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Author(s)	Shrestha, Dipti; Maharjan, Bhagwan; Oo, Nan Aye Thida; Isoda, Norikazu; Nakajima, Chie; Suzuki, Yasuhiko
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1 **Molecular analysis of streptomycin-resistance associating genes in *Mycobacterium tuberculosis***
2 **isolates from Nepal**

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4 **Dipti Shrestha^a, Bhagwan Maharjan^b, Nan Aye Thida Oo^{a,c}, Norikazu Isoda^{d,e}, Chie**
5 **Nakajima^{a,d}, Yasuhiko Suzuki^{a,d,*}**

6
7 ^aDivision of Bioresources, Hokkaido University Research Center for Zoonosis Control, Kita 20, Nishi
8 10, Kita-ku, Sapporo, 001-0020, Japan

9 ^bGerman Nepal Tuberculosis Project c/o Nepal Anti-Tuberculosis Association, Kalimati, Kathmandu,
10 Nepal, Post Box 1494

11 ^cBacteriology Research Division, Department of Medical Research, Ministry of Health and Sports,
12 Myanmar

13 ^dThe Global Station for Zoonosis Control, Global Institution for Collaborative Research and
14 Education (GI-Core), Hokkaido University, Kita 20, Nishi 10, Kita-ku, Sapporo, 001-0020, Japan

15 ^eUnit of Risk Analysis and Management, Hokkaido University Research Center for Zoonosis Control,
16 Kita 20, Nishi 10, Kita-ku, Sapporo, 001-0020, Japan

17
18 ***Corresponding author**

19 **Yasuhiko Suzuki**

20 Division of Bioresources, Hokkaido University, Research Center for Zoonosis Control, Kita 20, Nishi
21 10, Kita-ku, Sapporo, 001-0020, Japan

22 Tel: +81-11-706-9503, Fax: +81-11-706-7310

23 Email address: suzuki@czc.hokudai.ac.jp

24

25 **Abstract**

26 Mutation in *rpsL* (encoding ribosomal protein S12), *rrs* (encoding 16S ribosomal RNA) and *gidB*
27 (encoding 7-methylguanosine methyltransferase) are associated with resistance to streptomycin
28 (STR), which is used for the treatment of multi-drug resistant tuberculosis (MDR-TB) in Nepal. The
29 aim of our study is to analyze the correlation between mutations in the target genes and STR-
30 resistance in 197 *Mycobacterium tuberculosis* (MTB) isolates from Nepal. Mutations in *rpsL* was
31 harbored by 65.9% of isolates, in which the most common mutation in *rpsL* is caused by K43R
32 (58.8%) and were significantly associated with Beijing genotype ($P<0.001$). About 13.2% of isolates
33 harbored mutations in two highly mutable regions of *rrs*, the 530 loop and the 912 region. About
34 13.2% of *gidB* mutants do not show any mutation in *rpsL* and *rrs*, which might suggest the role of
35 *gidB* mutations in STR-resistance in MTB. In addition, 5.6% of isolates do not show any mutations
36 in three genes examined, suggesting the involvement of other mechanism in STR-resistance in MTB.
37 Our findings can be implemented for the establishment of molecular STR-susceptibility testing, in
38 which tuberculosis can be treated with appropriate drugs and can improve control strategies for DR-
39 TB.

40

41 Keywords: *Mycobacterium tuberculosis*, streptomycin, drug-resistance, *rpsL*, *rrs*, *gidB*

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), remains a major global health problem. According to the Global Tuberculosis Report (2019), World Health Organization (WHO) estimated 10.0 million cases and 1.5 million deaths in 2018 [1]. Drug-resistant TB (DR-TB) tends to increase worldwide, which causes difficulties in TB treatment. Multi-drug resistant TB (MDR-TB), which is defined as TB resistant to at least two potent anti-TB drugs, isoniazid (INH) and rifampicin (RIF), is a serious threat to fight against TB [2]. The WHO estimated that, worldwide in 2018, there were half a million new cases of rifampicin-resistant TB (RR-TB), and of this, 78% had MDR-TB [1]. Almost half (50%) of the global TB cases were in India (27%), China (14%), and the Russian Federation (9%).

National TB Program (NTP) Nepal registered 32,474 TB cases in 2018 and estimated annual case notification rate of 112/100,000 [3]. However, WHO estimated incidence rate of 151/100,000 and death rate of 19/100,000 for Nepal, meaning NTP Nepal is yet to identify the missing cases in the community and bring them to treatment [1].

Streptomycin (STR) has been an important drug for the treatment of pulmonary TB in human [4]. For the retreatment TB patients in Nepal, STR has been included in intensive phase for two months with other first line anti-TB drugs [5]. However, NTP no longer recommends STR to be used in retreatment regimen in Nepal [3]. According to the WHO recommended regimen as published in “WHO treatment guidelines for MDR and RR-TB, 2018 Pre-final test”[6] and updated guidelines in “WHO consolidated guidelines on drug-resistant tuberculosis treatment, 2019” [7], STR is categorized into Group C agents in which it may be included in the treatment of MDR/RR-TB patients aged 18 years or more on longer regimens when susceptibility has been demonstrated and adequate measures to monitor for adverse reaction can be ensured, if amikacin is not available [3]. To move into the new WHO recommendation, NTP will develop a comprehensive transition plan [3]. With an increment of MDR-TB cases, and in addition of MDR-TB isolates resistant to second-line drugs and newly discovered drugs, STR should be used in combination with other drugs for MDR-TB treatment regimen. Despite its high rate of side effects and risk of developing drug resistance in TB of its single use in a regimen [4], it is crucial to understand the molecular mechanism of resistance to STR in MTB from Nepal.

