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# The implications of mutations in multiple genes associated with ethambutol resistance among multidrug-resistant tuberculosis isolates from China

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## Abstract

**Objectives** To assess the mutation effects of ethambutol (EMB) resistance-associated genes, including *embCAB* operon, *ubiA*, *embR*, and *aftA*, on the EMB resistance levels among multidrug-resistant tuberculosis (MDR-TB) isolates from China.

**Methods** A total of 159 MDR-TB from China had their EMB MICs quantified, and the sequences of the four ethambutol resistance-associated regions were analyzed. A multivariate regression model was established to evaluate the effects of mutations on EMB resistance.

**Results** Our results showed that overall 95.6% (109/114 isolates) of EMB-resistant isolates harbored at least one mutation within the regions associated with EMB resistance. Most mutations were in *embB*, particularly in the *embB*300-500, and the *embC-embA* intergenic regions. Mutations in other genes were seldom seen and mainly occurred along with mutations in the *embB* or the *embC-embA* among the EMB-resistant isolates. DNA sequencing of the *embB*300-500 and the *embC-embA* was the most effective approach for detecting EMB resistance, with an accuracy of 91.2%. Nevertheless, some EMB-susceptible isolates still had a single mutation in the gene related to EMB resistance. Moreover, there was a significant correlation between EMB high-level resistance and multiple mutations.

**Conclusion** Distinct individual mutations, as well as multiple concurrent mutations, within EMB resistance-associated genes, contributed to variable levels of EMB resistance. These results have broadened our understanding of the molecular characteristics of EMB resistance in China.

**Keywords** Multidrug-resistant tuberculosis, Ethambutol, Resistance, Mutation

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## Introduction

Tuberculosis (TB) remains a great menace to global public health, with approximately 10.6 million new cases and 1.3 million deaths occurring annually [1]. The appearance of drug-resistant TB, especially multi-drug-resistant TB (MDR-TB), has been regarded as a major obstacle to effective TB control. In the previous year, there were an estimated 270, 000 incident cases of MDR-TB worldwide [1].

Ethambutol (EMB) is a crucial drug used in first-line anti-TB treatment and is also commonly recommended for treating drug-resistant TB, including MDR-TB. As an arabinose analogue, EMB effectively interferes with the arabinosyl transferase encoding by the *embCAB* operon, which is involved in polymerizing arabinose into the arabinan components of arabinogalactan and lipoarabinomannan [2]. Substantial reports demonstrated that mutations in the *embCAB* operon, particularly in *embB*, significantly develop EMB resistance in TB [3–5]. However, some studies also demonstrated that TB isolates could resist EMB without mutation in the entire *embCAB* genes [3–5]. Additionally, mutations in *embCAB* genes have been detected among EMB susceptible isolates [3–5]. These reports suggest that additional mechanisms and/ or genes may contribute to EMB resistance in TB.

EmbR has been reported to regulate the arabinosyl transferase activity [6, 7]. UbiA can control the decaprenylphosphoryl-D-arabinose (DPA) level, which is involved in cell wall synthesis [8, 9]. As a novel arabinofuranosyltransferase, AftA initiates the galactan for further elaboration by the arabinofuranosyltransferase [10, 11]. Mutations in the genes encoding these three proteins, EmbR, UbiA, and AftA, can influence the biosynthesis of mycobacterial cell walls, leading to different levels of EMB resistance in TB. However, most studies focused on the mutations within relatively limited regions of *embCAB*, especially *embB* [3, 5, 12, 13]. Information regarding the roles of mutations in more ethambutol-responsive genes, including the entire *embCAB*, *ubiA*, *embR*, and *aftA*, is limited. Therefore, this study aimed to investigate mutations in ethambutol resistance-related genes, including the *embCAB*, *ubiA*, *embR* and *aftA*, among the MDR-TB isolates in China and their impacts on the level of EMB resistance. This study is essential for comprehensively understanding the association between the phenotypic susceptibility to EMB and the mutation patterns in clinical MDR-TB isolates in China.

