Characterization of *embB* mutations involved in ethambutol resistance in multi-drug resistant *Mycobacterium tuberculosis* isolates in Zambia

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Abstract

Background: Ethambutol (EMB) is an important anti-tuberculosis drug used in the management of multi-drug resistant tuberculosis (MDR-TB). Mutations in *embB* are the major mechanism of resistance. This study investigated *embB* mutations among MDR-TB isolates and analyzed their correlations with phenotypic drug susceptibility testing (DST) in Zambia.

Method: A total of 132 MDR-TB isolates were collected from January 2014 to April 2017 and characterized using MGIT 960 systems, *embB* sequencing, and spoligotyping.

Results: Out of 61 phenotypically EMB resistant isolates, 53 had mutations in *embB*. Among the 71 EMB susceptible isolates, 47 had *embB* mutations. Sensitivity of *embB* mutations was 86.9% while specificity was 33.8%. CAS1_Kili (SIT21) had high odds of having *embB* mutations, particularly, G918A (Met306eIl) (Odds ratio 16.7, $p < 0.0001$).

Conclusion: Molecular EMB resistance testing by DNA sequencing can improve detection of EMB resistance among MDR-TB patients in Zambia. Additionally, CAS1_Kili was associated with *embB* amino acid substitution Met306Ile suggesting transmission. A detailed investigation to track and determine transmission hotspot area for MDR-TB could help optimize control strategies.

Key words: *Mycobacterium tuberculosis*, ethambutol, multi-drug resistance, *embB* mutations, Zambia

1. Introduction

The emergence and transmission of drug resistant tuberculosis (TB) is a major obstacle to the ongoing global efforts to control and end TB. In recent years, Zambia has seen an increasing trend of multi-drug resistant (MDR) TB and was recently included in the list of high MDR-TB burden countries in the world [1]. In 2020, 484 laboratory-confirmed cases of rifampicin resistant (RR)/MDR-TB were reported, an increase from the 196 laboratory-confirmed cases reported in 2015 [2][3]. An earlier study showed that the increasing cases of MDR-TB was due to local transmission of MDR-TB strains in Zambia ([4] in press). Undiagnosed and unsuspected or diagnosed but inadequately treated MDR-TB patients are the likely source of transmission in Zambia [5][6]. To control the spread of MDR-TB in Zambia, active case finding such as awareness programs, increasing TB suspicion index of health care workers, availability of rapid and accurate diagnostic tools, and adequate treatment is imperative [5]. The adoption and implementation of rapid molecular based diagnostic tools such as GeneXpert (Cepheid, Sunnyvale, CA) and Line Probe Assay (Hain Lifescience GmbH, Nehren, Germany) have improved MDR-TB case detection and subsequent treatment. However, Zambia has not yet adopted the use of molecular tools for resistance testing for some drugs used in MDR-TB treatment.

In Zambia, ethambutol (EMB) is an integral part of the first-line drug regimen as well as in the short course MDR-TB regimen. Additionally, EMB is among group C drugs recommended for inclusion in the longer individualized MDR/RR-TB treatment regimen depending on drug susceptibility testing (DST) results [7]. With the recent reports of laboratory-confirmed pre-extensively drug resistant (pre-XDR) TB in Zambia [8] , EMB will play an increasing role in longer MDR-TB treatment regimens. To effectively treat the emerging cases of MDR/pre-XDR-TB and avoid resistance amplification, it is imperative to accurately determine resistance profile of EMB before its inclusion in the MDR-TB regimen.

Ethambutol inhibits arabinosyltranferases embC, embA, and embB involved in the synthesis of cell wall components and subsequently compromising the cell wall integrity [9]. The *embA* and *embB* are involved in the synthesis of arabinogalactan while *embC* is involved in the synthesis of lipoarabinomannan [10]. Resistance to EMB has been attributed to mutations in the *embCAB* locus encompassing 3 contiguous genes *embC*, *embA,* and *embB* [11–13]. The *embB* gene mutations have the predominant role in EMB resistance, particularly at codons 306, 406, and 497, which are considered as hotspot resistance codons [9,12]. Codon 306 was shown

71 to be directly involved in EMB binding while codons 406 and 497 are not directly involved.
72 Nevertheless, mutations at codon 497 cause conformational changes that affect codon 327, one
73 of the EMB binding sites. Codon 406 mutations may also affect drug binding by causing
74 protein conformation changes [9].

