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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 *embB*306 mutants and 11.5% of *embB*406 or *embB*497 mutants were ethambutol susceptible. Among 39 ethambutol-resistant strains without *embB*306 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 *embB*406 and *embB*497 as hotspots, in addition to *embB*306, 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, *embB*306, *embB*497, ethambutol

Introduction

Multidrug-resistant (MDR) tuberculosis (TB) has an estimated 4.8% prevalence worldwide and poses a serious threat to global public health.[1] In China, the significantly high (9.3%) prevalence of MDR-TB makes the prevention and control of tuberculosis especially challenging.[2] 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.[3] 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.[4] Exchanging wild-type *embB*306, *embB*497 and *embB*406 with mutant codons increases the ethambutol minimal inhibition concentrations (MICs) of *Mycobacterium tuberculosis*.[5–7] Mutations at *embB*320 and *embB*324,[5,7] as well as mutations at *embB*397, *embB*445, *embB*1024 and *embC*13, have also been found to be associated with ethambutol resistance.[5]

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.[4,8–11] 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.[10] However, the detection of *embB*306 from ethambutol-susceptible clinical isolates questions the validity of this assertion.[12–15] Mokrousov *et al.*[14] 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.*[12] suggested that the *embB*306 mutation is associated with broad drug resistance rather than ethambutol resistance *per se*. Shen *et al.*[15] 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 laboratorial isolates,[5,6,9,16,17] 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,[18–20] including ethambutol resistance,[5] 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.[21,22]

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

DTM-PCR was performed to identify the Beijing family strains.[23] 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.[24,25] 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-VNTR*plus* (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.[26,27]

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.[28] 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*1-1 (5′-TCGACGATCGCCACGTACCT-3′) and *embB*1-2 (5′-CAGCAGCAGCCAGCA CACTA-3′); *embB*2-1 (5′-TATTCGGCTT CTGCTCTGG-3′) and *embB*2-2 (5′-CACACCGTAGCTGGAGACAT-3′); and *embB*3-1 (5′-GTTCCTGGC GGCGTTATTCT-3′) and *embB*3-2 (5′-AGCCTG ACGCTATGGACCAA-3′). The sequencing primers were *embB*(S)1 (5′-CGTCCTTGCCTTGCTTGGGT-3′) and *embB*(S)3 (5′-GCGTGGTATCTCCTGCCTAAG-3′); other sequencing primers were the same as *embB*1-2, *embB*2-1, *embB*2-2 and *embB*3-1. 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 association analysis were performed using SPSS for Windows® (version 10.0, SPSS, Inc., USA).

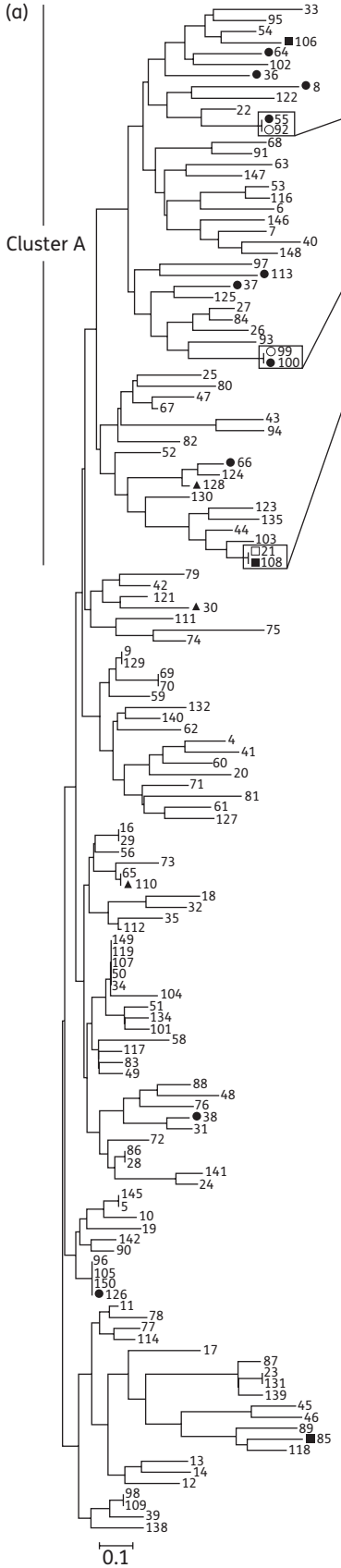
Results and discussion

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

(a)