## Material and methods

### Mycobacterium tuberculosis isolates

Overall, 159 MDR-TB isolates were obtained from 159 patients with pulmonary tuberculosis in China. These MDR-TB isolates were obtained from nine provincial tuberculosis hospitals, including Fujian (23 isolates), Guangxi (16 isolates), Guizhou (15 isolates), Hunan (22 isolates), Xizang (20 isolates), Gansu (10 isolates), Inner Mongolia (21 isolates), Xinjiang (11 isolates), and Jilin (21 isolates). H37Rv was used as a reference (ATCC 27294). All isolates were cultured on Lowenstein-Jensen (L-J) medium and freshly sub-cultured before being used for EMB susceptible testing.

### Minimum Inhibitory Concentration determination

Minimum Inhibitory Concentrations (MIC) for EMB were determined using the Sensititre® plates (Thermo Fisher Scientific Inc., Cleveland, Ohio, USA), and all steps were carried out in accordance with the manufacturer's instructions. According to previous reports [14–15], susceptibility, low-level resistance (LLR), and high-level resistance (HLR) to EMB were defined as MIC was $\leq 2$ μg/ml, MIC > 2 - < 5 μg/ml, and $\geq 5$μg/ml, respectively.

### Whole Genome Sequencing and bioinformatics and analyses

Genomic DNAs of all 159 MDR-TB isolates were extracted using the cetyltrimethylammonium bromide (CTAB) methods described in a previous report [14]. Then, they were sequenced on BGISEQ platforms with a depth of at least 100-fold coverage. After filtering the low-quality reads, the sequencing reads were mapped to the reference genome H37Rv (GenBank NC000962.3) using Bowtie2 (version 2.3.3.1) with default parameters to detect single-nucleic acid polymorphism (SNP).

Whole Genome Sequencing was employed to identify nonsynonymous mutations within the regions associated with EMB resistance, such as *embCAB*, *ubiA*, *embR* and *aftA*. These sequencing data were deposited in the NCBI Sequencing Read Archive (SRA) under the accession number: PRJNA1187775.

### PCR and DNA sequencing

All novel mutations observed within *embCAB*, and all mutations within *ubiA*, *embR* and *aftA* regions from genome data were confirmed with PCR and DNA sequencing. Nine primer sets (described in Table 1) were used for PCR amplification. All PCR amplicons were submitted for DNA sequencing, and mutations in the fragments were identified by aligning them to the H37Rv reference strain (GenBank accession number

**Table 1** PCR primers for amplification and sequencing

| Primer | Sequence (5′ to 3′) | Nucleotide position |
|---|---|---|
| EmbC-f1 | CGTTTCGTCGTCGAGGACATTG | 4239789–4240810 |
| EmbC-r1 | CCAGCGGTAGTAGTTGGCCATATAG | |
| EmbC-f2 | GATCTTTCGTGATCAGACCTTCGC | 4241320–4241806 |
| EmbC-r2 | CACACCGAAGTTGGACACGTAC | |
| EmbC-f3 | GCCAGCTACCTCAAAGACGACTG | 4242998–4244173 |
| EmbA-r1 | AGCTGGGCAAGCACCGATGTATACC | |
| EmbB-f1 | ATCAGGGCGCTGCCATGACACAG | 4246500–4247237 |
| EmbB-r1 | TGAGGAGGAGCTGGGCAATTGAG | |
| EmbB-f2 | CGACCATCGACACCCGGTTCTCCAC | 4247124–4248367 |
| EmbB-r2 | CACCGATGGGGATACCAACACCGTC | |
| EmbB-f3 | TTCATCATGTTGCGGCGCAAGCG | 4248176-424971 |
| EmbB-r3 | AACTTGCGCAGGGAACCCCAATCG | |
| Afta-f | TGGTACACCCTGTTGGTCGCCTAC | 4238742–4239829 |
| Afta-r | ACGAACGGGCCAATGTCCTCGAC | |
| Ubia-f | GTGAAGATGTGGTGACTCAACCTCCG | 4268937–4269829 |
| Ubia-r | AACAGCGGCCCCAACCGTTGCTATC-3 | |
| EmbR-f | GATTCTGCGTCAGCAACCGCTGGAT | 1416202–1416589 |
| EmbR-r | CGCGCTGATCTGGAACGTGAATTCA | |

NC_000962) using BioEdit v7.05.3. The raw sequencing data were also submitted to the NCBI SRA with the accession number: PRJNA1187775.

