Characteristics of embB mutations in multidrug-resistant Mycobacterium tuberculosis isolates in Henan, China

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Objectives: To determine the association between embB mutations and drug resistance, and to further investigate the mechanism of embB mutations involved in the development of ethambutol and multidrug resistance in Mycobacterium tuberculosis.

Methods: One hundred and thirty-eight multidrug-resistant clinical M. tuberculosis isolates, including 86 ethambutol-resistant and 52 ethambutol-susceptible strains, were analysed to characterize mutations within the entire coding region of the embB gene. Moreover, a two-step genotyping was performed to identify the genetic lineage.

Results: In total, 27 embB mutation types were detected in 19 distinct codons. Though a strong association was observed between embB mutations and ethambutol resistance, 19.2% of embB306 mutants and 11.5% of embB406 or embB497 mutants were ethambutol susceptible. Among 39 ethambutol-resistant strains without embB306 mutations, 51.3% harboured mutations at codons 406 or 497. Particularly, three pairs of isolates with identical embB mutations and genotyping features were identified with variant ethambutol susceptibility. Among 77 isoniazid, rifampicin, streptomycin and ethambutol quadruple drug-resistant isolates, 89.6% carried embB mutations and 83.1% could be identified by detecting 10 embB mutations.

Conclusions: Our results suggest embB mutations alone are not sufficient for the development of full resistance to ethambutol in M. tuberculosis and mutations other than embB are also needed. Our study confirms the importance of mutations at embB406 and embB497 as hotspots, in addition to embB306, for detecting ethambutol resistance. Ten selected mutations of embB, covered by a short PCR product, can be used as candidate markers for the prediction of quadruple resistance to isoniazid, rifampicin, streptomycin and ethambutol.

Keywords: genetic background, MIRU-VNTR, embB306, embB497, ethambutol

Introduction

Multidrug-resistant (MDR) tuberculosis (TB) has an estimated 4.8% prevalence worldwide and poses a serious threat to global public health. In China, the significantly high (9.3%) prevalence of MDR-TB makes the prevention and control of tuberculosis especially challenging. The rapid and reliable detection of drug resistance is critical for optimizing treatment regimens and for preventing the spread of tuberculosis. Ethambutol, which is an essential first-line drug in tuberculosis treatment, plays an important role in the chemotherapy of drug-resistant TB. Ethambutol inhibits mycobacterial arabinosyl transferases encoded by the embCAB operon, which includes three genes (embC, embA and embB). Amino acid substitutions encoded by embB are observed in non-tuberculous mycobacteria with intrinsic resistance to ethambutol. Exchanging wild-type embB306, embB497 and embB406 with mutant codons increases the ethambutol minimal inhibition concentrations (MICs) of Mycobacterium tuberculosis. Mutations at embB320 and embB324, as well as mutations at embB397, embB445, embB1024 and embC13, have also been found to be associated with ethambutol resistance.
The most commonly detected point mutation in ethambutol-resistant clinical strains of *M. tuberculosis* is in the *embB* gene at codon 306, which occurs in 30%–69% of ethambutol-resistant clinical strains. Initial studies indicated that the *embB*306 mutations were only observed in ethambutol-resistant strains and led to the proposal that the *embB*306 locus be considered as a diagnostic marker for ethambutol resistance. However, the detection of *embB*306 from ethambutol-susceptible clinical isolates questions the validity of this assertion. Mokrousov et al. first described this phenomenon as a genuine discrepancy between genotypic and phenotypic tests, and noticed that *embB*306 mutations in ethambutol-susceptible isolates were limited to the isolates already resistant to other drugs. Based on a study of 1020 clinical isolates, Hazbon et al. suggested that the *embB*306 mutation is associated with broad drug resistance rather than ethambutol resistance per se. Shen et al. also proposed the *embB*306 locus as a candidate marker for the detection of MDR and extensively drug-resistant *M. tuberculosis* isolates. Although the association between *embB*306 mutation and ethambutol resistance or broad drug resistance has been observed in several groups’ studies with both clinical or laboratory isolates, the exact role *embB*306 mutations play in the development of ethambutol resistance and multidrug resistance in *M. tuberculosis* is not fully understood. Mutations in *embB* other than *embB*306 were also detected in these studies, but the contribution of such mutations to the development of ethambutol resistance is similarly not clear. It is believed that variant genetic alterations that accumulate in epidemic *M. tuberculosis* lead to the development of drug resistance, including ethambutol resistance, but not much evidence has been obtained from studies of clinical isolates. Therefore, to further investigate the mechanism of *embB* mutations in the development of drug resistance and to evaluate the association between *embB* mutations and drug resistance, including ethambutol, multidrug and broad drug resistance, a relatively large population of MDR-TB isolated from Henan province, China was examined. In the present study, we characterized the mutations of the *embB* complete coding sequence and documented the variable number tandem repeat of mycobacterial interspersed repetitive units (MIRU-VNTR) genotypes of this MDR-TB population, to further analyse the mechanism underlying the development of ethambutol drug resistance in clinical *M. tuberculosis* isolates.

