTR* genotyping

219 *MIRU-VNTR* identified four different strain lineages: Cameroon (four isolates), Haarlem (four
220 isolates), LAM (two isolates), and S (one isolate).

221 Three of the Cameroon lineage isolates (isolates Alg 7, Alg 10 and Alg 11) share the same *MIRU-*
222 *VNTR* code and exhibit identical genotypic resistance patterns, even rare mutations (A190S *inhA*,
223 undescribed V139A *gidB*, D328Y *embB*, V155A *pncA*, E21Q *gyrA*). This suggests the transmission
224 of the same strain between these three patients. However, since they were not living in the same
225 area (Fig. 1) and were not epidemiologically related, this may suggest the diffusion of this clone in
226 a broader population.

227 The isolates Alg 2 and Alg 3 LAM lineage were also very similar since they have almost the same
228 *MIRU- VNTR* code (only 2 differences) and resistance genotypes were identical for *rpoB*, *katG*,
229 *inhA*, *rrs*, *gid* and *pncA*. However, the isolate Alg 3 evolved towards drug resistance since it
230 harbored additional mutations conferring ethambutol resistance (M306V *embB*) and ofloxacin
231 resistance (A90V *gyrA*) and a status of pre-XDR strain.

232 The four Haarlem lineage strains (Alg 5, Alg 6, Alg 8 and Alg 9) showed different *MIRU- VNTR*
233 code and different resistance genotypes, indicating that these strains were not related.

234

235 4. Discussion

236 Studies performed in different countries on drug resistant isolates of *MTB* have reported gene
237 mutations conferring resistance to anti-tuberculous drugs [23]. No studies were carried out in Algeria
238 before our study. There was only one study on 50 sputum positive cases of pulmonary TB from
239 Western Algeria submitted to GeneXpert *MTB/RIF* assay, which confirmed RIF resistance without
240 detailing the mutations [39].

241 We report the genotypes of 10 MDR and 1 INH-resistant MTB isolated from patients diagnosed with
242 pulmonary TB in Alger. All isolates showed at least one typical mutation known to confer resistance.
243 In addition, novel mutations associated to resistance and polymorphisms (gene mutations associated
244 to susceptibility) were described indicating that MTB strains circulating in Algeria are not the result
245 of the world endemic situation.

246 Several molecular methods are used in routine conditions for the detection and genetic
247 characterization of mutations in MDR-TB. The line probe assays (GenoType MTBDRplus and
248 MTBDRsl) recommended by WHO and easy to implement in the field, can detect classical mutations
249 associated with INH resistance such as *katG* S315T, promoter *inhA* c-15T but cannot detect mutations
250 at codon 126 and 496 in *katG* for instance, since there are out of the hybridization region tested. The
251 PCR-based DNA sequencing is still a highly reliable method able to detect and describe mutations
252 responsible for drug resistance [24] but Sanger equipment is disappearing from research labs. Next
253 generation sequencing used for determining the whole genome sequence (WGS) or amplicon
254 sequences is driven forward rapidly [25]. NGS is still more costly than commercial methods and
255 needs technical skills as well as bioinformatics tools and expertise when performed outside a research
256 laboratory or with low resources. Amplicon-NGS of loci involved in MTB drug resistance can appear
257 simpler compared to PCR Sanger sequencing since multiple loci can be screened in one sequencing
258 reaction, and can appear more feasible than WGS, since it can be done with crude DNA present in
259 specimens, as performed in this study [29].

260 Our results on MDR isolates from Algeria are consistent with previous studies for most of the
261 strains. All (10/10) RIF-resistant MTB isolates had mutations within the RRDR as shown for
262 isolates tested in India, (146/149, 97.98%) [26], and in Brazil, (79/82, 96,3%) [27], and the codons
263 531 or 526 were mainly involved [28]. All the 11 INH-resistant isolates harbored *katG* mutation at
264 codon 315 and some isolates had mutations in *inhA* promoter. In the clinical MTB strains of
265 patients living in an European country, *katG* mutations were the most prevalent mutation (85%) in
266 strains resistant to INH, specifically S315T in 96% of the cases [29]. In Tunisia, *katG* S315T