STR impairs translational proofreading, leading to the blockade of protein synthesis. Resistance to STR is associated with mutations in *rpsL* and *rrs* genes, encoding for ribosomal protein S12 and 16S ribosomal RNA, respectively. The most frequent mutations localize around two regions of *rrs*, namely the 530 loop and the 912 region, or at codons 43 or 88 within *rpsL* [8],[9]. Mutations within the *gidB* gene, encoding a conserved 7-methylguanosine methyltransferase specific for the 16S rRNA, have

76 been reported to confer low-level STR-resistance in MTB [10]. Since, STR-resistance associating
77 genes vary geographically, it is important to perform the molecular studies which will detect the
78 common mutations in target genes conferring STR-resistance among MTB strains [10],[11].

79 The time consuming conventional proportional method using Löwenstein-Jensen medium is the
80 only available drug susceptibility testing (DST) for STR in Nepal. Recently, NTP have adopted the
81 latest diagnostic tools such as Xpert MTB/RIF, Line probe assay (LPA), and Mycobacterium Growth
82 Indicator Tube (MGIT) [3]. Despite the high number of MDR-TB cases with STR-resistant MTB
83 strains, no genetic information regarding the molecular mechanism of STR-resistant MTB in Nepal
84 is currently available.

85 The main aim of this study is to characterize the molecular mechanism of STR-resistance of MTB
86 in Nepal; to analyze the correlation between mutations in the *rpsL*, *rrs* and *gidB* and STR-resistant
87 MTB isolates from Nepal; and to study the association of those mutations with different MTB
88 genotypes. To elucidate mutations responsible for the resistance to STR, we performed sequence
89 analysis of STR-resistance associating genes (*rpsL*, *rrs* and *gidB*) in MDR-MTB isolates from Nepal.

91 **2. Materials and Methods**

92 **2.1. Studied sample**

93 MDR-TB isolates with resistant to all four first line drugs INH, RIF, STR and ethambutol (EMB)
94 has been preserved at the German Nepal Tuberculosis Project (GENETUP), Nepal. A total of 197
95 representative STR-resistant MTB isolates from April 2009 to March 2012 were selected in this study.
96 These samples were subjected to genotyping and mutation analysis.

98 **2.2. Drug susceptibility test (DST)**

99 DST for INH, RIF, EMB (FatolArzneimittel GmbH, Schiffweiler, Germany) and STR (Sigma-
100 Aldrich, St. Louis, MO) were performed by the indirect proportional method on Löwenstein-Jensen
101 medium with the critical concentrations of 0.2, 40, 2 and 4 µg/ml, respectively [12],[13].

103 **2.3. DNA extraction**

104 Extraction of DNA was done for PCR following the manufacturers protocol using the Genotype
105 MTBDR_{plus} Ver2 (HainLifescienceNehren, Germany) [14].

107 **2.4. Spoligotyping**

108 The genotypes of the MTB isolates were identified using spoligotyping which was performed as
109 described previously [15],[16].

110

111 **2.5. PCR amplification and sequencing**

112 Amplification of the 437 bp, 590 bp and 870 bp fragments of *rpsL*, *rrs* and *gidB*, respectively, were
113 performed using primers shown in Table 1. PCRs were carried out in a thermal cycler (Bio-Rad
114 Laboratories, Inc., Hercules, CA) under the condition previously described [17]. The presence of
115 PCR products was confirmed by agarose gel electrophoresis. Purified PCR products were
116 sequenced using DNA sequencer, 3500 Genetic Analyzer (Thermo Fisher Scientific, Inc., Waltham,
117 MA) using both forward and reverse primers same as for amplification. The obtained sequences
118 were aligned with the corresponding sequences from the wild-type *M. tuberculosis* H37Rv using
119 Bio-Edit software (version 7.0.9) [18].

120

121 **2.6. Statistical analysis**

122 All data were analyzed using R version 3.6. Pearson's Chi-square test was used to compare
123 different variances and to determine the association between MTB lineages, drug-resistance profile
124 and mutations in the genes examined. A p-value less than 0.05 was considered statistically significant.

125

126 **2.7. Ethical approval**

127 This study proposal was submitted to Nepal Health Research Council (NHRC) with register no
128 136/2013 on 20th Sep 2013 and approved by Ethical Review Board on 29th Nov 2013.

129

130 **3. Results**

131 **3.1. *Mycobacterium tuberculosis* genotypes**

132 Through spoligotyping, the genotypes of 197 STR-resistant MDR-MTB isolates were detected as
133 11 lineage 1, 116 lineage 2, 47 lineage 3 and 23 lineage 4 (Table 2).

134

135 **3.2. Mutation in *rpsL*, *rrs* and *gidB***

136 Mutations in only *rpsL* were harbored by 65.9% (130/197) of isolates. Mutations conferring
137 amino acid substitution at *rpsL* codon 43 and 88 were found in 58.8% (116/197) and 7.1% (14/197)
138 of isolates, respectively (Table 3).

139 There were seven different substitutions found at seven different positions in the *rrs* gene.
140 Mutations in only *rrs* was harbored by 13.2% (26/197) of isolates (Table 3). Mutations at positions

141 A514C and C517T of *rrs* were found in 5.6% (11/197) and 2.5% (5/197) of isolates, respectively.
142 Mutations around 912 loop (A906G, A907C, A908C or A912C) of *rrs* were found in 5.1% (10/197)
143 of isolates. A co-mutation in *rpsL* and *rrs* (*rpsL* K43R, *rrs* A907C; *rpsL* K88R, *rrs* A644G; and *rpsL*
144 G118D, *rrs* A514C) was found in 1.5% (3/197) of isolates.

