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1 **NTF-RINT, a new method for the epidemiological**
2 **surveillance of MDR L2/Beijing strains *Mycobacterium***
3 ***tuberculosis***

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15 **Keywords :** atypical Beijing; typical Beijing; MDR-TB; molecular epidemiology

16

17 **Abstract**

18 The most widely discussed antibiotic-resistant tuberculosis strains (“W” and “B0/W148”,
19 “CAO”) belong to L2/Beijing Lineage and are characterized by IS6110 insertion sequences at
20 the NTF locus. We present a high-throughput, microbead-based method, called NTF/RINT
21 for detection of IS in NTF and Rifampicin and Isoniazid Typing. This method provides
22 tuberculosis diagnostic confirmation, screens for the so-called modern L2/Beijing sublineage
23 and detects mutations involved in resistance to Rifampicin (RIF) and Isoniazid (INH).

24 1. Introduction

25 *Mycobacterium tuberculosis* L2/Beijing is responsible of around one fifth of tuberculosis
26 cases around the world with the highest prevalence in Asia (50%) and Eastern Europe
27 (27.9%). In contrast, it is rare in South America and in Africa except for African coastal areas
28 and South Africa [1]. The L2/Beijing lineage harbors many *IS6110* copies and most strains
29 have a specific spoligotyping profile: absence of spacers 1-34 and presence of most of 35-43
30 spacers in the CRISPR region previously referred to as DR region [2]. The L2/Beijing is
31 subdivided in two major groups based on the *IS6110* copy numbers in a region usually
32 referred to as NTF region (between Rv3128c and Rv3129 genes, position 3494463-3493908
33 in H37Rv **AL123456.3**, GI:444893469) : “modern” (also referred to as “typical”) and
34 “ancient” (also referred to as “atypical”) Beijing strains [3].

35 Some of the largest most successful tuberculosis clusters belong to the so-called modern
36 Beijing sublineage characterized by 1) at least one *IS6110* insertion sequence in the 3’ part of
37 NTF region taken in its original orientation (**Fig. 1**), and 2) two mutations in 3R genes (*mutT2*
38 and *ogt* respectively at positions 58 and 12) [4]. The modern sublineage occurs worldwide
39 probably as a result of massive population migration, different immunogenic properties linked
40 to higher virulence [5, 6] and/or faster progression from latent infection to active tuberculosis
41 [7]. One of the reason for this increased virulence could be linked to PPE38 deletion, a
42 genotype preventing the secretion of several antigens that was found solely in modern Beijing
43 strains [8]. One of the first multi-drug resistant (MDR) epidemics reported was caused by a
44 modern Beijing strain termed “W”, characterized by an *IS6110* insertion in the 5’ end of NTF
45 locus in addition to the one on the 3’ end characteristic of modern sublineage [9]. More recent
46 L2/Beijing MDR outbreaks involve other modern isolates in Europe/Russia (B0/W148,
47 VNTR 100-32), and central Asia (CAO, VNTR 94-32) [10-12].

48 In this study, we aimed at developing a new high throughput technique to 1) diagnose
49 tuberculosis detecting *IS6110* insertion, 2) classify different Beijing genotype strains by
50 typing *IS6110* insertion in the NTF locus, and 3) identify drug resistance markers. We
51 previously developed the TB-SPRINT technique on Luminex technology that targets main
52 resistance mutations associated with Rifampicin (RIF) and Isoniazid (INH) resistance
53 simultaneously with spoligotyping (TB-SPRINT=TB-SPOL+TB-RINT, i.e. 43-Plex +16-Plex
54 [13]. We propose here “NTF/RINT” a technique that combines RINT to the detection of
55 *IS6110* in the NTF region. With this new technique, users will be able to classify strains in

56 different L2/Beijing clusters according to genotype and resistance profiles. This may
57 contribute to the improvement of the L2/Beijing epidemiological surveillance.