### Statistical analysis

A multivariate regression model evaluated the association between mutations and EMB MICs. The linear trend between the mutated type and EMB MICs category was assessed with the exact Cochran-Armitage test. A *P* value less than 0.01 was regarded as statistically significant. All statistical analysis was conducted using SAS (version 9.3) software (SAS Institute, Cary, NC).

## Results

### EMB MICs

Table 2 summarized the MIC distribution data of all 159 isolates for EMB. One hundred and fourteen isolates had EMB resistance. Sixty-four isolates had high-level EMB resistance, and 50 had low-level EMB resistance.

### Sequencing analysis of *embCAB*, *ubiA*, *embR*, and *aftA*

Among the 114 EMB-resistant isolates, 109 isolates carried nonsynonymous mutations in the EMB resistance-associated genes (Table 1). Specifically, mutations were found in 106 isolates, one isolate had insertions, and two isolates had deletions. Additionally, there were 12 EMB-susceptible isolates with mutations in the EMB resistance-related genes (Table 1).

Most mutations in the EMB resistance-related genes occurred in emb*B*. A total of 109 isolates, including 104 EMB-resistant isolates and 5 EMB-susceptible isolates, harbored at least one mutation in emb*B*. These mutations were located in 22 different positions of emb*B* and consisted of 33 mutated types, as shown in Table 2. Most mutations were concentrated in the region between codon 300 and codon 500. Codons 306, 406, and 497 were the most frequently mutated sites, present in 87 EMB-resistant isolates and 2 EMB-susceptible isolates. Mutations in *embB* outside codons 306, 406, and 497 were also found in codons 34, 50, 246, 300, 303, 319, 328, 330, 354, 402, 505, 563, 581, 603, 814, 907,1000, and 1024, detected in 23 EMB-resistant isolates and three EMB-susceptible isolates. Among them, six EMB-resistant isolates had an additional 306, 406, or 497 mutations. A single mutation at codons 319, 328, 330, 402, 581, and 1024 was only detected in EMB-resistant isolates.

The next most common mutations were mainly in the *embC-embA* intergenic region. In total, 22 isolates (including 19 EMB-resistant isolates and 3 EMB-susceptible isolates) had mutations at positions −75, −43, −29, −22, −16, −15, −12, −11 and −5 bp sites within this region. Most of these mutations were combined with additional mutations in other EMB resistance-related genes, especially *embB*. Only five isolates had a single *embC-embA* mutation ($n$=1 for -g5t, $n$=1 for -c15g, $n$=1 for -c16a and n=2 for -c16t). Two of them with nucleotide changes at the −16bp site were susceptible to EMB with MICs of 2 μg/ml.

The *ubiA* is also a relatively frequently mutated region in EMB resistance. A total of 12 isolates had *ubiA* mutations. Seven of these isolates, which also had mutations in *embB*, were resistant to EMB, while the other five isolates with only *ubiA* mutation were susceptible to EMB (MICs of 2 μg/ml). Additionally, one isolate had a mutation in *embR*, and three isolates harbored mutations in *embA*, *embC* or *aftA*. All these isolates also had other mutations in the *embB* or *embC-embA* intergenic region and belonged to the EMB-resistant group.

