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

Precious BWALYA[1,2], Eddie S. SOLO[2], Joseph Y. CHIZIMU[1,3], Dipti SHRESTHA[1,4], Grace MBULO[2], Jeewan THAPA[1], Chie NAKAJIMA[1,5], Yasuhiko SUZUKI[1,5,*]

[1] Division of Bioresources, Hokkaido University International Institute for Zoonosis Control, Sapporo, 001-0020, Japan

[2] Department of Pathology and Microbiology, University Teaching Hospital, Ministry of Health, Lusaka, 10101, Zambia

[3] Zambia National Public Health Institute, Ministry Health, Lusaka, 10101, Zambia

[4] Department of Microbiology, Kathmandu College of Science and Technology, Tribhuvan University, Kathmandu, Nepal.

[5] International Collaboration Unit, Hokkaido University International Institute for Zoonosis Control, Sapporo, 001-0020, Japan

* Correspondence: suzuki@czc.hokudai.ac.jp
Kita 20, Nishi 10, Kita-ku, Sapporo, 001-0020, Japan
Tel 011-706-9503/7315

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

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

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

2. Materials and Methods

2.1. Samples and phenotype drug susceptibility testing

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

2.2. DNA extraction

DNA was extracted by the boiling method as previously described [16]. The extracted DNA was stored at -20^{o}C until use.

2.3. DNA sequencing

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

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

2.4. Spoligotyping

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

2.5. Phylogenetic analysis

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

2.6. Data analysis

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

3. **Results**

3.1. Frequency of embB mutations in MDR-TB isolates

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

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

3.2. Occurrence of embB mutations in different spoligotypes

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

4. Discussion

In Zambia, routine phenotypic DST for EMB is performed using the MGIT 960 culture system. However, this method is considered unreliable and unreproducible [15]. Consequently, the external quality assurance for EMB DST often performs poorly. Unreliable results lead to 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

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

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

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

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

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

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

.

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

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

Acknowledgement

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

Funding

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

Ethical approval

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

References

[1] World Health Organization. WHO global lists of high burden countries for tuberculosis (TB), TB / HIV and TB (MDR / RR-TB), 2021-2025. Geneva, Switzeland: World Health organisation; 2021.

[2] World Health Organization. Global Tuberculosis Report 2016. Geneva, Switzeland: World Health organisation; 2016.

[3] World Health Organization. Global Tuberculosis Report 2021. Geneva, Switzeland: World Health organisation; 2021.

[4] Chizimu JY, Solo ES, Bwalya P, Kapalamula T, Akapelwa ML, Lungu P, et al. Genetic Diversity and Transmission of Multidrug Resistant Mycobacterium tuberculosis strains in Lusaka, Zambia. Int J Infect Dis n.d.

[5] Kagujje M, Chilukutu L, Somwe P, Mutale J, Chiyenu K, Lumpa M, et al. Active TB case finding in a high burden setting; comparison of community and facility-based strategies in Lusaka, Zambia. PLoS One 2020;15:1–12. doi:10.1371/journal.pone.0237931.

[6] Bates M, O'Grady J, Mwaba P, Chilukutu L, Mzyece J, Cheelo B, et al. Evaluation of the burden of unsuspected pulmonary tuberculosis and co-morbidity with non-communicable diseases in sputum producing adult inpatients. PLoS One 2012;7:e40774. doi:10.1371/journal.pone.0040774.

[7] The National Tuberculosis and Leprosy Control Program M. Guidelines for the Programmatic Management of Drug-resistant Tuberculosis in Zambia. 2017.

[8] World Health Organization. Tuberculosis profile: Zambia 2021. https://worldhealthorg.shinyapps.io/tb_profiles/?_inputs_&entity_type=%22country%22&lan=%22EN%22&iso2=%22ZM%22 (accessed January 21, 2022).

[9] Zhang L, Zhao Y, Gao Y, Wu L, Gao R, Zhang Q, et al. Structures of cell wall arabinosyltransferases with the anti-tuberculosis drug ethambutol. Science

2020;368:1211–9. doi:10.1126/science.aba9102.

[10] Abrahams KA, Besra GS. Mycobacterial cell wall biosynthesis: A multifaceted antibiotic target. Parasitology 2018;145:116–33. doi:10.1017/S0031182016002377.

[11] Sun Q, Xiao TY, Liu HC, Zhao XQ, Liu ZG, Li YN, et al. Mutations within *embCAB* are associated with variable level of ethambutol resistance in *Mycobacterium tuberculosis* isolates from China. Antimicrob Agents Chemother 2018;62:1–8. doi:10.1128/AAC.01279-17.