75 Despite the documented evidence of *embB* involvement in EMB resistance, there is an apparent
76 discord with conventional phenotypic DST. The high EMB critical concentration (5.0 µg/ml)
77 of MGIT 960 shows the low-level EMB resistance as susceptible[14]. In addition, phenotypic
78 DST is considered unreliable and unreproducible, thus WHO recommends molecular detection
79 of resistance for EMB[15]. The accurate determination of resistance is vital in clinical decision
80 to use a drug for MDR-TB treatment; therefore, it is important to investigate the mutations
81 responsible for EMB resistance in order to develop the strategy to use molecular based EMB
82 DST in Zambia. This study is the first in Zambia to describe *embB* mutations involved in EMB
83 resistance among MDR-TB and evaluate the concordance with phenotypic DST.

84 **2. Materials and Methods**

85 *2.1. Samples and phenotype drug susceptibility testing*

86 *Mycobacterium tuberculosis* (*Mtb*) isolated from patient samples referred to The University
87 Teaching Hospital Tuberculosis Reference Laboratory between January 2014 to April 2017
88 were included in this study. The DST was done as part of the routine testing for rifampicin
89 (RIF), isoniazid (INH), streptomycin (STR), and EMB at a critical concentration of 1.0 µg/ml,
90 0.1 µg/ml, 1 µg/ml, and 5.0 µg/ml, respectively using the MGIT M960 liquid culture systems
91 following manufacturer's instructions (BD BACTEC^{TM} MGIT^{TM} 960 SIRE kit). A total of 132
92 MDR-TB isolates were randomly selected. The isolates information was extracted from the
93 Laboratory information system.

94 *2.2. DNA extraction*

95 DNA was extracted by the boiling method as previously described [16]. The extracted DNA
96 was stored at -20°C until use.

97 *2.3. DNA sequencing*

98 The *embB* gene was amplified using the primers *embB*-F (5'-
99 CGACGCCGTGGTGATATTCG-3') and *embB*-R (5'- CGACGCCGTGGTGATATTCG-3').
100 The PCR reaction volume of 20µl contained DDW, 5x Go Tag buffer green (Promega Corp,
101 Madison, WI, USA), 25mM dNTP (Promega Corp), 25mM MgCl, 5M betaine, 10µM primers,

102 and GoTaq DNA polymerase (Promega Corp). The amplified product was purified using
103 ExoSAP-IT^{TM} Express PCR product cleanup (Thermo Fisher Scientific Inc., Santa Clara, USA)
104 as instructed by the manufacturer. Purified DNA was sequenced using the BigDye Terminator
105 V3.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) on an ABI 3500 genetic analyzer.
106 Bioedit software was used to align the sequences to the H37Rv reference sequence
107 (NC_000962.3) [17].

108 *2.4. Spoligotyping*

109 PCR targeting the direct repeat region (DR) was done using the DRa and DRb primers and the
110 resulting PCR products were hybridized on to a membrane as previously described [18][19].
111 The resulting hybridized spoligotype pattern was converted to the binary code and compared
112 to SpolD4 database for determination of the Spoligo-International Type (SIT) and spoligotypes
113 [20].

114 *2.5. Phylogenetic analysis*

115 The dendogram was generated using unweighted pair group method with arithmetic averages
116 (UPGMA) based on spoligotype patterns in BioNumerics version 7.6 (Applied Maths, Sint-
117 Martens-Latem, Belgium). A cluster was considered as 2 or more isolates having same
118 spoligotype pattern and same *embB* nucleotide substitution.