### Association between the EMB MICs and mutations

Since 23.3% of isolates (37/159 isolates) had more than one mutation within the EMB resistance-related genes, the association between EMB resistance and mutations was evaluated using multivariate regression (Table 3). Statistics analysis revealed that only mutations in *embB*, including *embB*Met306Ile, Met306Leu, Met306Val, Gly406Ala, Gly406Asp, and Gln497Arg, showed a significant association with EMB resistance. However, *embB*Met306Ile mutation was also found in two EMB-susceptible isolates with MICs of 2 μg/ml. In addition, some single mutations, such as *embB*Phe330Ile, Gly406Ser, and Asp1024Asn, which occurred exclusively

**Table 2** Nonsynonymous mutations in EMB resistance-associated genes among 159 multidrug-resistant isolates

| Mutations in: | | | | | | No of isolates | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Ethambutol MIC (μg/ml) | | | | | | |
| ***embA*** | ***embB*** | ***embC*** | ***ubiA*** | ***embR*** | ***aftA*** | ≤0.5 | 1 | 2 | 4 | 8 | 16 | ≥32 |
| -g75c* | Met306Ile | | | | | | | | 1 | | | |
| -g43gc | | | | 1041delC* | | | | | 1 | | | |
| -c29 del | Ile303Leu*, Thr581Ala* | | | | | | | | 1 | | | |
| -g22 del* | Val50Ala | | | | | | | 1 | | | | |
| -c16a | | | | | | | | 1 | | | | |
| -c16a | Val50Ala, Gln497Pro | | | | | | | | | 1 | | |
| -c16a | Gly406Ser | | | | | | | | | | 1 | |
| -c16g | Met306Ile | | | | | | | | | 1 | 1 | |
| -c16g | Ala505Val | | | | | | | | | | 1 | |
| -c16t | | | | | | | | 1 | 1 | | | |
| -c16t | Met306Ile | | | | | | | | | 1 | | |
| -c16t | Met306Val | | | | | | | | | | | 1 |
| -c16t | Asp328Gly | | | | | | | | 1 | | | |
| -c16t | Leu402Val | | | | | | | | | 1 | | |
| -c15g* | | | | | | | | | 1 | | | |
| -c12t | Val50Ala, Met306Ile | | | | | | | | | | 1 | |
| -c12t | Met306Ile | | | | | | | | | 1 | | |
| -c12t | | Asn60Ser*, Arg260Gln* | | | | | | | | 1 | | |
| -c11a | Gly34Glu*, Gly406Ala | | | | | | | | | | 1 | |
| -g5t* | | | | | | | | | 1 | | | |
| Asn37Lys*, Ala84Ser | Asp354Ala | | | | | | | | 1 | | | |
| Thr100Pro* | Met306Val | | | | | | | | | | | 1 |
| Gly220Glu* | Asp328Tyr, Asp814Asn*, Asp1024Asn | | | | | | | | | | 1 | |
| | Asp300Gly | | Ser173Ala | | | | | | | 1 | | |
| | Met306Ile | | | | | | | 2 | 17 | | | |
| | Met306Ile, Gln497Pro | Arg30Gln* | | | | | | | | | 1 | |
| | Met306Leu | | | | | | | | 1 | 1 | | |
| | Met306Leu | | Ala167Thr | | | | | | | 1 | | |
| | Met306Leu | | | | Phe355Leu* | | | | | 1 | | |
| | Met306Val | | | | | | | | 1 | 17 | 5 | |
| | Met306Val | | | | Arg333Gly* | | | | | 1 | | |
| | Met306Val, Gly246Arg | Ala543Thr* | | | | | | | | 1 | | |
| | Met306Val, Gly406Asp | | | | | | | | | | 1 | |