58

59 **2. Material and Methods**

60 *2.1. Material origin and characterization*

61 This study relies on two sets of strains. Forty-seven (n=47) clinical strains from New
62 York City (NYC), USA, were used to set up the method (sample set #1). Eighteen of them
63 belonged to the first large historical “W” MDR outbreak reported in NYC; double-checked
64 reference genotypes and partial phenotype characterizations were available for all these
65 strains. They were extracted by Cetyl-Trimethyl-Ammonium Bromide (CTAB) method as
66 described previously (van Soolingen, 1995). A second set (sample set #2) of 142 L2/Beijing
67 clinical strains from Kazakhstan was used as a validation set. Spoligotype pattern and full
68 genotype prediction as characterized by two methods (TB-SNPID, TB-SPRINT) were
69 available [14]. DNA was extracted by standard thermolysis on loops of solid cultures.

70 Genotype information concerning the *IS6110* insertions in the NTF locus for the set #1
71 was independently confirmed on agarose gel using slightly amended Plikaytis procedure: IS60
72 probe was changed to IS60B = 5' TGATGTGCTCCTTGAGTTCG 3' to amplify a 355 bp
73 fragment [9]. For set #2, it was indirectly studied by specific mutation at amino acid 58 in
74 *mutT2* included in TB-SNPID assay [15]. Genotype information concerning resistance loci
75 was obtained by TB-SNPID and by TB-SPRINT [13]. BCG Paris strain and a sample from
76 *Salmonella enterica* called “Sara8” were used as negative controls.

77 *2.2. NTF/RINT principle, primers and, probes*

78 For the *IS6110* typing in NTF, regarding amplifications, we used previously published
79 primers for detecting *IS6110* sequence [16] and designed new primers to amplify fragments
80 overlapping NTF insertion sites: NTF5'_Fw: GGCTGCCTACTACGCTCAAC; NTF5'_Rv1:
81 TCGACAACCCACTTCCCATG; NTF3'_Fw_new: TCACCACTGCTGCGTAATTTCG;
82 NTF3'_Rv_new: CCTGACATGACCCCATCCTTTC. For detection, we used a published
83 oligonucleotide sequence for *IS6110* sequence [17] and designed sequences straddling NTF
84 and *IS6110* sequences to detect the targeted insertions: NTF5'-probe
85 (GGCGGTTCAACATGGTGAC); NTF3'-probe (ATGAGGTCGTGAACCGCC). Probes
86 were coupled to microbeads as recommended by the manufacturer (www.luminex.corp).

87 The RINT part of NTF/RINT targets the same SNPs as those described by
88 Gomgnimbou et al. (2013): 1) the *rpoB* mutations 435GTC, 445GAC, 445TAC, 450TTG,
89 450TGG; 2) the *katG* mutations 315ACC and 315AAC; 3) the *inhA* promoter mutations -15T
90 and -8A. Please note that *rpoB* mutations are numbered according to their position in *M.*
91 *tuberculosis* H37Rv strain annotation GenBank **AL123456.3**, GI:444893469, in contrast with
92 previous studies referring to *E. coli* positions [13].

93 All primers and probes were obtained from integrated DNA Technologies (IDT,
94 Leuven, Belgium). The two spanning probes present in the TB-SPRINT assay development
95 were not included here.

96

97 2.3. Amplification and detection on Luminex

98 The amplification reaction mixture contained 2 μ L of DNA and 23 μ L of reaction
99 mixture (0.2 mM dNTPs, 1 μ M mix of primers, 0.25U GoTaq Flexi, 1.5mM MgCl₂, 1X
100 GoTaq buffer) as recommended by the manufacturer (Promega Corp., Fitchburg, WI). Cycles
101 consisted in 3 min at 96°C and 35 cycles of 30s at 96°C, 30s at 60°C, and 30s at 72°C. The
102 rest of the procedure was performed as for TB-SPRINT [13] and hybridized microbeads
103 samples were analyzed on a Luminex 200® device (Luminex, Austin, TX).