119 *2.6. Data analysis*

120 The data was described using proportions and the Odds ratio was used for statistical analysis.
121 A two-tailed *p* value was used, and significance was set at <0.05. Sensitivity and specificity
122 for *embB* sequencing method were calculated by comparing to MGIT 960 DST as the reference
123 standard.

124 3. **Results**

125 *3.1. Frequency of embB mutations in MDR-TB isolates*

126 The analysis of phenotypic DST results showed that 46.2% (61/132) isolates were EMB
127 resistant. Sequencing analysis of *embB* revealed mutations in 75.8% (100/132) of MDR-*Mtb*
128 isolates. Among EMB resistant isolates, 86.9% (53/61) had mutations in *embB* gene. EMB
129 resistant isolates had higher odds of having mutations in *embB* compared to susceptible isolates
130 (Odds ratio 3.4. $p = 0.0074$). Isolates with resistance to 4 drugs had higher odds of having *embB*
131 mutations (Odds ratio 5.93, $p = 0.0055$) (Table 1).

132 A total of 14 single nucleotide mutations resulting in 11 amino acid substitutions were observed
133 in *embB*. Codon 306 was the most mutated, accounting for 82% (82/100) of the isolates. Amino
134 acid substitution Met306Ile was the most predominant and found in 42% (42/100) of the
135 isolates. Among isolates with mutations leading to Met306Ile (G918A, G918C, G918T) amino
136 acid change, a transition mutation G918A was found in 38 of the 42 isolates. The second
137 dominant amino acid change was Met306Val and was found in 35% (35/100) of isolates,
138 followed by Gln497Arg and Met306Leu detected in 6% (6/100) and 5% (5/100) of isolates,
139 respectively. One mutation G982T (Asp328Tyr) and a double mutation G1215C and G1225C
140 (Glu405Asp and Ala409Pro) were exclusively found in EMB resistant isolates. The remaining
141 mutations were observed in either susceptible isolates only or both susceptible and resistant
142 isolates. Codon 306 (*embB306*) mutations were observed in both resistant and susceptible
143 isolates with exception of G918C observed only in susceptible isolates. Mutations at *embB306*
144 were significantly associated with EMB resistance (p value = 0.011). Codons 497 and 406 were
145 mutated in equal proportion. Table 2 summarizes the mutations detected among MDR-*Mtb*
146 isolates in this study.

147 *3.2. Occurrence of embB mutations in different spoligotypes*

148 Spoligotyping revealed 7 major genotypes (Table S2). Among these genotypes, CAS1_Kili
149 had high odds of acquiring *embB* mutations (Odds ratio 15.3, p =0.0086). Stratification of
150 spoligotype SITs and *embB* gene mutations revealed 14 clusters of isolates (figure 1). The
151 largest cluster had 26 isolates belonging to CAS1_Kili (SIT21) clade and harboring G918A
152 (Met306Ile) mutation (figure 1). One isolate of CAS1_Kili (SIT21) clade had wildtype *embB*
153 gene. We identified 6 clusters having mutation A916G (Met306Val). The 6 clusters included
154 13 isolates belonging to LAM11_ZWE (SIT59); 6 isolates to LAM11_ZWE (SIT815); 4
155 isolates each to T1 (SIT53), T2 (SIT52), and X2 (SIT137); and 2 isolates to LAM1 (SIT20).
156 Mutation A916G (Met306Val) was found in only one isolate of CAS1_Kili (SIT21) clade.
157 CAS1_Kili (SIT21) had significantly high odds of acquiring mutations leading to Met306Ile
158 amino acid substitution (Odds ratio 16.7, p <0.0001).

159 **4. Discussion**

160 In Zambia, routine phenotypic DST for EMB is performed using the MGIT 960 culture system.
161 However, this method is considered unreliable and unreproducible [15]. Consequently, the
162 external quality assurance for EMB DST often performs poorly. Unreliable results lead to
163 insufficient treatment of patients which can drive emergence, transmission, or amplification of

drug resistance. Detection of mutations in *embCAB* locus is used to infer resistance to EMB, but *embB* mutations accounts for the majority of isolates. The WHO recommends mutation analysis for inference of EMB resistance over phenotypic testing [15].