[12] Xu Y, Jia H, Huang H, Sun Z, Zhang Z. Mutations found in *embCAB*, *embR*, and *ubiA* genes of ethambutol-sensitive and -resistant *Mycobacterium tuberculosis* clinical isolates from China. Biomed Res Int 2015;2015:951706. doi:10.1155/2015/951706.

[13] Zhang Z, Wang Y, Pang Y, Kam KM. Ethambutol resistance as determined by broth dilution method correlates better than sequencing results with *embB* mutations in multidrug-resistant *Mycobacterium tuberculosis* isolates. J Clin Microbiol 2014;52:638–41. doi:10.1128/JCM.02713-13.

[14] Li MC, Chen R, Lin SQ, Lu Y, Liu HC, Li GL, et al. Detecting ethambutol resistance in *Mycobacterium tuberculosis* isolates in China: A comparison between phenotypic drug susceptibility testing methods and DNA sequencing of *embAB*. Front Microbiol 2020;11:1–7. doi:10.3389/fmicb.2020.00781.

[15] World Health Organization. Technical manual for drug susceptibility testing of medicines used in the treatment of tuberculosis. Geneva, Switzeland: World Health organisation; 2018.

[16] Bwalya P, Yamaguchi T, Solo ES, Chizimu JY, Mbulo G, Nakajima C, et al. Characterization of mutations associated with streptomycin resistance in multidrug-resistant *Mycobacterium tuberculosis* in Zambia. Antibiotics 2021;10:1169. doi:10.3390/antibiotics10101169.

[17] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1999;41:95–8. doi:citeulike-article-id:691774.

[18] Kamerbeek J, Schouls L, Kolk A, Agterveld M Van, Soolingen D van, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for

345 diagnosis and epidemiology. J Clin Microbiol 1997;35:907–14.
346 doi:10.1128/jcm.35.4.907-914.1997.

347 [19] Solo ES, Suzuki Y, Kaile T, Bwalya P, Lungu P, Chizimu JY, et al. Characterization
348 of *Mycobacterium tuberculosis* genotypes and their correlation to multidrug resistance
349 in Lusaka, Zambia. Int J Infect Dis 2021;102:489–96. doi:10.1016/j.ijid.2020.10.014.

350 [20] Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, et al.
351 *Mycobacterium tuberculosis* complex genetic diversity: Mining the fourth
352 international spoligotyping database (SpolDB4) for classification, population genetics
353 and epidemiology. BMC Microbiol 2006;6:1–17. doi:10.1186/1471-2180-6-23.

354 [21] Al-Mutairi NM, Ahmad S, Mokaddas EM. Molecular characterization of multidrug-
355 resistant *Mycobacterium tuberculosis* (MDR-TB) isolates identifies local transmission
356 of infection in Kuwait, a country with a low incidence of TB and MDR-TB. Eur J Med
357 Res 2019;24:1–13. doi:10.1186/s40001-019-0397-2.

358 [22] Safi H, Sayers B, Hazbón MH, Alland D. Transfer of *embB* codon 306 mutations into
359 clinical *Mycobacterium tuberculosis* strains alters susceptibility to ethambutol,
360 isoniazid, and rifampin. Antimicrob Agents Chemother 2008;52:2027–34.
361 doi:10.1128/AAC.01486-07.

362 [23] Safi H, Fleischmann RD, Peterson SN, Jones MB, Jarrahi B, Alland D. Allelic
363 exchange and mutant selection demonstrate that common clinical *embCAB* gene
364 mutations only modestly increase resistance to ethambutol in *Mycobacterium*
365 *tuberculosis*. Antimicrob Agents Chemother 2010;54. doi:10.1128/AAC.01288-09.

366 [24] Safi H, Lingaraju S, Amin A, Kim S, Jones M, Holmes M, et al. Evolution of high-
367 level ethambutol-resistant tuberculosis through interacting mutations in
368 decaprenylphosphoryl-β-D-arabinose biosynthetic and utilization pathway genes. Nat
369 Genet 2013;45:1190–7. doi:10.1038/ng.2743.

370 [25] Ahmad S, Jaber AA, Mokaddas E. Frequency of *embB* codon 306 mutations in
371 ethambutol-susceptible and -resistant clinical *Mycobacterium tuberculosis* isolates in
372 Kuwait. Tuberculosis 2007;87:123–9. doi:10.1016/j.tube.2006.05.004.