145 Mutations in *gidB* was harbored by 25.8% (51/197) of isolates (Table 5). The *gidB* mutants
146 showing mutations in *rpsL* and/or *rrs* were found in 12.7% (25/197) of STR-resistant isolates
147 (Supplementary Table). About 13.2% (26/197) of *gidB* mutants do not show any mutation in *rpsL* and
148 *rrs* (Supplementary Table). No mutations in three genes examined in this study was found in 5.6%
149 (11/197) of isolates (Supplementary Table).

150

151 **4. Discussion**

152 According to our knowledge, this is the first study on the molecular characterization conferring
153 STR-resistance associating genes in MTB isolates from Nepal. It has been determined that mutations
154 in *rpsL* and *rrs* genes are associated with STR-resistance in MTB [20]. In MDR-TB isolates, about
155 80.7% (159/197) of STR-resistant MTB isolates harbored mutations in either *rpsL* or *rrs*. This is
156 within the range, reported from Asian countries, 40% to 98% [19],[20],[21],[22],[23]. Mutations in
157 *rpsL* was harbored by 65.9% (130/197) of isolates (Table 3), which suggest that these mutations
158 strongly correlate to STR-resistance in MTB. The most common mutation in *rpsL* codon K43R was
159 harbored by 58.8% of *rpsL* mutants in this study. The detection rate of mutations in *rpsL* codon K43R
160 varies considerably between geographical areas (Table 4). The high detection rate of mutation in *rpsL*
161 K43R in Nepal might be due to its non-restrictive mutation leading to no fitness cost in MTB [24]
162 which can be widely transmitted; and the bad practice of STR mono-therapy usage for the treatment
163 of TB and other bacterial diseases in the past in Nepal [25]. The predominant mutation *rpsL* K43R
164 was found in 80% (92/117) of Beijing isolates. There was a significant association between Beijing
165 strains and mutation K43R compared with the group of STR-resistant non-Beijing isolates ($P<0.001$).
166 Sun et al reported that mutation in *rpsL* causing K43R showed strong association with Beijing
167 genotype in STR-resistance isolates [22]. However, in India and Mexico, where Beijing genotype was
168 not frequent, mutations in these two loci had no significant contribution to Beijing genotype [19],[21].
169 The Beijing genotype has been reported as more virulent than other MTB genotypes, and has a
170 propensity to acquire the *rpsL* K43R mutation [22]. The high mutation rate in *rpsL* K43R represents
171 the geographical areas predominate by Beijing genotype family. In contrast, mutation *rpsL* K88R was
172 not significantly associated with the Beijing family ($P=0.34$). Mutations in *rpsL* K43R and G71D
173 were found in one STR-resistant non-Beijing isolate. As mutation K43R of *rpsL* strongly contributes
174 to STR-resistance in MTB, it is doubtful that whether mutation G71D of *rpsL* contribute to STR-

175 resistance in MTB or not. Mutation conferring G71D found in this study, was reported by study [26]
176 with an isolate with mutation in *rpsL* G71S (GGC→AGC), compounded with mutation in *rpsL* K43R;
177 and might be of only minor importance.

178 About 13.2% (26/197) of isolates harbored *rrs* mutations (except co-mutation in *rpsL*) in our study,
179 which showed a frequency range of 0 to 12.5% (Table 4) in the Mideast and Southeast Asian countries
180 [27],[28],[29],[30],[31]. Mutations in *rrs* were found in two highly mutable regions of *rrs*, the 530
181 loop and the 912 region, which suggest these mutations contributed to STR-resistance in MTB as
182 reported [8],[10],[19],[20],[21],[27],[32],[33],[34],[35],[36].

183 Three strains showed co-mutations in both *rpsL* and *rrs*. Among them, one strain showed
184 mutations in *rpsL* causing K43R and *rrs* 912 region A907C. Meier et al had reported the similar
185 findings of co-mutations in *rpsL* and *rrs*, which described that the *rrs* mutation (A→G at position
186 904) presumably conferred as yet ill-defined partial resistance to STR in vivo, thereby increasing
187 the number of cells which could acquire a second mutation in the *rpsL* (alteration of K88),
188 conferring resistance to STR-induced misreading [33]. Similar co-mutations *rpsL* K43R/*rrs* C789T
189 [37]; and *rpsL* K88Q/*rrs* A905G and *rpsL* K43R/*rrs* A513C [38] have been reported. In contrast,
190 Jagielski et al., had reported that mutations in *rrs* and *rpsL* were mutually exclusive, that is isolates
191 with *rrs* mutations had no *rpsL* mutations and vice versa [39]. The discrepancy in our results might
192 be due to the presence of mixed population of *rpsL* K43R mutants and *rrs* A907C mutants. This was
193 confirmed to be 1:1 mixture of A and G at nucleotide position 128 in *rpsL* by the electropherogram
194 obtained. One strain harbored mutations in *rpsL* causing K88R and *rrs* A644G. One studies had
195 reported an isolate with mutation in *rpsL* K43R and a second mutation in *rrs* nucleotide 645 A
196 deletion [26]. A mutation in *rrs* A644G is a novel mutation and this mutation is located far away
197 from hot spot mutation regions of *rrs*, the 530 loop and 912 region, which is associated with the
198 STR-resistance in MTB. As mutation in *rpsL* causing K88R is enough to cause STR-resistance in
199 MTB, a mutation in *rrs* A644G might not be associated with the STR-resistance in MTB but be a
200 natural polymorphism. Other strain harbored mutations in *rpsL* causing G118D and *rrs* A514C. As
201 MTB has a single rRNA operon [40], a single mutation in *rrs* A514C is enough to cause STR-
202 resistance in MTB. Mutation G118D have not been previously reported and this mutation is located
203 far away from frequent mutations in *rpsL*, K43R and K88R, which cause STR-resistance in MTB. It
204 is unclear that the mutation in *rrs* A644G and mutation in *rpsL* causing G118D may affect the
205 mechanism of STR-resistance in MTB. So, further studies should be carried out to find out the exact
206 role of these mutations in causing STR-resistance in MTB.