**Table 2** (continued)

| Mutations in: | | | | | | No of isolates | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Ethambutol MIC (μg/ml) | | | | | | |
| ***embA*** | ***embB*** | ***embC*** | ***ubiA*** | ***embR*** | ***aftA*** | **≤0.5** | **1** | **2** | **4** | **8** | **16** | **≥32** |
| | Met306Val, Gln497His | | | | | | | | | | 1 | 1 |
| | Met306Val, Asp1024Asn | | | | | | | | | | 1 | |
| | Tyr319Cys | | | | | | | | 1 | | | |
| | Asp328Tyr | | | | | | | | | 1 | | |
| | Phe330Ile | | | | | | | | 2 | | | |
| | Asp354Ala | | | | | | | | 1 | | | |
| | Leu402Val | | | | | | | | 1 | | | |
| | Gly406Ala | | | | | | | | 3 | 4 | | |
| | Gly406Ala | | Ala237Thr | | | | | | | 1 | | |
| | Gly406Ala, Pro907Ser | | | | | | | | 1 | | | |
| | Gly406Asp | | | | | | | | 4 | | | |
| | Gly406Ser | | | | | | | | 2 | | | |
| | Gly406Ser | | Met180Ile | | | | | | 1 | | | |
| | Gln497Arg | | | | | | | | 1 | 5 | | |
| | Gln497Arg | | Ala38Thr | | | | | | | 1 | | |
| | Gln497Arg | | Val55Leu | | | | | | | 1 | | |
| | Gln497Arg | | Phe59Cys | | | | | | | 1 | | |
| | Ile563Leu | | | | | | 1 | | | | | |
| | Thr581Ala | | | | | | | | | 1 | | |
| | Gly603Arg | | | | | | | 1 | | | | |
| | Met1000Arg | | | | Leu372Arg | | | | 1 | | | |
| | Asp1024Asn | | | | | | | | 2 | | | |
| | | | Ala39Glu | | | | | 1 | | | | |
| | | | Val111Ala | | | | | 1 | | | | |
| | | | Ser173Ala | | | | | 1 | | | | |
| | | | Trp175Cys | | | | | 1 | | | | |
| | | | Val283Leu | | | | | 1 | | | | |
| NM | NM | NM | NM | NM | NM | 3 | 24 | 6 | 3 | 2 | | |

*NM* no mutation

* Mutation not previously reported

**Table 3** Logistic Regression multivariate model between mutations and EMB resistance

| Gene | Mutations | isolates | Median MIC (IQR) | OR | *P* |
|---|---|---|---|---|---|
| *embC-embA* | -c16g | 3 | 16 (8, 16) | 3.95 | 0.3619 |
| *embC-embA* | -c16t | 6 | 6 (4, 8) | 7.558 | 0.2361 |
| *embC-embA* | -c16a | 3 | 8 (2, 16) | 4.004 | 1 |
| *embC-embA* | -c12t | 3 | 8 (8, 16) | 3.806 | 0.3873 |
| *embB* | Met306Val | 31 | 8 (8, 16) | 127.426 | <.0001* |
| *embB* | Met306Ile | 26 | 4 (4, 4) | 32.933 | <.0001* |
| *embB* | Gly406Ala | 10 | 8 (4, 8) | 47.908 | <.0001* |
| *embB* | Gln497Arg | 9 | 8 (8, 8) | 42.97 | <.0001* |
| *embB* | Gly406Asp | 5 | 4 (4, 4) | 18.287 | 0.0068* |
| *embB* | Asp1024Asn | 4 | 10 (4, 16) | 8.433 | 0.1033 |
| *embB* | Gly406Ser | 4 | 4 (4, 10) | 14.928 | 0.0129 |
| *embB* | Met306Leu | 4 | 8 (6, 8) | 18.287 | 0.0068* |
| *embB* | Val50Ala | 3 | 8 (2, 16) | 4.004 | 1 |
| *embB* | Asp328Tyr | 2 | 12 (8, 16) | 3.549 | 0.4396 |
| *embB* | Asp354Ala | 2 | 4 (4, 4) | 8.433 | 0.1033 |
| *embB* | Gln497His | 2 | 24 (16, 32) | / | / |
| *embB* | Gln497Pro | 2 | 12 (8, 16) | 0.417 | 1 |
| *embB* | Leu402Val | 2 | 6 (4, 8) | 4.161 | 0.3305 |
| *embB* | Phe330Ile | 2 | 4 (4, 4) | 8.433 | 0.1033 |
| *embB* | Thr581Ala | 2 | 6 (4, 8) | 8.433 | 0.1033 |
| *ubiA* | Ser173Ala | 2 | 5 (2, 8) | 3.77 | 0.7759 |

* Significant at the 0.01 threshold.