104 2.4. Data interpretation

105 Median Fluorescent Intensities (MFI) reported by the Luminex 200® device were
106 analyzed as follows: for the presence/absence of *IS6110* and its insertions in NTF region, the
107 standard cut-off for presence (MFI=150) was used and adjusted when necessary based on
108 control samples values. For SNP genotyping in *rpoB*, *katG* genes and *inhA* promoter, the
109 genotypes were inferred as described in Klotoe et al (2018) using allele-call method and
110 maximal MFI [18] except when the DNA concentration was very low as evidenced by low
111 signals for all markers, or rare erroneous inference in some of the controls due to high
112 background (*inhA* promoter -8A probe and *rpoB* 435GTC probe). In those cases,
113 interpretation was manual.

114

115

116 3. Results

117 3.1. NTF/RINT test design

118 NTF/RINT test aims to identify likely epidemic MDR L2/Beijing strains through
119 targeting IS6110 sequence, its insertion at two specific locations in the NTF locus and the
120 mutations most frequently implicated in RIF and INH resistance (**Fig. 2**). Raw data are
121 Median Fluorescent Intensities (MFI) obtained through a Luminex platform that after
122 interpretation are converted to positive or negative values (black/white squares in **Fig. 2B**,
123 **Supplementary Material 1**).

124 3.2. Sensitivity and specificity on a reference laboratory sample

125 Applied on the reference sample set, NTF/RINT produced bimodal MFI values allowing
126 us to discriminate positive from negative signals. Genotypes were inferred accordingly. For
127 the *katG* 315 position, no allele produced a positive signal for NYC outbreak strains in
128 accordance with them carrying a double mutation (AGC>ACA; **Supplementary Material 1**;
129 **Table 1**). Three other genotypes were declared Not Determined (ND) although reference
130 method declared them “wt”. A single allele of one strain was discordant with the reference
131 method TB-RINT (locus *rpoB* 450 for strain 1595). Altogether, 347 over 376 loci were
132 assigned a genotype and 346 were concordant with the reference (99.7%). The method
133 detected the 18 outbreak strains, 18 modern L2/Beijing and 11 strains belonging to ancient
134 Beijing clade or to other lineages, and a total of 24 MDR (**Table 1**).

135 3.3. Evaluation on clinical strains from Kazakhstan

136 A set of 142 Beijing clinical strains from Kazakhstan independently characterized with TB-
137 SPRINT and TB-SNPID methods were subjected to NTF/RINT. All but one (n=141) were
138 detected as modern Beijing by NTF/RINT as compared to 142 as detected by the reference
139 methods (*mutT2* mutation at position 58 detected by TB-SNPID). Sensitivity to detect modern
140 L2/Beijing was thus 99.6% while specificity could not be evaluated due to the composition of
141 the sample (absence of ancient L2/Beijing). No strains were detected that would carry solely
142 an insertion at the 5' end of the NTF locus.

143 Regarding the detection of resistance mutations, 130 strains (91.5%) and 980 loci out of 994
144 data points (98.6%) retrieved genotypes in full accordance with the reference
145 (**Supplementary Material 1**). A total of 101 isolates were correctly predicted as MDR-
146 modern Beijing, corresponding to a specificity of 93.5% (**Table 2**). Thirty out of a total of 34

147 strains predicted to be non-MDR were detected as such by NTF/RINT (sensitivity=88%).
148 MDR prediction could not rely on chance (Cohen's Kappa =0.81).

149

150 4. Discussion and conclusion

151 We set up NTF/RINT method to follow-up resistant Beijing strains by identifying most
152 common RIF and INH resistance mutations and by identifying *IS6110* insertions in the NTF
153 locus. Many *IS6110* insertions have been described in Beijing strains but only the one inserted
154 on the 3' end of NTF has been linked to a group of clinical relevance, namely "modern
155 Beijing" sublineage. The NTF-RINT assay is directly useful to tuberculosis researchers
156 interested in bridging current standards to the more classical test set up by Plikaytis. The
157 resulting assay is both sensitive and specific for all the mutations targeted.

158 Altogether in this study, NTF/RINT identified 125 modern L2/Beijing MDR strains,
159 among which 18 belonged to the "W"-strain branch (from the NYC outbreak), and four MDR
160 ancient Beijing. We identified a total of sixteen clusters according to genotypes (10 of which
161 are represented in **Fig. 2**), and seven clusters regarding classification and phenotype: 1) MDR
162 W-strain modern Beijing (n = 18), 2) MDR modern Beijing other than W (n = 108), 3) INH^R
163 modern Beijing (n = 18), 4) susceptible modern Beijing (n = 29), 5) susceptible ancient
164 Beijing (n = 7), 6) MDR ancient Beijing (n = 4), 7) INH^R ancient Beijing (n=1).