207 We observed that 13.7% (27/197) of STR-resistant isolates showed many mis-sense mutations in
208 *gidB* that were close to active or part of the interacting amino acid residues between the GidB protein

209 and S-adenosylmethionine (SAM) which are critical for methylation at the position G517 of 16S
210 rRNA [27]. These alterations may have an impact on the function of GidB which leads to low affinity
211 of STR to 16S rRNA and hence contribute to STR-resistance in MTB. The missense mutations in
212 *gidB* codons causing amino acid substitutions A19G, R20P, A27P, A27V, V36L, A82P, L101E,
213 V112G, V124G, A133V, M218V.

214 The *gidB* mutants harboring co-mutations in *rpsL*/*rrs* genes were found in 12.7% (25/197) of
215 isolates. These co-mutations of in *rpsL*/*rrs* of *gidB* mutants might suggest the evolution of *rpsL*/*rrs*
216 mutations for its existence as a compensation for losing methylation function of GidB caused by
217 distortion of its structure and consequently results in high level resistance to STR [10],[36].

218 It was found that 13.2% (26/197) of isolates showed mutations in *gidB* without those in *rpsL* and
219 *rrs* genes. All 13 mis-sense mutations had an exclusive *gidB* mutation without mutations in *rpsL* and
220 *rrs* conferring these *gidB* mutations contribute to STR-resistance in MTB. Deletion mutations in *gidB*
221 nucleotide without those in *rpsL* and *rrs* (112delC, 254delA, 347delG and 372delG) and insertion
222 mutations in *gidB* nucleotide (243insGC) causing frameshift in *gidB* in STR-resistant isolates found
223 in this study which alter the GidB protein and consequently leading to low-level STR-resistance in
224 MTB. Deletions with variable sizes at different positions in *gidB* were also reported [10],[8],[30].
225 Frameshift mutations (112delC, 254delA, 347delG, 372delG, 384delG and 243insGC) were
226 previously reported. The deletion mutation in *gidB* delG at nucleotide 98 have also been reported in
227 both STR-susceptible and STR-resistant isolates [39],[41]. Mutations in *gidB* found exclusively in
228 STR-resistant strains play a role in STR-resistance in MTB. These 13 mis-sense mutations and 5
229 frameshift mutations in *gidB* were highly associated with STR-resistance in MTB.

230 There were four *gidB* polymorphisms phylogenetically specific to MTB lineages which were found
231 in 75.6% (149/197) of isolates. The missense mutation E92D conferred by 100% (116/116) of lineage
232 2 isolates which was significantly associated with the Beijing family of lineage 2 ($P<0.001$). Similar
233 indication of high specificity to Beijing strains have been reported in previous studies
234 [34],[35],[36],[42]. The silent mutation in *gidB* codon at V110 was significantly associated with EAI
235 strains ($P<0.001$), which is also supported by previous findings [34],[42]. Another silent mutation in
236 *gidB* at codon A205 was present in 100% (23/23) of the Euro-American strains of lineage 4 which
237 was significant ($P<0.001$). In contrast, polymorphisms in *gidB* codon at A205 was absent from almost
238 all of the Euro-American strains in which this allele may be used as a specific marker for
239 differentiating Euro-American strains from non-Euro-American strains [34],[42]. Finally, all LAM
240 strains conferred the allele mutation in *gidB* codon at L16 was significant ($P<0.01$) which is also
241 confirmed by the previous findings [36],[43],[44].

242 In total, 94.4% (186/197) of STR-resistant isolates harbored mutations in either *rpsL* or *rrs* or *gidB*

243 alone or in combinations. This suggests that mutations in *rpsL*, *rrs* and *gidB* play a significant role in
244 the mechanism of STR-resistance in MTB. In this study, we found 11 isolates (one lineage 1 strains,
245 two lineage 2 strains, seven lineage 3 strains and one lineage 4 strains) do not harbor mutation in any
246 of the genes studied, accounting for 5.6% of the STR-resistant strains. This suggests that there might
247 be an involvement of other genes in the mechanism of STR-resistance such as the efflux system, cell
248 membrane permeability barrier or aminoglycosides converting enzymes.

249 In conclusion, the mutations in *rpsL* and *rrs*; and some exclusive mutations in *gidB* identified in
250 this study are responsible for STR-resistance in MTB. The sequence analysis of mutations in *rpsL*,
251 *rrs* and *gidB* could be used for the establishment of rapid and effective molecular diagnostic tools to
252 detect STR-resistant MTB strains in Nepal. This study can be further implicated for designing rapid
253 diagnostic tools considering geographic specificity in which MDR-TB transmission can be interrupt
254 worldwide.