267 mutation was present in 85.7% of INH-resistant isolates [30]. Similarly, we found a majority of
268 INH-resistant isolates harboring *katG* S315T mutation (88.9 %). Mutations in promoter *inhA* are
269 less frequent [13] with 8-43% of INH-resistant MTB isolates in various countries [11][23] and
270 27.3% in our study. In our study, one INH-resistant isolate did not harbor the *katG*315 mutation but
271 a double *katG* mutation M126I and R496L. R496L was previously reported in combination with
272 *katG* S315T mutation in one MTB clinical strains resistant to INH [24] but never with *katG* M126I.
273 It is unclear whether R496L is lonely capable of conferring INH resistance but concomitantly with
274 M126I, it may [36]. The deletion of nine nucleotides from 1290-1298 (codons 512, 513 and 514) in
275 *rpoB* found in one MDR-TB strain was not been previously reported. Similar deletions but
276 uncomplete (512-513) or at different positions were reported in isolates from Korea: deletion of ten
277 nucleotides from 1287-1296 in one isolate XDR-TB and deletion of nine nucleotides from 1282-
278 1290 in one isolate MDR-TB [24].

279 For STR, 75 % of the STR-resistant isolates harbored mutations in *rpsL* (L43R mostly) or *rrs*,
280 described as conferring resistance [12][31]. We detected *rrs* mutations at the nucleotide 514 (loop
281 530) in 25% of STR-resistant isolates, which was similar to previous reports [31] [32]. These data are
282 similar to those of other studies strongly confirming that mutations in the *rrs* 912 region are rare [33].
283 From the few studies on *gidB* mutation associated with low level STR resistance [13], six variants
284 were identified but none of the four substitutions found in our isolates (G37V, E92A, V124G, V139A)
285 [13]. However, *gidB* V139A was observed co-existing with L43R in *rpsL* and *gidB* L16R with *rrs*
286 mutations at position 514 in STR-resistant isolates.

287 Classical mutations in *embB* [34], as M306I or V, were identified in only 6/10 MDR-TB isolates, but
288 other rare mutations were also previously described at codons 328 and 319, [34]. In our study, the
289 *embB* mutations involving these codons were D328Y and Y319S and were identified in EMB-
290 susceptible MDR-TB, suggesting that they do not confer resistance but may be polymorphisms
291 associated with MDR-TB. Since the phenotypic test for PZA was not performed in Algeria, the report
292 of *pncA* mutations in 80% of the MDR-TB isolates revealed that 8 isolates may be PZA-resistant [18],

293 which confirmed a recent report of a high prevalence of PZA resistance in Sub-Saharan Africa
294 countries [35]. The mutations we identified in *pncA* have been described earlier to confer resistance
295 to PZA (V155A and T142M) [18]. Lastly, natural *gyrA* polymorphisms (E21Q and S95T) were found
296 and confirmed they are not associated with resistance to fluoroquinolones [19].

297 The limitations of our study are the following: First, we studied only ten MDR-TB strains, but since
298 the rate of resistance in Algeria is low we preferred to study quite recent isolates than working on a
299 complete data set of several year collection. In addition, the difficulty to study strains isolated in
300 Algeria was also due to limitations in techniques and cost. With the collaboration with the NRC in
301 France, we could apply molecular tools that could not performed in the Algerian laboratory.
302 Therefore, this study will now allow the implementation of these genotyping techniques in the
303 tuberculosis reference laboratory of Algeria. The difficulty to exchange MDR-TB cultures explain
304 why we could not have a complete phenotypic DST for PZA and second line drugs. For the same
305 reason, we could not apply the WGS technique because only a small amount of crude DNA extract
306 can be brought from Algeria to France.

307 Since there are no publications about the genotype of MDR-TB strains from Algeria to refer to, we
308 had searched in the data base of the NRC in France and found 16 MDR-TB isolates from patients
309 born in Algeria but who were diagnosed in France, none of them were isolated in 2014 or 2015 We
310 did not include these data in the analysis since we cannot assess that the MTB infection was acquired
311 in Algeria, except for one strain isolated in 2007 that shares the same MIRU pattern than the 3
312 clustered isolates of genotype Cameroon. This shows that the transmission was already ongoing in
313 2007. Moreover, materials and methods were different from the 10 MDR-TB isolates cultivated in
314 Algiers and detailed in this paper.

315

316 **5. Conclusions**

317 Resistant MTB isolates from Algeria harbor similar resistance genotypes as other countries. However
318 MDR-TB isolates showed some rare patterns, which probably are the result from selection and

319 transmission processes inherent to the country, such as for instance the long use of streptomycin.
320 Tuberculosis has been monitored very well in Algeria [38][39] and some of the strains may circulate
321 from the 90's contrarily to what is observed in Asia and even other countries of Africa [3] [40]. This
322 kind of study can help physiologists and public health authorities to track transmission and recent
323 drug resistance selection.