**Table 4** Relationship between mutations and different levels of EMB resistance

| EMB resistance related regions | EMB resistance | | | *P* value |
|---|---|---|---|---|
| | Susceptible | Low level EMB resistance | High level EMB resistance | |
| Single mutation | 11 | 39 | 34 | 0.001* |
| Multiple mutations | 1 | 8 | 28 | |

* Significant at the 0.01 threshold.

in EMB isolates, were not associated with EMB resistance in the multivariate analysis.

Among the 37 isolates with multiple mutations, 35 had at least one mutation in *embB* and the remaining two had at least one mutation in *embC-embA*. Most multiple mutations were found in EMB HLR isolates. Of the 64 isolates with EMB HLR, 28 (43.8%) isolates had two or more mutations in EMB resistance-related regions. This proportion is significantly higher than that among EMB LLR isolates (16.0%, 8/50 isolates) and EMB susceptible isolates (2.2%, 1/45 isolates). Statistics analysis also showed that multiple mutations were related to EMB HLR (*P*=0.001). Notably, one EMB-susceptible isolate (with a MIC of 2 μg/ml) carried both *embC-embA* -g22 del and *embB*50 mutations (Table 4).

### Agreement between the phenotypic drug susceptibility and DNA sequencing

Table 5 summarizes the concordance between the phenotypic and genotypic tests for detecting EMB resistance using DNA sequencing based on different regions in resistance-associated genes. In this study, screening of *embC-embA* and the entire *embB* yielded the best prediction outcomes (92.5% accuracy), superior to the results from screening of *embC-embA* and *embB*300–500 (91.2% accuracy). Moreover, a notable discrepancy emerged among 12 isolates. The phenotypic susceptibility tests verified these isolates as EMB susceptible but contained at least one mutation in a resistance-associated gene.

## Discussion

In this study, we measured the EMB MICs of 159 MDR-TB from China, and analyzed the mutations present in the four ethambutol resistance-associated regions. Notably, in the course of conducting drug susceptibility tests, the determination of the critical concentration for EMB was crucial, yet it was mired in controversy. Some reports suggested that the previously applied critical concentration of 4 μg/ml could result in a greater disparity between genotypic and phenotypic tests for EMB [15, 16]. However, when the critical concentration was adjusted from 4 μg/ml to 2 μg/ml, the concordance between the genotypic and MIC significantly improved. Consequently, the Clinical and Laboratory Standards Institute (CLSI) recommended classifying the MIC of 4 μg/ml as the "inconclusive" category [17]. In our study, among all 50 isolates with a MIC of 4 μg/ml, 47 isolates had mutations in the analyzed genes. Most of the mutations in these isolates fell into group 1 (Associated with resistance) for EMB according to the mutation catalogue recommended by the World Health Organization (WHO) [18]. Thus, isolates with a MIC of 4 μg/ml were more appropriately classified as the low-level EMB-resistant group. This classification has also been utilized in some publications [19, 20].

It has been reported that EMB resistance is correlated with mutations in the *embCAB*, most commonly in *embB* [4, 5, 21]. Accordingly, we found that among 114 EMB-resistant isolates, 104 (91.2%) had mutations in *embB*. We also noticed 19 EMB-resistant isolates carrying *embC-embA* mutations, which supported the notion that mutations in the *embC-embA* contribute to EMB resistance [4, 22]. Some reports have suggested that the transcriptional regulators *embR*, *ubiA* and *aftA* are also associated