Materials and methods

*M. tuberculosis* clinical strains

One hundred and fifty MDR-TB strains were collected by sequentially screening 1605 clinical *M. tuberculosis* strains isolated from patients from Henan province in 2007–09. Meanwhile, 22 pan-susceptible strains were collected from the same location to be used as controls in this study.

Drug susceptibility testing (DST)

DST to four first-line antituberculosis drugs was performed in the Tuberculosis Reference Laboratory at Henan Provincial Centers for Disease Control and Prevention, China. The Löwenstein–Jensen (LJ) proportion method, recommended by WHO/International Union Against Tuberculosis and Lung Disease (IUATLD), was used to perform DST with the following critical drug concentrations: 0.2 mg/L isoniazid; 40.0 mg/L rifampicin; 2.0 mg/L ethambutol; and 4.0 mg/L streptomycin.

**RD105 deletion-targeted multiplex PCR (DTM-PCR) and MIRU-VNTR genotyping**

DTM-PCR was performed to identify the Beijing family strains. A China-specified MIRU-VNTR genotyping method (VNTR-7) was performed on all MDR-TB isolates with seven VNTR loci in this study, and additional nine VNTR loci (VNTR-9) was applied to the isolates with identical first seven VNTR loci. Samples with more than one band in the PCR product on any VNTR locus were considered as mixed strains and excluded from the studied population. The VNTR genotyping data, transformed into a distance matrix on the web site MIRU-VNTRplus (http://www.miru-vntrplus.org) by default setting, were treated as categorical variables and the phylogenetic analysis of the distance data was conducted using MEGA version 4.

**PCR amplification, sequencing and data analysis**

The full-length *embB* gene coding region of the studied strains was amplified with three overlapped fragments. Chromosomal DNA was extracted using the boiling method. Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes, Finland), an ultrahigh-fidelity DNA polymerase, was used for the amplification. The primers synthesized by Sangon Biochemical for DNA amplification were: *embB*-1 (5′-TCGAGATGCGACGTACTT-3′) and *embB*-2 (5′-CAGAACAGCCGACGACGACTA-3′); *embB*-1 (5′-TCTGGGCTTCTGCTCTGG-3′) and *embB*-2 (5′-CACCCGTAGCGACGACT-3′); and *embB*-3 (5′-GGCGTGATTCT-3′) and *embB*-2 (5′-AGCCTGACGCTATGGACCAA-3′). The sequencing primers were *embB*-1S (5′-CGTCCTTCGCTTGGTGG-3′) and *embB*-3S (5′-CGTCCTTCGCTTGGTGG-3′) and *embB*-1 (5′-GCGTGGATCTCCGCTTGG-3′); other sequencing primers were the same as *embB*-1, *embB*-2, *embB*-2 and *embB*-3. PCR products were sequenced by Sinogenomax Co. Ltd. Sequence data were assembled by Seqman pro (version 7.1, DNASTar Lasergene), and mutations were determined by comparing with the H37Rv sequence of *embB* from Tuberculist (http://genolist.pasteur.fr/Tuberculist/) and the GenBank database (http://www.ncbi.nih.gov/; gene). The frequency calculation and analysis were performed using SPSS for Windows® (version 10.0, SPSS, Inc., USA).