373 [26] Cuevas-Córdoba B, María Juárez-Eusebio D, Almaraz-Velasco R, Muñiz-Salazar R,
374 Laniado-Laborin R, Zenteno-Cuevas R. Mutation at *embB* codon 306, a potential

marker for the identification of multidrug resistance associated with ethambutol in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2015;59:5455–62. doi:10.1128/AAC.00117-15.

[27] Hazbón MH, Bobadilla Del Valle M, Guerrero MI, Varma-Basil M, Filliol I, Cavatore M, et al. Role of *embB* codon 306 mutations in *Mycobacterium tuberculosis* revisited: A novel association with broad drug resistance and IS6110 clustering rather than ethambutol resistance. Antimicrob Agents Chemother 2005;49:3794–802. doi:10.1128/AAC.49.9.3794-3802.2005.

[28] Payne JL, Menardo F, Trauner A, Borrell S, Gygli SM, Loiseau C, et al. Transition bias influences the evolution of antibiotic resistance in *Mycobacterium tuberculosis*. PLOS Biol 2018;17:e3000265. doi:10.1371/journal.pbio.3000265.

[29] Phelan JE, O'Sullivan DM, Machado D, Ramos J, Oppong YEA, Campino S, et al. Integrating informatics tools and portable sequencing technology for rapid detection of resistance to anti-tuberculous drugs. Genome Med 2019;11:1–7. doi:10.1186/s13073-019-0650-x.

[30] Katale BZ, Mbelele PM, Lema NA, Campino S, Mshana SE, Rweyemamu MM, et al. Whole genome sequencing of *Mycobacterium tuberculosis* isolates and clinical outcomes of patients treated for multidrug-resistant tuberculosis in Tanzania. BMC Genomics 2020;21:1–15. doi:10.1186/s12864-020-6577-1.

[31] Park YK, Ryoo SW, Lee SH, Jnawali HN, Kim CK, Kim HJ, et al. Correlation of the phenotypic ethambutol susceptibility of *Mycobacterium tuberculosis* with *embB* gene mutations in Korea. J Med Microbiol 2012;61:529–34. doi:10.1099/jmm.0.037614-0.

[32] World Health Organisation. Global Tuberculosis Report 2020. Geneva, Switzeland: World Health Organisation; 2020.

[33] Dookie N, Sturm AW, Moodley P. Mechanisms of first-line antimicrobial resistance in multi-drug and extensively drug resistant strains of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa. BMC Infect Dis 2016;16:609. doi:10.1186/s12879-016-1906-3.

[34] Tulyaprawat O, Chaiprasert A, Chongtrakool P, Suwannakarn K, Ngamskulrungroj P. Distribution of *embB* mutations of Thai clinical isolates of ethambutol-resistant

Mycobacterium tuberculosis. J Glob Antimicrob Resist 2019;18:115–7. doi:10.1016/j.jgar.2019.05.033.

[35] Zhang D, Liu B, Wang Y, Pang Y. Rapid molecular screening for multidrug-resistant tuberculosis in a resource-limited region of China. Trop Med Int Heal 2014;19:1259–66. doi:10.1111/tmi.12359.

[36] Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya I. Evolution and transmission of drug-resistant tuberculosis in a Russian population. Nat Genet 2014;46:279–286. doi:10.1038/ng.2878.

[37] Bakuła Z, Napiórkowska A, Bielecki J, Augustynowicz-Kopeć E, Zwolska Z, Jagielski T. Mutations in the *embB* gene and their association with ethambutol resistance in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from Poland. Biomed Res Int 2013;2013:167954. doi:10.1155/2013/167954.

[38] Zhao LL, Sun Q, Liu HC, Wu XC, Xiao TY, Zhao XQ, et al. Analysis of *embCAB* mutations associated with ethambutol resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates from China. Antimicrob Agents Chemother 2015;59:2045–50. doi:10.1128/AAC.04933-14.

Author contribution

Conceptualization: Precious Bwalya, Eddie S. Solo, Chie Nakajima, and Yasuhiko Suzuki. Methodology: Precious Bwalya, Dipti Shrestha, and Chie Nakajima. Investigation: Precious Bwalya and Dipti Shrestha. Formal analysis: Precious Bwalya, Jeewan Thapa, Chie Nakajima, and Yasuhiko Suzuki. Data resources: Grace Mbulo, Eddie S. Solo, Chie Nakajima, and Yasuhiko Suzuki. Data curation: Precious Bwalya and Joseph Y. Chizimu. Writing - original draft: Precious Bwalya, Joseph Y. Chizimu, Jeewan Thapa and Eddie S. Solo. Writing – review and editing: Jeewan Thapa, Eddie S. Solo, Grace Mbulo, Chie Nakajima, and Yasuhiko Suzuki. Visualization: Precious Bwalya, Chie Nakajima, and Yasuhiko Suzuki. Supervision: Jeewan Thapa, Chie Nakajima, and Yasuhiko Suzuki. Project administration: Chie Nakajima and Yasuhiko Suzuki. Funding acquisition: Yasuhiko Suzuki