This study revealed phenotypic EMB resistance in 46.2% of MDR-TB isolates whereas sequencing found *embB* mutations in 75.8% of the total isolates. In Kuwait, EMB resistance in MDR-TB was found in 44.1% of the isolates, while *embB* mutations were detected in 81.7% of the total MDR-*Mtb* isolates [21]. These results show that fewer isolates are determined as EMB resistance by phenotypic DST as compared to *embB* mutation analysis. Safi and colleagues demonstrated that *embB* mutations are involved in EMB resistance and they raise EMB MIC, albeit modestly [22,23]. For some isolates with high-level EMB resistance, another study showed that the acquisition of additional mutations in other genes such as *ubiA* and *embC* is required [24]. Thus, *embB* gene mutations are considered as the initial step to acquiring high-level EMB resistance and should be treated as clinically resistant isolates, although additional studies linking mutations to clinical outcomes would be needed.

Previous studies have revealed that *embB* mutations are significantly associated with resistance to RIF, INH STR, and/or EMB [25,26]. In agreement with these findings, our study also found that mutations in *embB* were more likely to occur in isolates with additional resistance to RIF, INH, and STR and less likely to occur in isolates with only RIF and INH resistance. This shows that *embB* mutations predispose to drug resistance amplification [27] and underscores the need for adopting a more reliable and rapid method of EMB resistance testing to receive appropriate treatment.

Among the three amino acid changes at *embB306* (Met306Ile, Met306Leu, and Met306Val), Met306Val was more likely to be found in EMB resistant isolates (Odds ratio 6.4, $p = 0.0002$) and Met306Ile was more likely to be found among EMB susceptible isolates (Odds ratio 2.9, $p = 0.0121$). This conformed to the previous results from an allelic exchange experiment that showed that the mutations G918A and G918C producing amino acid change Met306Ile, raises MIC close to the break-point of EMB resistance (5 to 7.5 µg/ml) [22]. Therefore, Met306Ile would more likely appear among susceptible isolates in the MGIT 960 system which has a critical concentration value of 5 µg/ml for resistance determination. Among the three nucleotide substitutions leading to amino acid change Met306Ile observed in our study, a transition mutation G918A was more frequent (90.5%) than the transversions G918C and G918T. This disproportionately high occurrence of transition mutation at this codon, can be

196 explained in part by the translation bias previously described in the genome of *Mtb*, wherein,
197 ATG>ATA translation was 1.8 times more frequent than the transversions ATC and ATT [28].
198 In addition, the high frequency of the transition mutation seen in this study compared to the
199 reported transition to transversion ratio, could reflect clonal expansion.

200 Mutation G1217A leading to amino acid change Gly406Asp was seen only in susceptible
201 isolates. Nonetheless, this mutation had been proven to raise EMB MIC by 5 fold in a previous
202 study and thus can be considered significant in eventual evolution to high-level EMB resistance
203 [23,24].

204 Mutations at *embB306* account for the majority of mutations in *embB* with an estimated global
205 frequency of 47.5% among MDR-TB isolates, followed by codon 406 at 11.3% and then codon
206 497 at 7.9%, respectively [29] (Table S1). In Tanzania, Mexico, and South Korea, where the
207 burden of MDR-TB is low, *embB306* mutations were found in 20.8%, 27.8%, and 38.5% of
208 MDR-TB isolates, respectively [26,30–32]. In high MDR-TB burden countries of South Africa,
209 Thailand, and China, *embB306* mutations were detected in 60%, 50%, and 30.3% of MDR-TB
210 isolates, respectively [33–35]. In Russia, a high MDR-TB burden country, *embB306* mutations
211 were detected in 30.7% of phenotypically determined MDR-TB isolates [36]. In South Africa
212 where the frequency of *embB306* mutations was high, most isolates were clustered MDR-*Mtb*
213 isolates [33]. In this study, mutations at this codon were detected in 62.1% of the MDR-TB
214 isolates, higher than the global estimate and the frequency reported in high MDR-TB countries,
215 but comparable to that reported in South Africa suggesting the clonal expansion of EMB
216 resistant MDR-TB isolates in Zambia. Mutations at codons 406 and 497 were both observed at
217 a frequency of 4.5% and were below global frequency.