324

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328

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334 **Ethical Approval:** Not required

335

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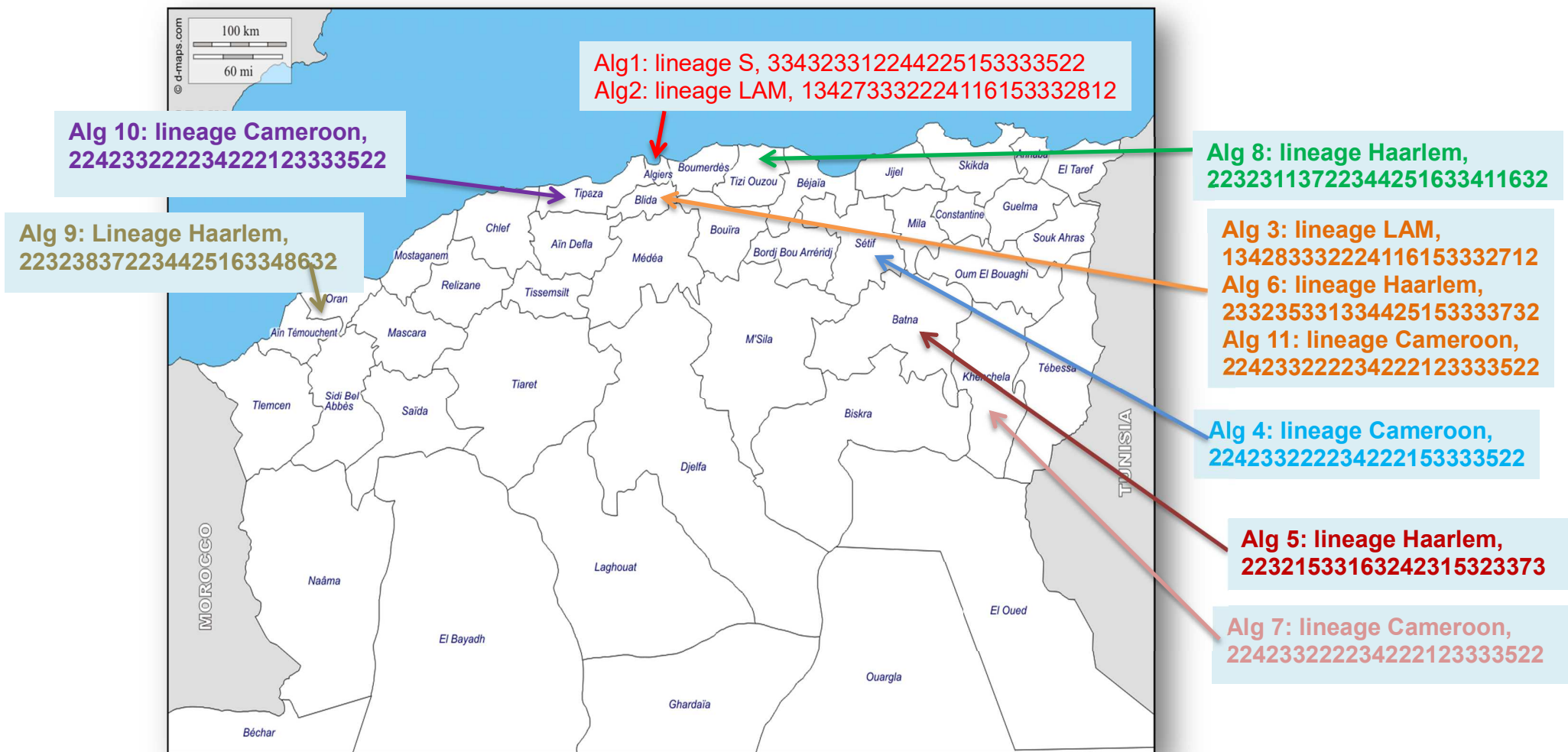


Fig. 1. Results of MIRU-VNTR for the 11 *Mycobacterium tuberculosis* isolates from Algeria.

1 **Table 1**

2 Genotypic resistance patterns of the 11 *Mycobacterium tuberculosis* clinical isolates detailed for rifampicin and isoniazid resistance.