**Results and discussion**

**General profile of genotyping, drug resistance and *embB* mutations**

Twelve isolates (8%) of the primary study population, which were identified as a mixture of different individual MDR-TB strains by a two-step genotyping method of VNTR-7 and VNTR-9, were not included in the following analysis. In total, 138 MDR isolates included in the study showed 116 unique and 11 clustered genotyping patterns based on VNTR-7 genotyping (Figure 1a). Most of the MDR isolates (95%, 131/138) were identified as Beijing family *M. tuberculosis* strains by DTM-PCR. Among the 138 clinical MDR-TB isolates, 9 presented no additional resistance (isoniazid/rifampicin resistant), 43 were isoniazid/rifampicin/streptomycin resistant, 9 were isoniazid/rifampicin/ethambutol resistant and 77 were isoniazid/rifampicin/ethambutol/streptomycin resistant. There were 86 ethambutol-resistant and 52 ethambutol-susceptible isolates.

One hundred and thirty-eight MDR-TB strains were screened for *embB* mutations. A total of 119 *embB* mutations representing...
ID  VNTR-9 genotyping  VNTR-9 result  Amino acid change  Nucleotide change  Drug resistance
55  1 2 3 3 5 4 2 4  C  Met306Ile  ATG→ATT  HRS
92  1 2 3 3 5 4 2 4  -  Met306Ile  ATG→ATT  HRS
99  1 2 3 3 5 4 2 4  C  Met306Ile  ATG→ATA  HRE
100 1 2 3 3 5 4 2 4  C  Met306Ile  ATG→ATA  HR
21  3 2 3 3 6 1 1 4  C  Gln497Arg  CAG→CGG  HRES
108 3 2 3 3 6 1 1 4  C  Gln497Arg  CAG→CGG  HRS
9  3 2 3 3 5 4 2 4  C  Gln497Arg  CAG→CGG  HRS
129 3 2 3 3 5 4 2 4  C  Gln497Arg  CAG→CGG  HRE
69  2 2 3 3 5 4 2 4  U  Met306Ile, Gln497Pro  ATG→ATA, CAG→CGG  HRE
70  5 2 2 3 1 4 2 4  U  Gln497Arg  CAG→CGG  HRES
16  4 2 3 3 5 4 2 4  U  Gly406Ala  GCC→GCG  HRES
29  3 2 3 3 5 4 2 4  U  Met306Val, Gly406Ala  ATG→GTC, GCC→GCG  HRES
65  3 2 3 3 5 4 2 4  C  —  —  —  HRS
110 3 2 3 3 5 4 2 4  C  Gly406Asp  GCC→GAC  HRS
149 3 2 3 3 5 4 0 5  U  Met306Ile  ATG→ATA  HRS
119 3 2 3 3 5 4 2 4  U  Met306Ile  ATG→ATT  HRES
107 3 2 3 3 5 4 2 4  U  —  —  —  HRS
50  2 2 3 3 5 4 1 4  U  Met306Ile  ATG→ATA  HRES
34  2 2 3 3 5 4 1 4  U  Met306Val  ATG→GTG  HRES
86  3 2 3 3 5 4 2 4  C  Met306Val  ATG→GTG  HRES
28  3 2 3 3 5 4 2 4  C  —  —  —  HRE
145 3 2 3 3 5 4 2 4  U  —  —  —  HRS
5  2 2 3 3 5 4 2 4  U  Met306Ile  ATG→ATC  HRS
96  2 2 3 3 5 4 2 4  C  Met306Ile  ATG→ATC  HRES
105 2 2 3 3 5 4 2 4  C  Met306Val  ATG→GTG  HRE
150 3 2 3 3 5 4 2 4  U  —  —  —  HRS
126 3 2 3 3 5 2 2 4  U  Met306Ile  ATG→ATA  HRS
23  3 2 3 3 5 4 2 4  C  Met306Leu  ATG→CTG  HRES
131 3 2 3 3 5 4 2 4  C  Met306Leu  ATG→CTG  HRES
98  3 2 3 3 5 4 2 4  U  Met306Val  ATG→GTG  HRES
109 3 2 3 3 5 3 2 4  U  —  —  —  HR

(a) (b) (c)
27 mutations types were detected in 19 distinct codons, including 4 synonymous mutation types at codons 304, 424, 534 and 539 (Table 1). Ninety-four mutations were detected in 86 ethambutol-resistant MDR-TB strains. Twenty-five mutations were detected in 52 ethambutol-susceptible MDR-TB strains. Of the 138 MDR-TB isolates studied, 38 (27.5%) showed wild-type embB sequences while 100 (72.5%) showed mutated sequences (Table 2).