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

437

438

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				

440

441

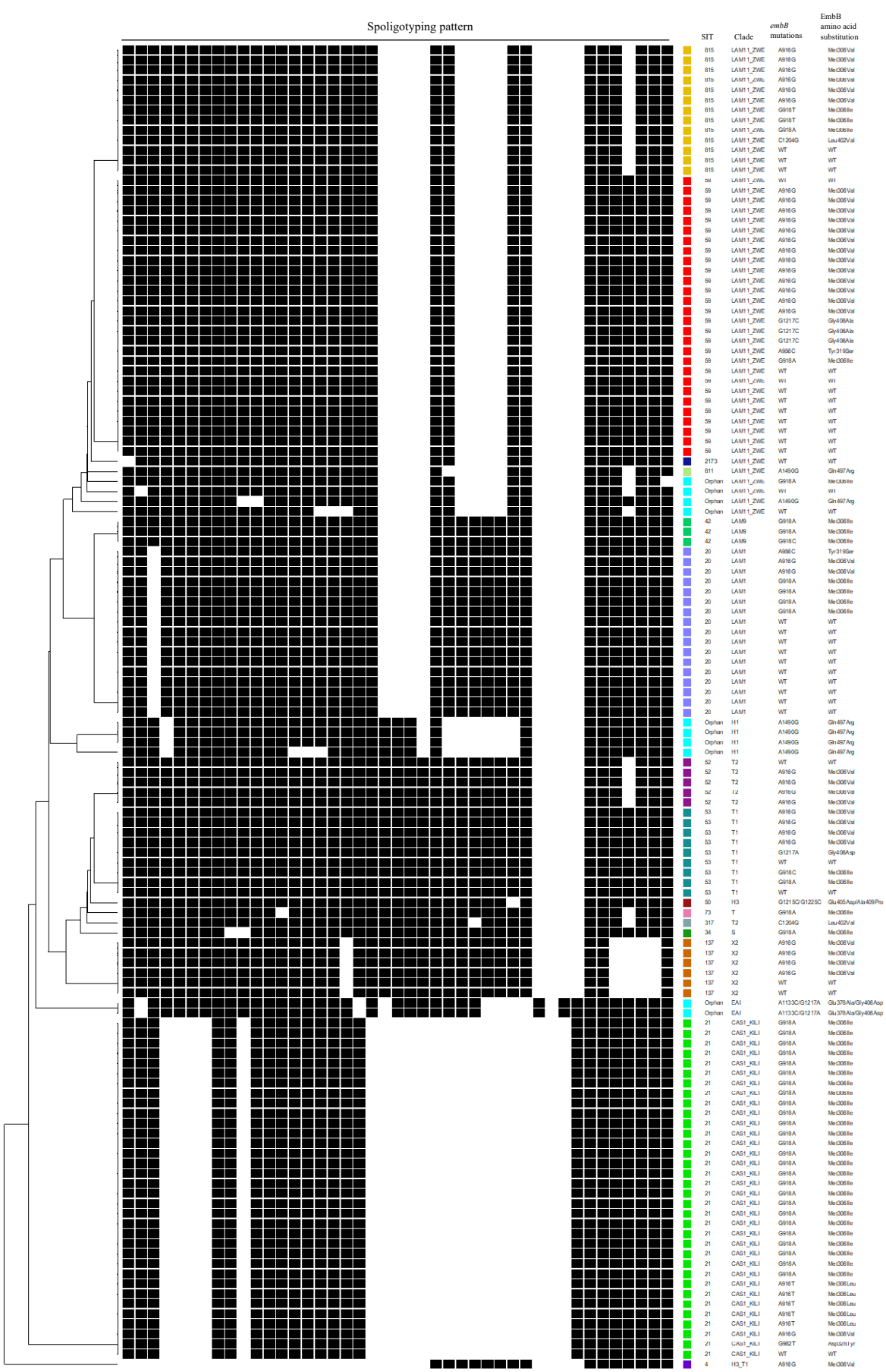

Figure 1: Dendogram of 132 MDR-TB isolates constructed based on spoligotype patterns by UPGMA.