Isolates	pAST	RIF		pAST	INH				
		<i>rpoB</i> gene			<i>katG</i> gene		p <i>inhA</i>		<i>inhA</i> gene
		MTBDRplus	Sanger sequencing		MTBDRplus	Sanger Sequencing	MTBDRplus	Sanger Sequencing	Sanger Sequencing
Alg 1	R	MUT2B	H526D (CAC-GAC)	R	MUT1	S315T (AGC-ACC)	WT	WT	WT
Alg 2	R	MUT3	S531L (TCG-TTG)	R	MUT1	S315T (AGC-ACC)	MUT1	c-15t	WT
Alg 3	R	MUT3	S531L (TCG-TTG)	R	MUT1	S315T (AGC-ACC)	MUT1	c-15t	WT
Alg 4	R	MUT3	S531L (TCG-TTG)	R	MUT1	S315T (AGC-ACC)	WT	WT	WT
Alg 5	R	WT7-,WT2- /WT3-	H526Q (CAC-CAG)/ Del 512-513-514*	R	MUT1	S315T (AGC-ACC)	MUT1	c-15t	WT
Alg 6	S	WT	WT	R	MUT1	S315T (AGC-ACC)	WT	WT	WT
Alg 7	R	MUT3	S531L (TCG-TTG)	R	MUT1	S315T (AGC-ACC)	WT	WT	A190S (GCC-TCC)
Alg 8	R	MUT1	D516V (GAC-GTC)	R	MUT-	S315R (AGC-AGG)	WT	WT	WT
Alg 9	R	MUT3	S531L (TCG-TTG)	R	WT	M126I (ATG-ATA)/ R496L (CGC-CTC)	WT	WT	WT
Alg 10	R	MUT3	S531L (TCG-TTG)	R	MUT1	S315T (AGC-ACC)	WT	WT	A190S (GCC-TCC)
Alg 11	R	MUT3	S531L (TCG-TTG)	R	MUT1	S315T (AGC-ACC)	WT	WT	A190S (GCC-TCC)

3
4 Mutations not previously reported are marked with an asterisk (*)

5 Corresponding E.coli numbering was used for *rpoB*

6 pAST, phenotypic antibiotic susceptibility testing; R: Resistant, S: sensible, RIF: rifampicin, INH: isoniazid

7
8

9 **Table 2**
 10 Genotypic resistance patterns of the 11 *Mycobacterium tuberculosis* clinical isolates detailed for aminoglycoside, ethambutol, pyrazinamide, ofloxacin,
 11 amikacin and kanamycin resistance.
 12

Isolates	STR			EMB		PZA		OFL		AMK/KAN		
	pAST	Sanger sequencing		pAST	Sanger sequencing	Sanger sequencing	pAST	MTBDRsl	Sanger sequencing	MTBDRsl	Sanger Sequencing	
		<i>rpsL gene</i>	<i>rrs gene</i>	<i>gidB gene</i>	pAST	<i>embB gene</i>	<i>pncA gene</i>		<i>gyrA gene</i>		<i>rrs gene</i>	
Alg 1	R	WT	WT	V124G (GTG-GGC)*	S	WT	Ins GG 391	S	WT	<u>E21Q</u> (GAG-CAG) <u>S95T</u> (AGC-ACC)	WT	WT
Alg 2	R	WT	a514c	L16R (CTT-CGT)	S	WT	T142M (ACG-ATG)	S	WT	<u>E21Q</u> (GAG-CAG) <u>S95T</u> (AGC-ACC)	WT	WT
Alg 3	R	WT	a514c	L16R (CTT-CGT)	R	M306V (ATG-GTG)	T142M (ACG-ATG)	R	MUT1	<u>E21Q</u> (GAG-CAG) A90V (GCG-GTG) <u>S95T</u> (AGC-ACC)	WT	WT
Alg 4	R	K43R (AAG-AGG)	WT	WT	S	Y319S (TAT-TCT)	V139L (GTG-CTG)	S	WT	<u>E21Q</u> (GAG-CAG)	WT	WT
Alg 5	S	WT	WT	E92A (GAA-GCA)*	R	M306I (ATG-ATA)	P62R (CCG-CGG)	S	WT	<u>E21Q</u> (GAG-CAG) <u>S95T</u> (AGC-ACC)	WT	WT
Alg 6	R	WT	WT	Del Int115	S	WT	WT	S	WT	<u>E21Q</u> (GAG-CAG) <u>S95T</u> (AGC-ACC)	WT	WT
Alg 7	R	K43R (AAG-AGG)	WT	V139A (GTG-GCG)*	S	D328Y (GAT-TAT)	V155A (GTG-GCG)	S	WT	<u>E21Q</u> (GAG-CAG)	WT	WT
Alg 8	S	WT	WT	WT	S	WT	WT	S	WT	P8A* (CCT-GCT) <u>E21Q</u> (GAG-CAG) <u>S95T</u> (AGC-ACC)	WT	WT
Alg 9	S	WT	WT	G37V (GGA-GTA)*	S	WT	WT	S	WT	P8A*(CCT-GCT) <u>E21Q</u> (GAG-CAG) <u>S95T</u> (AGC-ACC)	WT	WT
Alg 10	R	K43R (AAG-AGG)	WT	V139A (GTG-GCG)*	S	D328Y (GAT-TAT)	V155A (GTG-GCG)	S	WT	<u>E21Q</u> (GAG-CAG)	WT	WT
Alg 11	R	K43R (AAG-AGG)	WT	V139A (GTG-GCG)*	S	D328Y (GAT-TAT)	V155A (GTG-GCG)	S	WT	<u>E21Q</u> (GAG-CAG)	WT	WT