Overall, 16 isolates carried more than one mutation in the entire coding region of embB, including 7 isolates with two non-synonymous embB mutations. Thirteen isolates were detected to have double embB mutations, of which 5 isolates carried two non-synonymous mutations. Among the seven isolates carrying two non-synonymous embB mutations, six were resistant to ethambutol (isoniazid/rifampicin/ethambutol resistant or isoniazid/rifampicin/ethambutol/streptomycin resistant). Particularly, among those ethambutol-resistant isolates with multiple mutations, three isolates contained mutation combinations involving codons 306, 406 and 497 (embB306 plus embB406; embB306 plus embB497), which has not been documented in previous studies.8,10,29

### Characteristics of embB306 mutation

One of the major mutations was embB306, accounting for the highest proportion of all mutations detected in our study. Altogether, 41.3% (57/138) of MDR-TB isolates carried the embB306 mutation. The proportion of embB306 mutants among ethambutol-resistant MDR strains (54.7%, 47/86) was

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**Table 1.** Mutation pattern of the embB gene in isolates with different phenotypes

| Locus    | ethambutol-susceptible MDR | ethambutol-resistant MDR | Number of mutations |
|----------|-----------------------------|--------------------------|--------------------|
|          | isoniazid/rifampicin | isoniazid/rifampicin/streptomycin | total | isoniazid/rifampicin/ethambutol | isoniazid/rifampicin/ethambutol/streptomycin | total |
| embB306  | 3                           | 7                        | 10   | 4                                  | 43                                   | 47  |
| embB497  | 3                           | 3                        | 3    | 3                                  | 13                                   | 16  |
| embB406  | 3                           | 3                        | 1    | 6                                  | 7                                    |
| embB354  | 2                           | 2                        | 2    | 2                                  | 2                                    |
| embB534a | 1                           | 1                        | 3    | 8                                  | 11                                   |
| embB304a | 0                           | 1                        | 1    | 1                                  |
| embB328  | 0                           | 1                        | 1    | 1                                  |
| embB330  | 0                           | 1                        | 1    | 1                                  |
| embB424a | 0                           | 1                        | 1    | 1                                  |
| embB439  | 0                           | 1                        | 1    | 1                                  |
| embB469  | 1                           | 1                        | 1    | 1                                  |
| embB508  | 1                           | 1                        | 1    | 1                                  |
| embB539a | 0                           | 1                        | 1    |
| embB627  | 0                           | 1                        | 2    | 3                                  |
| embB651  | 1                           | 1                        | 2    | 3                                  |
| embB667  | 1                           | 1                        | 1    | 0                                  |
| embB1000 | 0                           | 1                        | 1    |
| embB1002 | 0                           | 1                        | 1    | 1                                  |
| embB1024 | 2                           | 2                        | 0    | 0                                  |
| Total    | 3                           | 22                       | 25   | 13                                 | 81                                   |

*aSynonymous mutations.

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**Figure 1.** Phylogenetic analysis of MDR-TB isolates, and characteristics of VNTR-9 genotyping, embB mutations and drug resistance patterns of isolates with the clustered VNTR-7 genotypes. (a) Phylogenetic map generated by the neighbour-joining (NJ) method, based on VNTR-7 genotyping data of 138 MDR-TB isolates. Numbers indicate strain ID. Filled circles indicate ethambutol-susceptible embB306 mutants, while open circles indicate ethambutol-resistant counterparts. Filled squares indicate ethambutol-susceptible embB497 mutants, while open squares indicate ethambutol-resistant embB497 counterparts. Filled triangles indicate ethambutol-susceptible embB406 mutants. (b) VNTR-9 genotypes, embB mutations and drug resistance patterns of isolates showing the clustered VNTR-7 genotypes. Blocks highlight three pairs of isolates with identical VNTR-16 genotypes, but opposite ethambutol resistance. C indicates isolates with identical VNTR-9 genotyping patterns. U indicates isolates with unique VNTR-9 genotyping patterns. H, isoniazid; R, rifampicin; E, ethambutol; S, streptomycin. (c) Radial NJ phylogeny based on VNTR-7 genotyping data of MDR-TB isolates, which shows the genetic background of six EmbB Met306Leu mutants. Filled circles represent strains with a Met→Leu amino acid substitution.
Table 2. Characteristics of embB mutants within the MDR-TB isolates