(b)

ID	VNTR-9 genotyping									VNTR-9 result	Amino acid change	Nucleotide change	Drug resistance
55	1	2	3	3	2	5	4	2	4	C	Met306Ile	ATG→ATT	HRS
92	1	2	3	3	2	5	4	2	4		Met306Ile	ATG→ATT	HRES
99	1	2	3	3	4	5	4	2	4	C	Met306Ile	ATG→ATA	HRE
100	1	2	3	3	4	5	4	2	4		Met306Ile	ATG→ATA	HR
21	3	2	3	3	3	6	1	1	4	C	Gln497Arg	CAG→CGG	HRES
108	3	2	3	3	3	6	1	1	4		Gln497Arg	CAG→CGG	HRS
9	3	2	3	3	2	5	4	2	4	C	—	—	HRS
129	3	2	3	3	2	5	4	2	4		Gln497Arg	CAG→CGG	HRE
69	2	2	3	3	2	5	4	2	4	U	Met306Ile, Gln497Pro	ATG→ATA, CAG→CCG	HRE
70	5	2	2	3	2	5	4	1	4	U	Gln497Arg	CAG→CGG	HRES
16	4	2	3	3	2	5	4	2	4	U	Gly406Ala	GGC→GCC	HRES
29	3	2	3	3	2	5	4	2	2	U	Met306Val, Gly406Ala	ATG→GTG, GGC→GCC	HRES
65	3	2	3	3	2	5	4	2	4	C	—	—	HRS
110	3	2	3	3	2	5	4	2	4		Gly406Asp	GGC→GAC	HRS
149	3	2	3	3	2	5	4	0	5	U	Met306Ile	ATG→ATA	HRES
119	3	2	3	3	2	5	4	2	4	U	Met306Ile	ATG→ATT	HRES
107	3	2	3	3	2	4	4	2	4	U	—	—	HRS
50	2	2	3	3	2	5	4	2	4	U	Met306Ile	ATG→ATA	HRES
34	2	2	3	3	2	5	4	1	4	U	Met306Val	ATG→GTG	HRES
86	3	2	3	3	2	5	4	2	4	C	Met306Val	ATG→GTG	HRES
28	3	2	3	3	2	5	4	2	4		—	—	HRE
145	3	2	3	3	2	5	4	2	4	U	—	—	HRS
5	2	2	3	3	2	5	4	2	4	U	Met306Ile	ATG→ATC	HRES
96	2	2	3	3	2	5	4	2	4	C	Met306Ile	ATG→ATC	HRES
105	2	2	3	3	2	5	4	2	4		Met306Val	ATG→GTG	HRE
150	3	2	3	3	2	5	4	2	4	U	—	—	HRS
126	3	2	3	3	2	5	2	2	4	U	Met306Ile	ATG→ATA	HRS
23	3	2	3	3	2	5	4	2	4	C	Met306Leu	ATG→CTG	HRES
131	3	2	3	3	2	5	4	2	4		Met306Leu	ATG→CTG	HRES
98	3	2	3	3	2	5	4	2	4	U	Met306Val	ATG→GTG	HRES
109	3	2	3	3	2	5	3	2	4	U	—	—	HR

(c)