13 Mutations/polymorphisms not previously reported are marked with an asterisk (*)

14 Mutation E21Q and S95T in *gyrA* are known as natural polymorphisms

15 pAST, phenotypic antibiotic susceptibility testing; R: Resistant, S: Sensible, STR: streptomycin, EMB: ethambutol, PZA: pyrazinamide, OFL:

16 ofloxacin AMK: amikacin, KAN: kanamycin

17 **Table 3**

18 Comparison of genotypic results obtained by Sanger PCR sequencing and by amplicon-based Next -Generation Sequencing.

Gene	<i>Mycobacterium tuberculosis</i> Alg 3			<i>Mycobacterium tuberculosis</i> Alg 5		
	Sanger	NGS	Percentages of mutations NGS (%)	Sanger	NGS	Percentages of mutation NGS (%)
<i>rpoB</i>	S531L	S531L	98,73%	H526Q/Delcodon512-513-514	H526Q/ Delcodon512-513-514	100%/100%
<i>KatG</i>	S315T	S315T	100%	S315T	S315T	100%
Promoter <i>inhA</i>	c-15t	c-15t	100%	C-15T	C-15T	100%
<i>inhA</i>	WT	WT		WT	WT	
<i>rpsL</i>	WT	WT		WT	WT	
<i>rrs</i>	a514c	a514c	100%	WT	WT	
<i>gidB</i>	L16R	L16R	100%	E92A	E92A	100%
<i>embB</i>	M306V	No detected		M306I	M306I	100%
<i>pncA</i>	T142M	T142M	100%	P62R	P62R	97,07%
<i>gyrA</i>	E21Q	E21Q	98.46%	E21Q	E21Q	100%
	A90V	A90V	100%/	S95T	S95T	100%
	S95T	S95T	100%			
<i>gyrB</i>	-	WT		-	WT	
<i>ethA</i>	-	WT		-	WT	
<i>ethR</i>	-	WT		-	WT	
<i>tlyA</i>	-	WT		-	WT	
<i>rplC</i>	-	WT		-	WT	

19

20 - Not tested

21

- **Supplementary Table**

22

Primers used for PCR amplification, DNA sequencing and amplicon based next generation sequencing (NGS).