**Table 5** Summary of sequence analysis of mutated locus and phenotypic drug susceptibility testing

| Locus | No. of isolates | | | | *P* value | Sensitivity (%) | Specificity (%) | Accuracy (%) |
|---|---|---|---|---|---|---|---|---|
| | Resistant | | Susceptible | | | | | |
| | Mutation | No mutation | Mutation | No mutation | | | | |
| *embC-embA* | 19 | 95 | 3 | 42 | 0.128 | 16.7 | 93.3 | 38.4 |
| *embB* | 104 | 10 | 5 | 40 | 0.000* | 91.2 | 88.9 | 90.6 |
| *embB*300–500 | 99 | 15 | 2 | 43 | 0.000* | 86.8 | 95.6 | 89.3 |
| *embB*800–1024 | 6 | 108 | 0 | 45 | 0.268 | 5.3 | 100.0 | 32.1 |
| *embA* | 3 | 111 | 0 | 45 | 0.559 | 2.6 | 100.0 | 30.2 |
| *embC* | 3 | 111 | 0 | 45 | 0.559 | 2.6 | 100.0 | 30.2 |
| *ubiA* | 7 | 107 | 5 | 40 | 0.462 | 6.1 | 88.9 | 29.6 |
| *aftA* | 3 | 111 | 0 | 45 | 0.559 | 2.6 | 100.0 | 30.2 |
| *embR* | 1 | 113 | 0 | 45 | 1.000 | 0.9 | 100.0 | 28.9 |
| *embC-embA* and *embB* | 109 | 5 | 7 | 38 | 0.000* | 95.6 | 84.4 | 92.5 |
| *embA* and *embB* | 104 | 10 | 5 | 40 | 0.000* | 91.2 | 88.9 | 90.6 |
| *embC* and *embB* | 105 | 9 | 8 | 37 | 0.000* | 92.1 | 82.2 | 89.3 |
| *ubiA* and *embB* | 104 | 10 | 10 | 35 | 0.000* | 91.2 | 77.8 | 87.4 |
| *aftA* and *embB* | 104 | 10 | 5 | 40 | 0.000* | 91.2 | 88.9 | 90.6 |
| *embR* and *embB* | 105 | 9 | 5 | 40 | 0.000* | 92.1 | 88.9 | 91.2 |
| *embCAB* | 109 | 5 | 7 | 38 | 0.000* | 95.6 | 84.4 | 92.5 |
| *embCAB* and *ubiA* | 109 | 5 | 12 | 33 | 0.000* | 95.6 | 73.3 | 89.3 |
| *embCAB*, *ubiA*, *aftA*, and *embR* | 109 | 5 | 12 | 33 | 0.000* | 95.6 | 73.3 | 89.3 |
| *embC-embA* and *embB*300–500 | 105 | 9 | 5 | 40 | 0.000* | 92.1 | 88.9 | 91.2 |

* Significant at the 0.01 threshold

with EMB-resistant TB [9, 11, 23]. However, our study revealed that among EMB-resistant isolates, mutations within these three genes were always accompanied by a mutation in *embB* or *embC-embA*. Single mutations among EMB-resistant TB were observed only in *embB* and *embC-embA*, while single *ubiA* mutations were only observed in EMB-susceptible isolates. This implied that mutations in *embB* or *embC-embA* remain the most critical factor for EMB resistance.

Numerous reports showed that mutations at *embB* codons 306, 406, and 497 are common in EMB-resistant isolates [12, 13, 24]. Correspondingly, the multivariate regression model in this study also indicated that mutations including *embB*Met306Ile, Met306Leu, Met306Val, Gly406Ala, Gly406Asp, and Gln497Arg were correlated with EMB resistance. According to the WHO catalogue of mutations and their association with drug resistance in TB, these six mutations belonged to group 1 (Associated with resistance) for EMB, which could be utilized as critical markers for identifying EMB resistance. The fact that multiple mutated sites of the *embB* are associated with EMB resistance also implies that some rapid diagnostic methods, like the GenoType MTBDR*sl* assay, which only detects mutations at the *embB306* locus, have relatively limited diagnostic sensitivity for EMB resistance in China. In addition, some single mutations, such as *embB*Phe330Ile, Gly406Ser, and Asp1024Asn, only occurred in EMB-resistant isolates. They were not detected by multivariate analysis, possibly due to the limited sample size in this study. Therefore, additional studies with more isolates carrying these mutations are needed.