218 We found that 86.9% of phenotypically EMB resistant isolates and 66.2% of EMB susceptible
219 isolates had *embB* mutations. Another study using the MGIT M960 method for phenotypic
220 testing same as current study found *embB* mutations in 73.1% of EMB susceptible MDR-TB
221 isolates [21]. In contrast, studies from South Korea, Poland, China, and Thailand using the
222 Lowenstein Jensen (LJ) proportion method found *embB* mutations in 30%, 42.5%, 45% and
223 45.5%, respectively, of EMB susceptible MDR-TB isolates [31,34,37,38]. The MGIT 960
224 culture system was previously shown to produce the lowest agreement (77.1%) with
225 sequencing, as compared to the LJ proportion method (81.4%) which has a critical
226 concentration of 2 µg/ml and the microtiter alamarBlue assay (MABA) (84.7%). In our study,
227 the sensitivity of *embB* mutations was 86.9% but the specificity was very low at 33.8% (Table

228 S3). The poor specificity of sequencing in our study is caused by the limitation of the
229 phenotypic testing method using MGIT M960. The LJ proportion method slightly improves
230 EMB resistance detection. However, phenotypic DST is not reproducible and is unreliable,
231 thus not recommended by WHO [15]. Therefore, the reliance on phenotypic testing alone for
232 EMB DST in Zambia would fail to detect resistance in a considerable number of MDR-TB
233 patients and expose these patients to inadequate treatment. In addition to phenotypic DST, we
234 recommend the adoption of a molecular testing method such as DNA sequencing for more
235 accurate EMB susceptibility results in Zambia. Additionally, data from both methods should
236 continuously be gathered to associate with clinical outcomes and for evaluation of EMB critical
237 concentration.

238 Interestingly, mutations in *embB* were significantly associated with CAS1_Kili (SIT21) in this
239 study, particularly with Met306Ile amino acid change (Odds ratio 16.7, $p < 0.0001$), with the
240 odds of acquiring Met306Val being 0.06 ($p = 0.0057$). Although both isoleucine and valine
241 are hydrophobic amino acids with only a methyl group difference, the substitution of
242 methionine with isoleucine at *embB306* produces low to moderate-level resistance to EMB
243 compared to valine [22], and was associated with susceptible isolates in this study. This means
244 that at the current EMB breakpoint of 5µg/ml, strains with this amino acid substitution would
245 be undetected as resistant, inadequately treated, acquire resistance to additional drugs, and
246 continue to silently spread. In fact, the largest cluster of 26 isolates identified in this study
247 belonged to CAS1_Kili (SIT21) clade and had a G918A (Met306Ile) transition mutation. The
248 size of this cluster suggests clonal expansion and may reflect increased transmissibility of
249 CAS1_Kili (SIT21) in Zambia. It is, therefore, urgent to adopt molecular detection of EMB
250 resistance in addition to phenotypic method to improve resistance detection. Previous reports
251 have associated CAS1_Kili (SIT21) with MDR-TB and streptomycin resistance in Zambia
252 [17][19]. The association of CAS1_Kili (SIT21) with drug resistance and increased
253 transmission in Zambia, makes this genotype a major concern and should be prioritized for
254 tracking and identification of hotspot regions of transmission. The second largest cluster
255 belonged to LAM11_ZWE (SIT59) followed by LAM11_ZWE (SIT815) both having
256 Met306Val amino acid change. Several smaller clusters were also identified and may have the
257 potential to expand. This reveals multi-clonal transmission events happening in Zambia.

258 .