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266 REFERENCES

- 267 [1] World Health Organization. Global Tuberculosis Report 2019. WHO;2019.
- 268 [2] Report MW. Prevention and control of tuberculosis in correctional and detention facilities:
 269 recommendations from CDC. Endorsed by the Advisory Council for the Elimination of
 270 Tuberculosis, the National Commission on Correctional Health Care, and the American
 271 Correcti. vol. 55. 2006;55/RR-9.
- 272 [3] National Tuberculosis Program Nepal-Annual-Report-2074-75-Up 2018;75.
- 273 [4] Diel R. Treatment of tuberculosis. *Pneumologie* 2019;16:117–30. doi:10.1007/s10405-019-
 274 0234-x.
- 275 [5] MoH/DoHS/NTC. National Tuberculosis Program - Nepal: Annual report 2073/74 (2017).
 276 Natl Tuberc Center, Nepal 2017;74:1–128.
- 277 [6] WHO | Global tuberculosis report 2018. 2018.
- 278 [7] Sharma SK, Dheda K. What is new in the WHO consolidated guidelines on drug-resistant
 279 tuberculosis treatment? vol. 149. 2019. https://doi.org/10.4103/ijmr.IJMR_579_19.
- 280 [8] Spies FS, Almeida Da Silva PE, Ribeiro MO, Rossetti ML, Zaha A. Identification of mutations
 281 related to streptomycin resistance in clinical isolates of *Mycobacterium tuberculosis* and
 282 possible involvement of efflux mechanism. *Antimicrob Agents Chemother* 2008;52:2947–9.
 283 <https://doi.org/10.1128/AAC.01570-07>.
- 284 [9] Via LE, Cho SN, Hwang S, Bang H, Park SK, Kang HS, et al. Polymorphisms associated with
 285 resistance and cross-resistance to aminoglycosides and capreomycin in *Mycobacterium*
 286 *tuberculosis* isolates from south Korean patients with drug-resistant tuberculosis. *J Clin*
 287 *Microbiol* 2010;48:402–11. <https://doi.org/10.1128/JCM.01476-09>.
- 288 [10] Okamoto S, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, Tokuyama S, et al. Loss of a
 289 conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin
 290 resistance in bacteria. *Mol Microbiol* 2007;63:1096–106. [https://doi.org/10.1111/j.1365-](https://doi.org/10.1111/j.1365-2958.2006.05585.x)
 291 [2958.2006.05585.x](https://doi.org/10.1111/j.1365-2958.2006.05585.x).

- 292 [11] Tracevska T, Jansone I, Nodieva A, Marga O, Skenders G, Baumanis V. Characterisation of
 293 rpsL, rrs and embB mutations associated with streptomycin and ethambutol resistance in
 294 *Mycobacterium tuberculosis*. Res Microbiol 2004;155:830–4.
 295 doi:10.1016/j.resmic.2004.06.007.
- 296 [12] Canetti G, Fox W, Khomenko A, Mahler HT, Menon NK, Mitchison DA, et al. Advances in
 297 techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in
 298 tuberculosis control programmes. Bull World Health Organ 1969;41:21–43.
- 299 [13] Edition F. Guidelines for surveillance of drug resistance in tuberculosis. WHO
 300 Geneva/IUATLD Paris. International Union Against Tuberculosis and Lung Disease. Int J
 301 Tuberc Lung Dis 1998;2:72–89.
- 302 [14] Hain Lifescience. Geno Type MTBDRplus Ver 2.0. Hain Lifescience 2015:1–10.
- 303 [15] Maharjan B, Nakajima C, Isoda N, Thapa J, Poudel A, Shah Y, et al. Genetic diversity and
 304 distribution dynamics of multidrug-resistant *Mycobacterium tuberculosis* isolates in Nepal. Sci
 305 Rep 2018;8:1–10. <https://doi.org/10.1038/s41598-018-34306-w>.
- 306 [16] Kamerbeek J, Schouls L, Kolk A, Van Agterveld M, Van Soolingen D, Kuijper S, et al.
 307 Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis
 308 and epidemiology. J Clin Microbiol 1997;35:907–14.
- 309 [17] Tekwu EM, Sidze LK, Assam JPA, Tedom JC, Tchatchouang S, Makafe GG, et al. Sequence
 310 analysis for detection of drug resistance in *Mycobacterium tuberculosis* complex isolates from
 311 the Central Region of Cameroon. BMC Microbiol 2014;14:1–10. doi:10.1186/1471-2180-14-
 312 113.
- 313 [18] Hall T.A, BioEdit: a user-friendly biological sequence alignment editor and analysis program
 314 for Windows 95/98/NT. Oxford University Press 1999; Nucleic acid Symposium Series No-
 315 42:95-98.
- 316 [19] Cuevas-Córdoba B, Cuellar-Sánchez A, Pasissi-Crivelli A, Santana-álvarez CA, Hernández-
 317 Illezcas J, Zenteno-Cuevas R. Rrs and rpsL mutations in streptomycin-resistant isolates of

318 *Mycobacterium tuberculosis* from Mexico. J Microbiol Immunol Infect 2013;46:30–4.
 319 <https://doi.org/10.1016/j.jmii.2012.08.020>.

320 [20] Fukuda M, Koga H, Ohno H, Yang B, Hirakata Y, Maesaki S, et al. Relationship between
 321 genetic alteration of the. J Antimicrob Chemother 1999;43:281–4.

322 [21] Siddiqi N, Shamim M, Hussain S, Kumar Choudhary R, Ahmed N, Prachee, et al. Molecular
 323 characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients
 324 in North India. Antimicrob Agents Chemother 2002;46:443–50. doi:10.1128/AAC.46.2.443-
 325 450.2002.

326 [22] Sun YJ, Luo JT, Wong SY, Lee ASG. Analysis of rpsL and rrs mutations in Beijing and non-
 327 Beijing streptomycin-resistant *Mycobacterium tuberculosis* isolates from Singapore. Clin
 328 Microbiol Infect 2010;16:287–9. <https://doi.org/10.1111/j.1469-0691.2009.02800.x>.