| Locus               | Nucleotide change     | Amino acid change           | No. of isolates (percentage of 138 MDR isolates) |
|---------------------|-----------------------|----------------------------|-------------------------------------------------|
| embB306             | atg→Ctg               | Met→Leu                    | 46 (33.3)                                       |
|                     | atg→Gtg               | Met→Val                    |                                                 |
|                     | atg→atA               | Met→Ile                    |                                                 |
|                     | atg→atC               | Met→Ile                    |                                                 |
|                     | atg→atT               | Met→Ile                    |                                                 |
| embB306 and embB534 | atg→atC and gac→gaA   | Met→Ile and Asp→Asp        | 6 (4.3)                                         |
|                     | atg→Gtg and gac→gaA   | Met→Val and Asp→Asp        |                                                 |
| embB306 and embB354 | G inserts between AT and gac→gCc | frameshift and Asp→Ala | 1 (0.7)                                         |
| embB306 and embB406 | atg→Gtg and ggc→gCc   | Met→Val and Gly→Ala        | 1 (0.7)                                         |
| embB306 and embB424 | atg→Gtg and cgg→cga   | Met→Val and Arg→Arg        | 1 (0.7)                                         |
| embB306 and embB497 and embB304 | atg→Gtg and cag→cCg and ctg→Ttg | Met→Leu and Gin→Pro and Leu→Leu | 1 (0.7) |
| embB306 and embB497 and embB534 | atg→atA and cag→cCg and gac→gaA | Met→Ile and Gin→Pro and Asp→Asp | 1 (0.7) |
| embB328             | gat→Tat               | Asp→Tyr                    | 1 (0.7)                                         |
| embB330             | ttc→Tcc               | Phe→Ser                    | 1 (0.7)                                         |
| embB354             | gac→Gcc               | Asp→Ala                    | 3 (2.2)                                         |
| embB406             | ggc→Agc               | Gly→Ser                    | 8 (5.8)                                         |
| embB406 and embB534 and embB539 | ggc→Gac and gac→gaA and cgg→cgaA | Gly→Asp and Asp→Asp and Arg→Arg | 1 (0.7) |
| embB439             | gco→Aca               | Ala→Thr                    | 1 (0.7)                                         |
| embB469             | cgt→cAt               | Arg→His                    | 1 (0.7)                                         |
| embB497             | caq→cCc               | Gin→Pro                    | 15 (10.9)                                       |
| embB497 and embB627 | caq→cCg and T inserts between CG | Gin→Arg and frameshift   | 1 (0.7)                                         |
| embB497 and embB8124 | caq→cCg and gac→Aac | Gin→Pro and Asp→Asn      | 1 (0.7)                                         |
| embB508             | gtt→Ttt               | Val→Phe                    | 1 (0.7)                                         |
| embB534             | gac→gaT               | Asp→Asp                    | 2 (1.4)                                         |
| embB534 and embB1000 | gac→gaT and atg→agG | Met→Arg                    | 1 (0.7)                                         |
| embB534 and embB1002 | gac→gaT and cag→cGc | His→Arg                    | 1 (0.7)                                         |
| embB627             | T inserts between CG  | frameshift                  | 2 (1.4)                                         |
| embB651             | aqc→Acc               | Ser→Thr                    | 1 (0.7)                                         |
| embB667             | aco→Aa                | Thr→Lys                    | 1 (0.7)                                         |
| embB8124            | gac→Aac               | Asp→Asn                    | 1 (0.7)                                         |
| Mutant isolates     |                       |                            | 100 (72.5)                                      |
| Wild-type isolates  |                       |                            | 38 (27.5)                                       |