259 The primary limitation of our study was an inability to perform MIC tests to correlate with
260 detected mutations. In addition, clustering and transmissibility were only inferred from
261 spoligotyping and *embB* mutations. This may overestimate clustering due to low sensitivity.
262 We also did not sequence other genes such as *ubiA*, *embA* and *embC* known to contribute to
263 EMB resistance.

264 In conclusion, our study highlights the high number of MDR-TB cases with mutations in *embB*,
265 undetected by the MGIT 960 culture system. These mutations can predispose progression to
266 high-level EMB resistance and should thus be considered clinically resistant to EMB. We
267 therefore recommend the adoption of genotypic testing to improve EMB resistance detection
268 and management of MDR-TB patients and an evaluation of genotypic testing and clinical
269 outcome of patients. Genotype CAS1_Kili (SIT21) was associated with *embB* mutations,
270 particularly G918Aand had a large cluster of isolates having Met306Ile amino acid substitution.
271 This suggests increased transmission and we recommend tracking this genotype, as well as
272 further investigation to determine hot spot areas of transmission for optimized interventions.

273

274 **Acknowledgement**

275 We thank Ms. Kasakwa Kunda and Mr. Kaemba Kunkuta Mwale for assisting in sample
276 retrieval from storage and Ms. Fukushima Yukari for the technical support.

277

278 **Funding**

279 This work was supported in part by a grant from the Ministry of Education, Culture,
280 Sports, Science and Technology (MEXT), Japan, and the Joint Research Program of the
281 Research Center for Zoonosis Control, Hokkaido University to YS, and in part by Japan
282 Agency for Medical Research and Development (AMED) under Grant Number
283 JP21wm0125008, JP21jm0510001, and JP21jk0210005 to YS.

284

285 **Ethical approval**

286 Ethical clearance for this work was obtained from ERES CONVERGE study reference number
287 2019-Oct-014.

288

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435 Table 1: Drug resistance profiles and demographic characteristics of the MDR-TB isolates

Characteristic	*embB* mutations	No *embB* mutations	Total	Odd ratio	95% CI	p value
Drug resistance						
INH, RIF, EMB, STR	38	3	41	5.93	1.69 to 20.79	**0.0055**
INH, RIF, EMB	15	5	20	0.95	0.32 to 2.87	0.9316
INH, RIF, STR	37	9	46	1.50	0.63 to 3.59	0.3609
INH, RIF	10	15	25	0.13	0.049 to 0.33	**< 0.0001**

436 INH-isoniazid, RIF-rifampicin, EMB-ethambutol, STR-streptomycin

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439 Table 2: Mutations detected in 132 MDR-*Mtb* isolates

Nucleotide substitution	Amino acid substitution	EMB resistant (n=61)		EMB susceptible (n=71)		Sensitivity	specificity	Accuracy
		Mutation	No mutation	Mutation	No mutation			
G918A	Met306Ile	15	46	23	48	24.6	67.6	47.7
G918C	Met306Ile	0	61	2	69	0.0	97.2	52.3
G918T	Met306Ile	1	60	1	70	1.6	98.6	53.8
A916T	Met306Leu	1	60	4	67	1.6	94.4	51.5
A916G	Met306Val	28	33	7	64	45.9	90.1	69.7
A956C	Tyr319Ser	1	60	1	70	1.6	98.6	53.8
G982T	Asp328Tyr	1	60	0	71	1.6	100.0	54.5
C1204G	Leu402Val	1	60	1	70	1.6	98.6	53.8
G1215C/G1225C	Glu405Asp/Ala409Pro	1	60	0	71	1.6	100.0	54.5
G1217C	Gly406Ala	1	60	2	69	1.6	97.2	53.0
G1217A	Gly406Asp	0	61	3	68	0.0	95.8	51.5
A1490G	Gln497Arg	3	58	3	68	4.9	95.8	53.8
WT	WT	8	53	24	47			
Total		61		71				