329 [23] Zhao Y, Xu S, Wang L, Chin DP, Wang S, Jiang G, et al. National survey of drug-resistant
 330 tuberculosis in China. N Engl J Med 2012;366:2161–70. doi:10.1056/NEJMoa1108789.

331 [24] Bottger EC, Springer B, Pletschette M, Sander P. Fitness of antibiotic-resistant microorganisms
 332 and compensatory mutations [3]. Nat Med 1998;4:1343–4. doi:10.1038/3906.

333 [25] Thapa G, Pant ND, Khatiwada S, Iekhak B, Shrestha B. Drug susceptibility patterns of the
 334 *Mycobacterium tuberculosis* isolated from previously treated and new cases of pulmonary
 335 tuberculosis at German-Nepal tuberculosis project laboratory, Kathmandu, Nepal. Antimicrob
 336 Resist Infect Control 2016;5:1–7. doi:10.1186/s13756-016-0129-0.

337 [26] Shi R, Zhang J, Li C, Kazumi Y, Sugawara I. Detection of streptomycin resistance in
 338 *Mycobacterium tuberculosis* clinical isolates from China as determined by denaturing HPLC
 339 analysis and DNA sequencing. Microbes Infect 2007;9:1538–44.
 340 doi:10.1016/j.micinf.2007.08.009.

341 [27] Verma JS, Gupta Y, Nair D, Manzoor N, Rautela RS, Rai A, et al. Evaluation of gidB alterations
 342 responsible for streptomycin resistance in *Mycobacterium tuberculosis*. J Antimicrob
 343 Chemother 2014;69:2935–41. doi:10.1093/jac/dku273.

- 344 [28] Ali A, Hasan Z, McNerney R, Mallard K, Hill-Cawthorne G, Coll F, et al. Whole genome
345 sequencing based characterization of extensively drug-resistant *Mycobacterium tuberculosis*
346 isolates from pakistan. PLoS One 2015;10. doi:10.1371/journal.pone.0117771.
- 347 [29] Sun H, Zhang C, Xiang L, Pi R, Guo Z, Zheng C, et al. Characterization of mutations in
348 streptomycin-resistant *Mycobacterium tuberculosis* isolates in Sichuan, China and the
349 association between Beijing-lineage and dual-mutation in *gidB*. Tuberculosis 2016;96:102–6.
350 doi:10.1016/j.tube.2015.09.004.
- 351 [30] Thida Oo NA, San LL, Thapa J, Aye KS, Aung WW, Nakajima C, et al. Characterization of
352 mutations conferring streptomycin resistance to multidrug-resistant *Mycobacterium*
353 *tuberculosis* isolates from Myanmar. Tuberculosis 2018;111:8–13.
354 doi:10.1016/j.tube.2018.05.003.
- 355 [31] Hlaing YM, Tongtawe P, Tapchaisri P, Thanongsaksrikul J, Thawornwan U, Archanachan B,
356 et al. Mutations in streptomycin resistance genes and their relationship to streptomycin
357 resistance and lineage of *Mycobacterium tuberculosis* Thai isolates. Tuberc Respir Dis (Seoul)
358 2017;80:159–68. doi:10.4046/trd.2017.80.2.159.
- 359 [32] Katsukawa C, Tamaru A, Miyata Y, Abe C, Makino M, Suzuki Y. Characterization of the *rpsL*
360 and *rrs* genes of streptomycin-resistant clinical isolates of *Mycobacterium tuberculosis* in
361 Japan. J Appl Microbiol 1997;83:634–40. doi:10.1046/j.1365-2672.1997.00279.x.
- 362 [33] Meier A, Kirschner P, Bange FC, Vogel U, Bottger EC. Genetic alterations in streptomycin-
363 resistant *Mycobacterium tuberculosis*: Mapping of mutations conferring resistance.
364 Antimicrob Agents Chemother 1994;38:228–33. doi:10.1128/AAC.38.2.228.
- 365 [34] Smittipat N, Juthayothin T, Billamas P, Jaitrong S, Rukseree K, Dokladda K, et al. Mutations
366 in *rrs*, *rpsL* and *gidB* in streptomycin-resistant *Mycobacterium tuberculosis* isolates from
367 Thailand. J Glob Antimicrob Resist 2016;4:5–10. doi:10.1016/j.jgar.2015.11.009.
- 368 [35] Villellas C, Aristimuño L, Vitoria MA, Prat C, Blanco S, De Viedma DG, et al. Analysis of
369 mutations in streptomycin-resistant strains reveals a simple and reliable genetic marker for

370 identification of the *Mycobacterium tuberculosis* Beijing genotype. J Clin Microbiol
371 2013;51:2124–30. doi:10.1128/JCM.01944-12.

372 [36] Spies FS, Ribeiro AW, Ramos DF, Ribeiro MO, Martin A, Palomino JC, et al. Streptomycin
373 resistance and lineage-specific polymorphisms in *Mycobacterium tuberculosis* *gidB* gene. J
374 Clin Microbiol 2011;49:2625–30. doi:10.1128/JCM.00168-11.

375 [37] Sreevatsan S, Pan X, Stockbauer KE, Williams DL, Kreiswirth BN, Musser JM.
376 Characterization of *rpsL* and *rrs* mutations in streptomycin-resistant *Mycobacterium*
377 *tuberculosis* isolates from diverse geographic localities. Antimicrob Agents Chemother
378 1996;40:1024–6.