Multiple mutations within ethambutol resistance-associated genes were more likely to be found in EMB HLR isolates. Statistical analysis also showed a significant association between them. These results suggested that EMB HLR is more complex, involving multiple mutations in one or several genes. Interestingly, multiple mutations usually included at least one mutation within *embB* or *embC-embA*. Still, fewer mutations within *embA*, *embC* *embR*, *ubiA*, or *aftA* imply that mutations occurring in these genes might have compensatory effects in EMB resistance.

Our results also confirmed some EMB-susceptible isolates carrying mutations in *embB* and/ or *embC-embA* or *ubiA*. Notably, almost all of these isolates had MICs of 2 μg/ml, which is very close to the MIC of EMB LLR, indicating that these mutations could cause a slight increase in the EMB MIC. In addition, this phenomenon, the strains that exhibit drug-sensitive

phenotypes yet carry some drug-resistant associated mutations, is also related to heteroresistance [25]. Heteroresistance, common in TB, is regarded as an initial, yet potentially crucial step in developing EMB resistance [26].

Compared to the phenotypic MICs, the accuracy of detecting EMB resistance by DNA analysis of a single *embB* in our study was 90.6%. Adding *embC-embA* to the molecular diagnosis increased the test accuracy to 92.5%. Adding *embR*, *ubiA*, or *aftA* to the molecular diagnosis could result in unchanged or even decreased test accuracy. Thus, DNA analysis of the combination of *embB* and *embC-embA* achieved the best accuracy for diagnosing EMB resistance. Since the entire *embB* gene was long and difficult for PCR amplification and sequencing, selectively targeting *embB*300–500 and *embC-embA* could also yield better detection accuracy for EMB resistance.

Furthermore, some novel mutations were observed in this study. Only a few single mutations, such as *embC-embA* -c15g and -g5t, occurred in the EMB-resistant isolates, expanding our understanding of mutations associated with EMB resistance. The other mutations occurred alongside known mutations in either *embB* or the *embC-embA* regions. Thus, the role of these novel mutations requires further investigation. Although this study explored mutations within several EMB resistance-associated genes, five EMB-resistant isolates with no mutation were still in the analyzed regions. This suggests that these isolates might have mutations outside the analyzed area, or the resistance may be due to other mechanisms [27], such as efflux pumps [28],.

In conclusion, mutations within *embB* or *embC-embA* are the most crucial for EMB resistance. Almost all EMB-resistant isolates had at least one mutation within these two regions. DNA sequencing of these regions could achieve the best diagnostic efficiency for EMB resistance. However, mutations in *embA, embC*, *embR*, *ubiA* or *aftA* will likely play compensatory roles in EMB resistance. These mutations were always accompanied by *embB* or *embC-embA* mutations occurring in EMB-resistant isolates or occurred alone (such as single *ubiA* mutation) in EMB-susceptible isolates. These results are beneficial for expanding our knowledge of the molecular characteristics of EMB resistance in China and for developing rapid molecular diagnostic methods.

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**Clinical trial number**
Not applicable.

**Authors' contributions**
LL Zhao and KL Wan conceived and designed the study. LL Zhao, TY Xiao, and HC Liu analyzed the data. MC Li, W Wang, H Hang, XL Bo, XT Nan, and XQ Zhao performed the laboratory experiments. HC Liu, MC Li, TY Xiao, C Qian, XT Fan, and GL Li contributed to the data collection and analysis. LL Zhao and SQ Lin wrote the manuscript. All authors read and approved the final manuscript.

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**Data availability**
All the DNA sequencing data which support the findings of this study are presented under the accession number: PRJNA1187775.

## Declarations

**Ethics approval and consent to participate**
This study was approved by the Ethics Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. All of the participants in the study had signed the informed consent. Every procedure involving human participants was performed in accordance with the Declaration of Helsinki.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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