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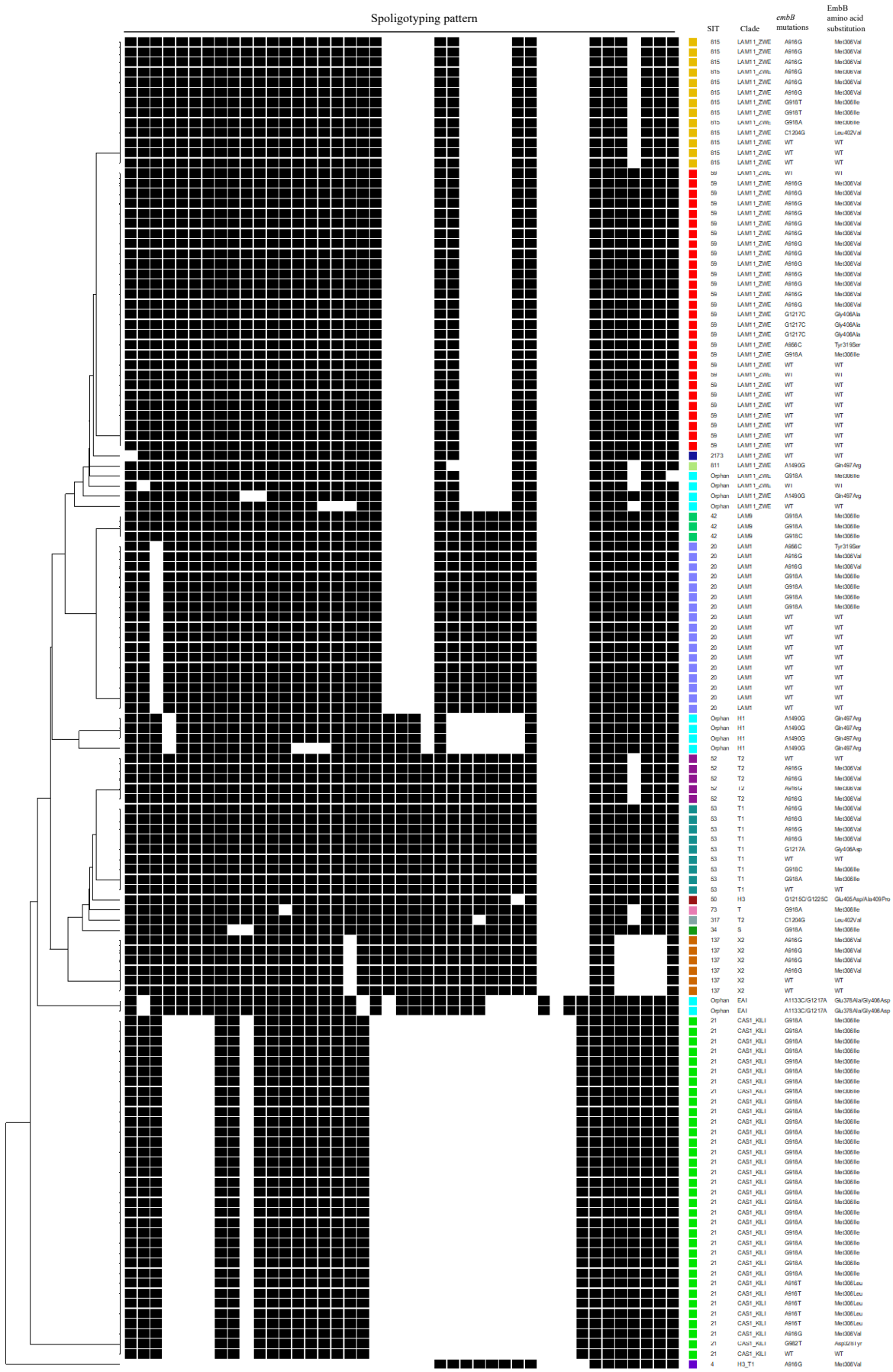

Figure 1: Dendogram of 132 MDR-TB isolates constructed based on spoligotype patterns by UPGMA.