379 [38] Dobner P, Bretzel G, Rüscher-Gerdes S, Feldmann K, Rifai M, Löscher T, et al. Geographic
380 variation of the predictive values of genomic mutations associated with streptomycin
381 resistance in *Mycobacterium tuberculosis*. Mol Cell Probes 1997;11:123–6.
382 doi:10.1006/mcpr.1996.0086.

383 [39] Jagielski T, Ignatowska H, Bakula Z, Dziewit Ł, Napiórkowska A, Augustynowicz-Kopeć E,
384 et al. Screening for streptomycin resistance-conferring mutations in *Mycobacterium*
385 *tuberculosis* clinical isolates from Poland. PLoS One 2014;9.
386 doi:10.1371/journal.pone.0100078.

387 [40] Suzuki Y, Yoshinaga K, Ono Y, Nagata A, Yamada T. Organization of rRNA genes in
388 *Mycobacterium bovis* BCG. J Bacteriol 1987;169:839–43. doi:10.1128/jb.169.2.839-843.1987.

389 [41] Nhu NTQ, Lan NTN, Phuong NTN, Van V Chau N, Farrar J, Caws M. Association of
390 streptomycin resistance mutations with level of drug resistance and *Mycobacterium*
391 *tuberculosis* genotypes. Int J Tuberc Lung Dis 2012;16:527–31. doi:10.5588/ijtld.11.0202.

392 [42] Feuerriegel S, Oberhauser B, George AG, Dafaie F, Richter E, Rüscher-Gerdes S, et al. Sequence
393 analysis for detection of first-line drug resistance in *Mycobacterium tuberculosis* strains from
394 a high-incidence setting. BMC Microbiol 2012;12. doi:10.1186/1471-2180-12-90.

395 [43] Bauskenieks M, Pole I, Skenders G, Jansone I, Broka L, Nodieva A, et al. Genotypic and

396 phenotypic characteristics of aminoglycoside-resistant *Mycobacterium tuberculosis* isolates in
397 Latvia. Diagn Microbiol Infect Dis 2015;81:177–82. doi:10.1016/j.diagmicrobio.2014.12.004.
398 [44] Perdigão J, Macedo R, Machado D, Silva C, Jordão L, Couto I, et al. GidB mutation as a
399 phylogenetic marker for Q1 cluster *Mycobacterium tuberculosis* isolates and intermediate-
400 level streptomycin resistance determinant in Lisbon, Portugal. Clin Microbiol Infect 2014;20.
401 doi:10.1111/1469-0691.12392.
402

403 **Table 1 Primers used for PCR amplification and sequencing of streptomycin-resistance**
 404 **associated genes in *M. tuberculosis***

Locus	Primers	Nucleotide sequence (5'-3')	Product size (bp)
<i>rpsL</i>	Forward (ON-1505)	GGCCGACAAACAGAACGT	437
	Reverse (ON-1506)	CTTCACCAACTGGGTGAC	
<i>rrs</i>	Forward (ON-1507)	GATGACGGCCTTCGGGTTGT	590
	Reverse (ON-1508)	AGGCCACAAGGGAACGCCTA	
<i>gidB</i>	Forward (ON-1509)	CGCCGAGTCGTTGTGCT	870
	Reverse (ON-1510)	AGCCTGGCCCGACCGTTA	

405 bp represents base pair

406 **Table 2 Distribution of 197 streptomycin-resistant *Mycobacterium tuberculosis* isolates into**
407 **different lineages**

Lineages	Number of isolates
1	11
2 (Beijing)	116
3	47
4	23
Total	197

408

409 **Table 3 Distribution of mutations in *rpsL* and *rrs* genes among different genotypes of 197**
410 **streptomycin-resistant *Mycobacterium tuberculosis* isolates**

<i>rpsL</i> nucleotide (amino acid substitution)	<i>rrs</i> nucleotide	Lineage 1	Lineage 2	Lineage 3	Lineage 4	No. of isolates
A128G (K43R)	-	2	91	13	9	115
A128G (K43R)	A907C		1			1
A128G (K43R), G212A (G71D)*	-			1		1
A263G (K88R)	-		12	2		14
A263G (K88R)	A644G*		1			1
G353A (G118D)*	A514C				1	1
-	A514C	1	5	2	3	11
-	A517T	1	1	2	1	5
-	A906C			1		1
-	A907C		3			3
-	A908C			3	2	5
-	A912G			1		1
-	-	7	2	22	7	38
Total		11	116	47	23	197

411 Fs denotes frameshift mutation

412 No. represents number

413 * denotes mutation previously not reported

414 - denotes no mutation.

415

Table 4 Detection rate of mutations in *rpsL*, *rrs* and *gidB* between countries

Countries	This study	India	Pakistan	China	Myanmar	Thailand
Mutations	(N=197)	(N=52)	(N=32)	(N=227)	(N=141)	(N=46)
<i>rpsL</i> codon K43R	58.8%	32.6%	34.3%	47.5%	64.0%	69.5%
<i>rpsL</i> codon K88R	7.1%	19.2%	3.1%	12.3%	6.0%	2.1%
<i>rrs</i>	13.2%	5.7%	12.5%	5.7%	3.5%	0%
<i>gidB</i>	13.2%	25.0%	21.8%	12.7%	2.7%	2.1%
Reference	This study	[27]	[28]	[29]	[30]	[31]

416

417 **Table 5 Distribution of mutations in *gidB* among different genotypes of 197 streptomycin-**
418 **resistant *Mycobacterium tuberculosis* isolates**