165 Regarding Beijing classification detection and sublineage prevalence, the dominance of
166 modern strains matches previous observations with a low prevalence of ancestral Beijing
167 except in Asia and South-Africa [10, 12, 19]. We detected no ambiguous cases where strains
168 would carry solely an insertion at the 5' end of the NTF locus. This was also true for the
169 L2/Beijing subsets from Peru, Mozambique, Bulgaria, Pakistan (data not shown). Merker et al
170 (2015) have reported L2/Beijing strains with this type of insertion but their analysis reported
171 any type of insertion whereas NTF/RINT targets only sequences straddling over *IS6110* at the
172 exact position described by Plikaytis et al (1994). The large study by Shitikov et al. also
173 observed no such case, and in contrast, identified a perfect link between the *IS6110* insertion
174 at the 3' end of NTF and the affiliation to the modern subbranch of Beijing [4]. Concerning
175 "W"-strain subbranch, its typical signature was detected solely in samples from the USA. This
176 matches its historical localisation and indicates no spreading of the epidemics.

177 Regarding resistance levels, we detected a very high prevalence of resistance among
178 modern Beijing strains as described on the complete study on Kazakhstan [14]. These strains
179 actually belong to the already well-known 94-32 epidemic cluster.

180 Limitations of this test in detecting MDR result from the detection of only the most
181 common antibiotic resistance mutations. We screened RIF Resistance due to 445 and 450
182 *rpoB* mutations that have shown to represent 80-91% of all RIF Resistance and INH
183 resistance due to *katG* 315ACC and 315AAC mutations responsible for 70% of INH
184 resistance [13]. Noteworthy, mutations other than the targeted ones lead to low signals
185 causing either indeterminations or erroneous assignation. As an example, the single case of
186 discordance in the set #1 which concerned *rpoB* 450 locus correlated with the 452CCG
187 mutation also conferring resistance. Altogether, NTF/RINT test may be useful for first-line
188 screening for any first-line drugs resistance [15].

189 As compared to other genotyping methods predicting resistance and/or classification,
190 NTF/RINT is relatively cost-effective (~4.5 euros of reagents for our laboratory in France in
191 2017-2019 including expenses for controls) due to the use of a restricted number of only 17
192 targets and the absence of need for the more costly xTAG® Luminex microbeads as used by
193 TB-SNPID [15]. Another characteristic of this technique is its high throughput since in one
194 working day, 90 samples can be processed. In contrast, testing smaller batches of samples
195 with this high-throughput method does not reduce turn-around time and increases the cost per
196 sample as the same number of controls is needed irrespective of the number of samples. This
197 test thus rather suits batch testing for retrospective studies for exploration or quality-control.
198 This test is best suited to settings where the circulating MDR-Beijing belong to several of the
199 subgroups detected by the test (W-strains, modern Beijing, ancient Beijing). This is the case
200 mainly in USA, South-Africa, Pacific region, former Soviet Union countries, and even more
201 in China where ancient- Beijing varies between ~10% to more than 44% [18]. In that case,
202 our method reaches a high discriminatory power for a very high throughput and low cost. It
203 can also serve as a control for species identification and check for resistance mutation
204 detection. The robustness of the test in trained hands makes it a priori suitable to further uses
205 of cartridges DNA remnants as can be retrieved from Genexpert's to confirm and/or refine
206 screening [20]. Studies measuring sensitivity and reproducibility in different settings to
207 evaluate robustness of the whole protocol should be performed before large scale
208 implementation. In addition to providing the detection of IS insertions in NTF, this test serves
209 as a proof-of-concept for a new type of assay as compared to SNP or spacer detection

210 previously set-up on microbead-based technique, namely the detection of insertions or
211 deletions. This concept may later serve detecting other types of insertions/deletions, such as
212 deletion of PE-PPE genes involved in modern Beijing strains virulence [20]. The NTF-RINT
213 test is also meant to be expanded to a 50-Plex *IS6110* and SNPs-based method dedicated to
214 L2 genotyping. This second step remains pending.