much higher than in the ethambutol-susceptible MDR strains (19.2%, 10/52). While the association between embB306 mutation and ethambutol resistance is statistically significant (odds ratio = 5.1, \( \chi^2 = 16.7, P < 0.0001 \)), our data suggest that embB306 is not the sole causative mutation of ethambutol resistance, but is a sensitive candidate marker for ethambutol resistance analysis. Our finding that embB306 mutations exist in both ethambutol-resistant and -susceptible clinical M. tuberculosis isolates differs from the work of Plinke et al., in which they showed no embB306 mutation in ethambutol-susceptible MDR strains, but agrees with the observations of others. One possible explanation for this inconsistency between phenotypic and genotypic testing results is that embB306 mutations confer M. tuberculosis variable ethambutol MICs and clinical strains with low to moderate levels of resistance may readily show opposite ethambutol susceptibility results using different testing methods. The occurrence of the embB306 mutations has been compared in different lineages and genotypes to identify the association between mutations and genetic structures. However, in MDR strains, it is not well addressed whether the genetic background contributes to the levels of ethambutol resistance in embB306 mutants. In this study, after locating the ethambutol-susceptible isolates with embB306 mutations into the phylogenetic map, we found that
although 80% (8/10) of ethambutol-susceptible $embB306$ mutants niched in a major cluster (indicated as ‘cluster A’ in Figure 1a), the genetic background of these strains was diverse and failed to identify an obvious relationship of any specific genetic lineage with pheno-genotype discordance of $embB306$ mutants (Figure 1a). Interestingly, two pairs of $embB306$ mutants sharing identical VNTR-16 genotyping patterns but with different ethambutol susceptibility for each paired isolates clearly indicates that ethambutol resistance and susceptibility can exist in clinical isolates with highly similar genetic background (Figure 1a). Different types of $embB306$ mutation have been reported to affect the ethambutol MICs for $M.\ tuberculosis$ and the results of ethambutol susceptibility testing. However, the pair 1 isolates (isolate 55 versus isolate 92) both carried a single $embB306$ mutation (ATG$\rightarrow$ATT), of which isolate 92 was ethambutol resistant and isolate 55 was ethambutol susceptible. The pair 2 isolates (isolate 99 versus isolate 100) carried another single $embB306$ mutation (ATG$\rightarrow$ATA), of which isolate 99 was ethambutol resistant while isolate 100 was ethambutol susceptible (Figure 1b). Thus, the pheno-genotype discordance of $embB306$ mutation and ethambutol resistance is not likely related to different types of $embB306$ mutation or multiple mutations of $embB$. In addition, this finding is unlikely related to additional antibiotic resistances, as observed between multidrug resistance and ethambutol resistance, because each of the paired isolates share identical first-line drug resistance patterns (isoniazid/rifampicin resistant for pair 1 and isoniazid/rifampicin/streptomycin resistant for pair 2). The inconsistency between ethambutol DST results and $embB306$ mutation is more likely related to other mutations occurring outside the $embB$ gene in the genome of these clinical strains. Several previous works indicate that ethambutol resistance is a multigene mutation process that requires mutations in the $embB$ gene and other currently unknown loci. Our findings clearly suggest that the determinant of ethambutol resistance may invoke more than one gene variation including $embB$ gene, and that concurrent mutations incident at different sites of the bacterial genome are needed to confer overt ethambutol resistance of $M.\ tuberculosis$.

Interestingly, the ATG$\rightarrow$CTG mutation at $embB306$, which was associated with a higher ethambutol MIC and conferred a growth advantage under sub-MICs of isoniazid or rifampicin, was detected in six strains with quadruple first-line drug resistance (isoniazid/rifampicin/ethambutol/streptomycin resistant) in our study (Table S1, available as Supplementary data at JAC Online) (Figure 1c).