Gene	Primer name	Sequence (5'-3')	Amplicon size (pb)	Tm(°)	Sanger	NGS
<i>rpoB</i>	rpoB1-F	GAGGGTCAGACCACGATGAC	375	54	+	+
	MTR-R	TCGATCGGGCACATCCGG				
<i>katG</i>	katG1-F	AACACCAACTCCTGGAAGGA	395	51	+	+
	KatG1-R	TCGGGCCAGCTGTTAAGCGG				
	katG2-F	ATCCGGATGGCGTGGCACGC	375	60	+	+
	KatG2-R	GGTCCGGGTTGCCGTTCCGGC				
	katG3-F	AGATGGGGCTGATCTACGTG	363	53	+	+
	katG3-R	CCGTCCTTGGCGGTGTATTG				
	katG4-F	GAGTTCGCCAAGGCCTGGTA	356	53	+	+
	katG4-R	ACTTTGATGTTCCCCGGCG				
	katG5-F	TGCGTCGCAGGAACAAACCG	375	55	+	+
	katG5-R	ATAGACCTCGACAAGCGCCC				
<i>Promoter inhA</i>	pinhA-F	CGGCGGGAAGATCCGCGTCC	375	55	+	+
	pinhA-R	ATGGGGGTTTGGCCCTTCA				
<i>inhA</i>	INH1-F	CACAAGGACGCACATGACA	371	51	+	+
	inhA1-R	CCTTGCCATCGAAGCATAC				
	INH2-F	TGCATTCGATTGGGTTCATG	288	55	+	
	INH2-R	AGATTCGAACGCACACCGT				
	INH3-F	CGTATTCGTATGCTTCGATG	589	55	+	
	INH3-R	CGAAATGCAGGTAGTGCTC				
	inhA2-F	GCATGGACTTCGACCCGAGC	391	57		+
	inhA2-R	CGGGCCCTTCCGGTCCGGCCC				
<i>embB</i>	embB1-F	CGACGCCGTGGTGATATTCG	375	55	+	+
	embB1-R	ACATAGGTGACCAGCGAGC				
	embB2-F	AGCAAACCCGCCTACTGGGC	355	55	+	+
	embB2-R	TTGGCGCGAACCCCTGGTGGC				
	embB3-F	CTGGTCGCGCAACCGGATGG	375	57	+	+
embB3-R	ATCAGGCTCGACGAGTACGT					
<i>pncA</i>	pnca1-F	GGCGTCATGGACCTATATC	380	56	+	+
	pnca1-R	TGCCGTTCTCGTCTGACTCCT				
	pnca2-F	TCTCCGGCACACCGGACTAT	396	58	+	+
	pnca2-R	CCAACAGTTCATCCCGGTTC				
<i>rpsL</i>	rpsL1-F	CAACCATCCAGCAGCTGGTC	375	55	+	+

	rpsL1-R	CGTAGACCGGGTCGTTGACC				
<i>rrs</i>	TB53-F	GATGACGGCCTTCGGGTTGT	384	54	+	+
	rrs2-R	GGAAACCCACACCTAGTACC				
	rrs3-F	GAACAGGATTAGATACCCTG	395	57	+	+
	rrs3-R	GGGCATGATGACTTGA				
	rrs1-F	ATGACGTCAAGTCATCATGC	392	50	+	+
	rrs1-R	GGCTCTCGCCCACTACAGAC				
<i>gidB</i>	gidB-F	GTCCCTCCACTCGCCATC	400	58	+	+
	gidB2-R	GCCATTGGCGATAGCGCGGC				
	gidB1-F	CAGCGTCGCTGCCGCCCAAT	378	58	+	+
	gidB-R	GTCCCTCCACTCGCCATC				
<i>gyrA</i>	gyrA-F	GATGTCTAACGCAACCCT	376	50	+	+
	gyrA-R	CGGTGGGTCATTGCCTGGCG				
<i>gyrB</i>	gyrB-F	GAGTTGGTGCGGCGTAAGAG	375	50		+
	gyrB-R	ACACATGCCCCGTTCTC				
<i>rplC</i>	rplC-F	CTGCGGCTGGACGACTCGGA	385	55		+
	rplC-R	CTTCTGCTCTTGCGCAGCCA				
<i>tlyA</i>	tlyA1-F	GGAGAAGGGTTGAGTGCGGC	400	57		+
	tlyA1-R	TGTGAGGCCACGTGCGTTGG				
	tlyA2-F	GCTGCGCAACGATCCTCGGG	400	60		+
	tlyA2-R	GGCCCTCGCTAATCGCACGG				
<i>ethA</i>	ethA1-F	GTTGACGGCCTCGACATTAC	396	58		+
	ethA1-R	GAATTCGCAGGTGAGGGCGC				
	ethA2-F	CTACGACGAGGGCTACTCGC	395	58		+
	ethA2-R	TGTAGTGCGGGCCGAAGTGC				
	ethA3-F	CAGACCGGACGGCATCGCC	396	60		+
	ethA3-R	ATAGTCGCCGTCGCCCCACC				
	ethA4-F	AGGGCATGATGCTTTC	375	50		+
	ethA4-R	TCATCGTCGTCTGACTATGG				
<i>ethR</i>	ethR1-F	CGCTGGCCGATATCTCGGTC	651	55		+
	ethR1-R	AGCGTCCGCTCGTTCATCAG				

23

24

25