215

216 **Acknowledgements**

217 François-Xavier Weill is acknowledged for giving Salmonella DNA sample Sara8 to serve as
218 negative control for *IS6110* detection. Sarah Sengstake is acknowledged for contribution to
219 the genotyping of set #2 by TB-SNPID method.

220

221 **Conflict of interest**

222 Bernice Klotoe was partly founded by Beamedex®, a society that sells microbead-based kits.
223 The society had however no part in the decision of what new tests could be set-up and this
224 was entirely decided by public researchers Christophe Sola, Guislaine Refrégier, with the
225 consent of Natalia Kurepina and Barry N. Kreiswirth. Guislaine Refrégier acknowledges one
226 travel grant by Luminex® on another project regarding SNP detection. Luminex® is a
227 manufacturer of microbead-based instruments and microbeads.

228

229 Figure legends

230

231 Figure 1- NTF structure showing IS6110 positions and orientations compared to H37Rv
232 reference genome (**AL123456.3**, GI:444893469).

233

234 Figure 2 - Evolutionary scenario of IS6110 in NTF the locus of Beijing Strain (A) and
235 corresponding identified clusters according to allele presence/absence (B).

236

237 Table legends

238 Table 1- Concordance between NTF/RINT and controls on the reference samples set.

239 Table 2- Prediction of modern MDR Beijing on a Kazakhstan sample by NTF/RINT versus
240 TB-SPRINT/TB-SNPID

241

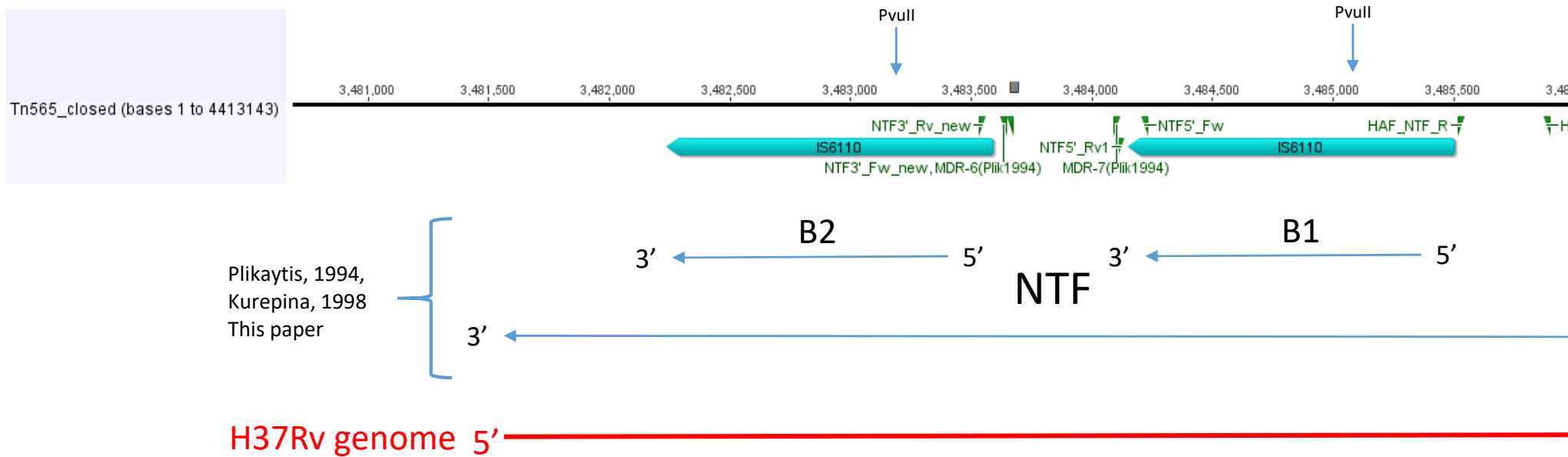


Figure 1 : NTF structure showing *IS6110* positions and orientations compared to H37Rv reference genome.