**Characteristics of $embB497$ and $embB406$ mutations**

The major $embB$ mutations detected in our isolates include not only $embB306$, but $embB497$ and $embB406$ as well. Mutations in $embB497$ were found in 16/86 (18.6%) ethambutol-resistant strains and 3/52 (5.8%) ethambutol-susceptible strains. Seven of 86 (8.1%) ethambutol-resistant strains and 3/52 (5.8%) ethambutol-susceptible isolates carried an $embB406$ mutation. However, a low frequency of mutations $embB497$ and $embB406$ (≤6%) has been reported in ethambutol-resistant strains by a limited number of studies. A recent study, which analysed all variations in the entire $embCAB$ operon of ethambutol-resistant isolates without an $embB306$ mutation, showed that 10/34 (29.4%) non-$embB306$-mutated ethambutol-resistant isolates carried $embB497$ mutations and 8/34 (23.5%) carried $embB406$ mutations, while our results showed 14/39 (35.9%) and 6/39 (15.4%), respectively. The high proportion of $embB497$ and $embB406$ mutations that occurred in ethambutol-resistant isolates lacking an $embB306$ mutation in our study indicates the importance of $embB497$ and $embB406$ mutations as additional hotspots to $embB306$ for the rapid detection of ethambutol resistance using molecular assays, especially in Henan, China. Although $embB406$ mutations had been detected in ethambutol-susceptible isolates in two previous studies, $embB497$ mutations have not been found in ethambutol-susceptible strains until now. The reason may be that $embB497$ mutations are located out of the common region for detecting $embB306$ mutations and such regions were not examined by many existing studies.

Similar to the finding in $embB306$ mutants, discordant ethambutol susceptibility of $embB497$ mutants with identical genetic background was also observed. Pair 3 isolates (isolate 21 versus isolate 108) sharing an identical VNTR-16 genotype and sole $embB497$ mutation (CAG$\rightarrow$CGG) showed different drug susceptibility; isolate 21 is ethambutol resistant while isolate 108 is ethambutol susceptible (Figure 1a and b). This result reveals that the contradiction between ethambutol drug susceptibility and $embB497$ mutation testing results is probably related to other mutations occurring outside the $embB$ gene in the genome of $embB497$ mutants, and provides more evidence that the development of full ethambutol resistance may require certain mutations occurring at multiple genes and $embB497$ is one such mutation site.

**$embB$ mutations and broad drug resistance**

We observed that $embB$ mutations among clinical MDR-TB strains from Henan are common, with 100/138 (72.5%) of MDR-TB isolates carrying at least one mutation. To determine whether these 27 mutation types of $embB$ are specific to drug resistance, 22 pan-susceptible clinical $M.\ tuberculosis$ isolates from Henan patients were adopted as controls for the rapid detection of ethambutol resistance using molecular assays, especially in Henan, China. Although $embB406$ mutations had been detected in ethambutol-susceptible isolates in two previous studies, $embB497$ mutations have not been found in ethambutol-susceptible strains until now. The reason may be that $embB497$ mutations are located out of the common region for detecting $embB306$ mutations and such regions were not examined by many existing studies.

Similar to the finding in $embB306$ mutants, discordant ethambutol susceptibility of $embB497$ mutants with identical genetic background was also observed. Pair 3 isolates (isolate 21 versus isolate 108) sharing an identical VNTR-16 genotype and sole $embB497$ mutation (CAG$\rightarrow$CGG) showed different drug susceptibility; isolate 21 is ethambutol resistant while isolate 108 is ethambutol susceptible (Figure 1a and b). This result reveals that the contradiction between ethambutol drug susceptibility and $embB497$ mutation testing results is probably related to other mutations occurring outside the $embB$ gene in the genome of $embB497$ mutants, and provides more evidence that the development of full ethambutol resistance may require certain mutations occurring at multiple genes and $embB497$ is one such mutation site.