<i>gidB</i> nucleotide (amino acid substitution)	Lineage 1	Lineage 2	Lineage 3	Lineage 4	No. of isolates
G28C (A10P)			2		2
G28C (A10P), A119G (E40G)	1				1
C56G (A19G)*, γ, θ				1	1
G59C (A20P)*, β	1				1
G79C (A27P)*			1		1
C80T (A27V)*, α		1			1
G106C (V36L)*			1		1
C142A (H48N), γ				2	2
T236G (L79Y)			1		1
G244C (A82P)*			1		1
T302G (L101E)*			1		1
T335G (V112G)*			1		1
T371G (V124G)*			1		1
C398T (A133V)*, α		1			1
C413T (A138V), γ				1	1
G471A (G157G)*, α		2			2
A487G (K163E)			1		1
C492T (G164G)*			1		1
T583C (Y195H), α		7			7
G630A (G210G)*, α		1			1
A652G (M218V)*, γ				2	2
243insGC (Fs)*, γ				1	1
98delG (R33Fs)			6		6
98delG (R33Fs), β	3				3
98delG (R33Fs), γ				3	3
112delC (P38Fs)*, β	1				1
112delC (P38Fs)*, γ				1	1
254delA (D85Fs)*, γ				1	1
347delG (R116Fs)			1		1
372delG (V124Fs)*			1		1
384delG (L128Fs)			2		2
A	1	104			105
B	4				4
Γ				8	8
γ, θ				3	3
-			26		26
Total	11	116	47	23	197

419 A276C (E92D)=α, G330T (V110V)=β, G615A (A205A)=γ and T47G (L16R)=θ

420 Fs denotes frameshift mutation

421 No. represents number

422 * denotes mutation previously not reported

423 - denotes no mutation

424 del denote deletion mutation

425

426 Supplementary Table: Distribution of mutations in *rpsL*, *rrs* and *gidB* genes among different genotypes of 197
 427 streptomycin (STR)-resistant *Mycobacterium tuberculosis* isolates

<i>rpsL</i> nucleotide (codon)	<i>rrs</i>	<i>gidB</i> nucleotide (codon)	Beijing	EA I	Euro-American	CAS	Other	No. of isolates
A128C(K43R)	-	α	77	2	1			80
A128C(K43R)	-	α , C398T(A133V)*	1					1
A128C(K43R)	-	α , T583C(Y195H)	7			1		8
A128C(K43R)	-	A445C(S149R), γ	1					1
A128C(K43R)	-	α , G630A(G210G)*	1					1
A128C(K43R)	-	112delC (P38Fs)*, β		1				1
A128C(K43R)	-	C286T (R96C)*	1					1
A128C(K43R)	-	G106C (V36L)*				1		1
A128C(K43R)	-	G28C (A10P)				2		2
A128C(K43R)	-	β	1					1
A128C(K43R)	-	γ	1		6			7
A128C(K43R)	-	A652G (M218V)*, γ			1			1
A128C(K43R)	-	γ , θ			1			1
A128C(K43R)	-	-	1			9		10
A128C(K43R)	A907C	α	1					1
A128C(K43R), G212A(G71D)*	-	-				1		1
A263G(K88R)	-	α	10					10
A263G(K88R)	-	G471A (G157G)*, α	2					2
A263G(K88R)	-	C492T (G164G)*				1		1
A263G(K88R)	-	-				1		1
A263G(K88R)	A644G*	α	1					1
G353A(G118D)*	A514C	98delG (R33Fs), γ			1			1
-	A514C	α	4					4
-	A514C	C80T (A27V)*, α	1					1
-	A514C	98delG (R33Fs), γ			2			2
-	A514C	β		1				1
-	A514C	γ , θ			1			1
-	A514C	-		1				1
-	A517T	α	1					1
-	A517T	β		1				1
-	A517T	γ , θ			1			1
-	A517T	-		1		1		2
-	A906G	-				1		1
-	A907C	α	3					3
-	A908C	C142A (H48N), γ			2			2
-	A908C	384delG (L128Fs)		1				1
-	A908C	-		1		1		2
-	A912G	384delG (L128Fs)				1		1
-	-	243insGC (Fs)*, γ					1	1
-	-	254delA (D85Fs)*, γ			1			1
-	-	α	2					2
-	-	A487G (K163E)				1		1
-	-	C413T (A138V), γ			1			1
-	-	112delC (P38Fs)*, γ					1	1
-	-	G244C (A82P)*				1		1
-	-	347delG (R116Fs)				1		1
-	-	372delG (V124Fs)*				1		1
-	-	G59C (A20P)*, β		1				1
-	-	γ			1			1
-	-	A652G (M218V)*, γ			1			1
-	-	G79C (A27P)*				1		1
-	-	98delG (R33Fs)		1		5		6
-	-	98delG (R33Fs), β		3				3
-	-	G28C(A10P), A119G(E40G)					1	1
-	-	β		1				1
-	-	T236G (L79Y)				1		1
-	-	T302G (L101E)*				1		1
-	-	T335G (V112G)*				1		1
-	-	T371G (V124G)*				1		1
-	-	C56G (A19G)*, γ , θ			1			1
-	-	-	1	1		6		8
Total			117	16	21	40	3	197

428 A276C (E92D)= α , G330T (V110V)= β , G615A (A205A)= γ and T47G (L16R)= θ

429 EAI, East-African Indian; CAS, Central Asian strain; and other, orphan and X2.

430 Fs denotes frameshift mutation.

431 * denotes frameshift mutation.

432 - denotes no mutation.

433 del denote deletion mutation.

434 Ins denote insertion mutation.

435 α , β , γ and θ represent A276C (E92D), G330T (V110V), G615A (A205A) and T47G (L16R), respectively.