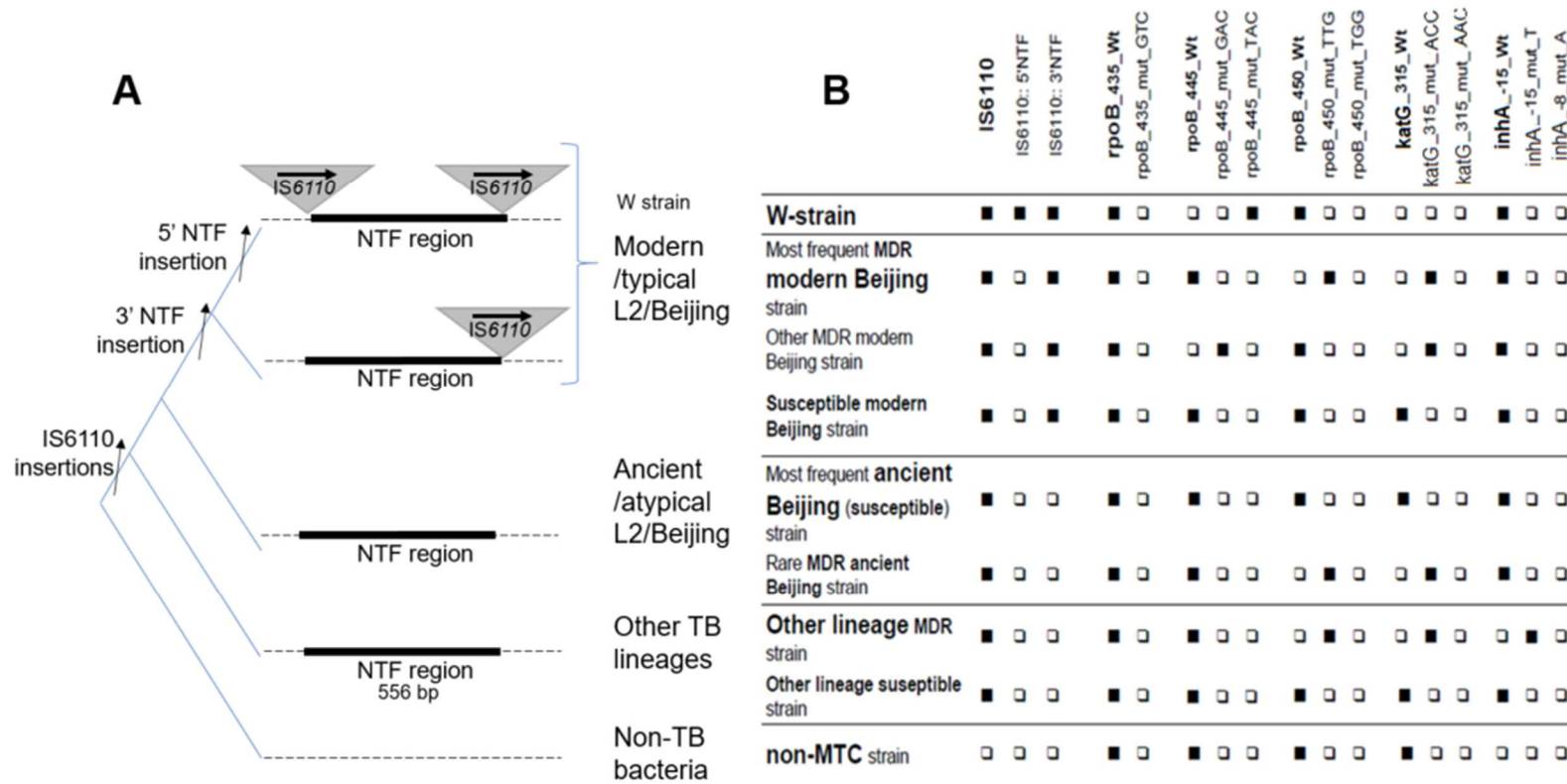


Figure 2 : Evolutionary scenario of *IS6110* in NTF the locus of Beijing Strain (A) and corresponding identified clusters according to allele presence/absence (B).