**Characteristics of $embB497$ and $embB406$ mutations**

The major $embB$ mutations detected in our isolates include not only $embB306$, but $embB497$ and $embB406$ as well. Mutations in $embB497$ were found in 16/86 (18.6%) ethambutol-resistant strains and 3/52 (5.8%) ethambutol-susceptible strains. Seven of 86 (8.1%) ethambutol-resistant strains and 3/52 (5.8%) ethambutol-susceptible isolates carried an $embB406$ mutation. However, a low frequency of mutations $embB497$ and $embB406$ (≤6%) has been reported in ethambutol-resistant strains by a limited number of studies. A recent study, which analysed all variations in the entire $embCAB$ operon of ethambutol-resistant isolates without an $embB306$ mutation, showed that 10/34 (29.4%) non-$embB306$-mutated ethambutol-resistant isolates carried $embB497$ mutations and 8/34 (23.5%) carried $embB406$ mutations, while our results showed 14/39 (35.9%) and 6/39 (15.4%), respectively. The high proportion of $embB497$ and $embB406$ mutations that occurred in ethambutol-resistant isolates lacking an $embB306$ mutation in our study indicates the importance of $embB497$ and $embB406$ mutations as additional hotspots to $embB306$ for the rapid detection of ethambutol resistance using molecular assays, especially in Henan, China. Although $embB406$ mutations had been detected in ethambutol-susceptible isolates in two previous studies, $embB497$ mutations have not been found in ethambutol-susceptible strains until now. The reason may be that $embB497$ mutations are located out of the common region for detecting $embB306$ mutations and such regions were not examined by many existing studies.

Similar to the finding in $embB306$ mutants, discordant ethambutol susceptibility of $embB497$ mutants with identical genetic background was also observed. Pair 3 isolates (isolate 21 versus isolate 108) sharing an identical VNTR-16 genotype and sole $embB497$ mutation (CAG$\rightarrow$CGG) showed different drug susceptibility; isolate 21 is ethambutol resistant while isolate 108 is ethambutol susceptible (Figure 1a and b). This result reveals that the contradiction between ethambutol drug susceptibility and $embB497$ mutation testing results is probably related to other mutations occurring outside the $embB$ gene in the genome of $embB497$ mutants, and provides more evidence that the development of full ethambutol resistance may require certain mutations occurring at multiple genes and $embB497$ is one such mutation site.
To assess the possibility of using only embB mutations to predict drug resistance, we performed trend analysis correlating any embB mutation and the number of first-line drug resistances. The statistically significant association ($\chi^2$ for trend = 26.5, $P<0.0001$) between embB mutations and resistance to increasing numbers of antituberculosis reagents (33.3% (3/9) of two-drug-resistant isolates (isoniazid/rifampicin resistant) carried embB mutations, 53.8% (28/52) of three-drug-resistant isolates (isoniazid/rifampicin/streptomycin resistant or isoniazid/rifampicin/ethambutol resistant) carried embB mutations and 89.6% (69/77) of four-drug-resistant isolates (isoniazid/rifampicin/ethambutol/streptomycin resistant) carried embB mutations) strongly suggests that embB mutations might be sensitive candidate markers for the prediction of concurrent resistance to isoniazid, rifampicin, streptomycin and ethambutol in M. tuberculosis clinical isolates. Moreover, 83.1% of these four drug-resistant isolates can be detected by sequencing a short fragment (606 bp) of the embB gene, which covers 10 mutation sites at codons 306, 328, 330, 354, 406, 424, 439, 469, 497 and 508. While it is yet unproven why mutations affecting embB-encoded arabinosyl transferases may predict resistance to other drugs, this study supports the findings of others that embB306 is a predictor of multidrug resistance.12,15,24 In addition, our findings may lead to the development of a simple molecular test to rapidly identify isolates that are likely to express resistance to four first-line drugs in Henan, China, based on only a limited number of embB mutations. Further studies would be required to examine such a strategy in clinical practice and in other geographic locations around the world.

In conclusion, our results demonstrated the mutation characteristics of the entire embB gene in clinical MDR-TB isolates, an undertaking not previously reported from China. Our results clearly suggest that the ethambutol resistance of M. tuberculosis depends on concurrent multiple mutations in the genome and that embB mutations alone are not sufficient for the development of full resistance to ethambutol. The three pairs of clinical isolates identified with identical VNTR genotypes and matched embB mutations but different ethambutol phenotypic susceptibility warrants further study. Using genome-wide single nucleotide polymorphism analysis of the three pairs of isolates, novel mutations contributing to ethambutol resistance are likely to be discovered.

**Supplementary data**

Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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