Table 1- Concordance between NTF/RINT and controls on the reference samples set.

N°	#	NY	NTF/RINT profiles			Control profiles		
			IS6110 profile	R. loc. profile	GDST	IS6110 on gel	TB_RINT or seq	PhDST
1	2015	YES	2 IS6110::NTF	435?/445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
2	2706	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
3	3452	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
4	3267	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
5	6138	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
6	8121	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
7	16852	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
8	1582	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
9	2470	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
10	15183	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
11	3542	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
12	4953	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
13	19556	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
14	13223	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
15	19996	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
16	1858	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	MDR
17	19962	YES	2 IS6110::NTF	445TAC/315?	MDR	2 IS6110::NTF	445TAC/315C	ND
18	13250	YES	2 IS6110::NTF	445TAC/315?	R ^R	2 IS6110::NTF	445TAC/315C	ND
19	11455	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	SUSC
20	14031	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	SUSC
21	14907	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	SUSC
22	15803	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	SUSC
23	16377	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	SUSC
24	7716	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	ND
25	7718	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	ND
26	20935	NO	1 IS6110::NTF	445?/WT	ND	1 IS6110::NTF	WT/WT	ND
27	12783	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	ND
28	6595	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	ND
29	20251	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	ND
30	20217	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	ND
31	17316	NO	1 IS6110::NTF	WT/WT	SUSC	1 IS6110::NTF	WT/WT	S ^R
32	15550	NO	1 IS6110::NTF	450TTG/-15T	MDR	1 IS6110::NTF	450TTG/-15T	ND
33	16870	NO	1 IS6110::NTF	450TTG/315C	MDR	1 IS6110::NTF	450TTG/315C	ND
34	3219	NO	1 IS6110::NTF	WT/315C	I ^R	1 IS6110::NTF	WT/315C	SI ^R
35	10598	NO	1 IS6110::NTF	450TTG/315C	MDR	1 IS6110::NTF	450TTG/315C	SI ^{E^R}
36	11877	NO	1 IS6110::NTF	435?/315C	ND/ I ^R	1 IS6110::NTF	WT/315C	ND
37	12766	NO	IS6110	WT/WT	SUSC	IS6110	WT/WT	SUSC
38	12360	NO	IS6110	WT/WT	SUSC	IS6110	WT/WT	SUSC
39	20082	NO	IS6110	WT/WT	SUSC	IS6110	WT/WT	ND
40	22661	NO	IS6110	WT/WT	SUSC	IS6110	WT/WT	ND
41	33452	NO	IS6110	WT/WT	SUSC	IS6110	WT/WT	ND
42	18842	NO	IS6110	435?/WT	ND/WT	IS6110	WT/WT	ND
43	12551	NO	IS6110	WT/WT	SUSC	IS6110	WT/WT	ND
44	10545	NO	IS6110	450TTG/315C	MDR	IS6110	450TTG/315C	MDR
45	11265	NO	IS6110	450TTG/315C	MDR	IS6110	450TTG/315C	MDR
46	1595	NO	IS6110	<u>450TGG/315C</u>	MDR	IS6110	WT/315C	ND
47	4948	NO	IS6110	WT/315C	I ^R	IS6110	WT/315C	ND

Outb=outbreak; GDST=Genotypic Drug Susceptibility Testing (DST); PhDST=Phenotypic DST; ND=Not Determined. I=Isoniazid; R=Rifampicin; S=Streptomycin; E=Ethionamide; SUSC=Susceptible; ^R/R.=Resistance; loc=locus. Seq=sequencing. The single true discordance is underlined.

Table 2- Prediction of modern MDR Beijing on a Kazakhstan sample by NTF/RINT versus TB-SPRINT/TB-SNPID

NTF/RINT	Reference (TB-SNPID, TB-SPRINT, Plikaytis)		
	MDR-modern Beijing	non-MDR modern Beijing	Total
MDR-anc. Beijing	1	0	1
MDR-modern Beijing	101	4	105
non-MDR modern Beijing	6	30	36
Total	108	34	142

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