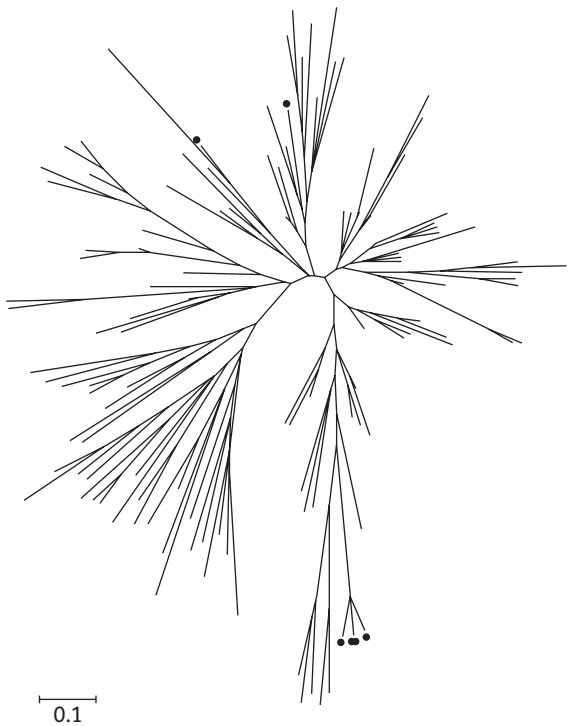


Table 1. Mutation pattern of the *embB* gene in isolates with different phenotypes

Locus	Number of mutations						
	ethambutol-susceptible MDR			ethambutol-resistant MDR			
	isoniazid/ rifampicin	isoniazid/rifampicin/ streptomycin	total ethambutol susceptible	isoniazid/ rifampicin/ ethambutol	isoniazid/rifampicin/ ethambutol/ streptomycin	total ethambutol resistant	pan-susceptible
*embB*306	3	7	10	4	43	47	0
*embB*497		3	3	3	13	16	0
*embB*406		3	3	1	6	7	0
*embB*354		2	2		2	2	0
*embB*534[a]		1	1	3	8	11	1
*embB*304[a]			0		1	1	0
*embB*328			0		1	1	0
*embB*330			0		1	1	0
*embB*424[a]			0		1	1	0
*embB*439			0		1	1	0
*embB*469		1	1			0	0
*embB*508		1	1			0	0
*embB*539[a]			0	1		1	0
*embB*627			0	1	2	3	0
*embB*651		1	1			0	0
*embB*667		1	1			0	0
*embB*1000			0		1	1	0
*embB*1002			0		1	1	0
*embB*1024		2	2			0	0
Total	3	22	25	13	81	94	1

[a]Synonymous mutations.

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 (Table 2). There were three isolates that carried triple *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 (*embB*306 plus *embB*406; *embB*306 plus *embB*497), which has not been documented in previous studies.[8,10,29]

Characteristics of embB306 mutation

One of the major mutations was *embB*306, accounting for the highest proportion of all mutations detected in our study. Altogether, 41.3% (57/138) of MDR-TB isolates carried the *embB*306 mutation. The proportion of *embB*306 mutants among ethambutol-resistant MDR strains (54.7%, 47/86) was

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 *embB*306 mutants, while open circles indicate ethambutol-resistant counterparts. Filled squares indicate ethambutol-susceptible *embB*497 mutants, while open squares indicate ethambutol-resistant *embB*497 counterparts. Filled triangles indicate ethambutol-susceptible *embB*406 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 and *embB* mutations, 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)
*embB*306	atg→Ctg	Met→Leu	46 (33.3)
	atg→Gtg	Met→Val	
	atg→atA	Met→Ile	
	atg→atC	Met→Ile	
	atg→atT	Met→Ile	
*embB*306 and *embB*534	atg→atC and gac→gaT	Met→Ile and Asp→Asp	6 (4.3)
	atg→Gtg and gac→gaT	Met→Val and Asp→Asp	
*embB*306 and *embB*354	G inserts between AT and gac→gCc	frameshift and Asp→Ala	1 (0.7)
*embB*306 and *embB*406	atg→Gtg and ggc→gCc	Met→Val and Gly→Ala	1 (0.7)
*embB*306 and *embB*424	atg→Gtg and cgg→cgA	Met→Val and Arg→Arg	1 (0.7)
*embB*306 and *embB*497 and *embB*304	atg→Ctg and cag→cCg and ctg→Ttg	Met→Leu and Gln→Pro and Leu→Leu	1 (0.7)
*embB*306 and *embB*497 and *embB*534	atg→atA and cag→cCg and gac→gaT	Met→Ile and Gln→Pro and Asp→Asp	1 (0.7)
*embB*328	gat→Tat	Asp→Tyr	1 (0.7)
*embB*330	ttc→tCc	Phe→Ser	1 (0.7)
*embB*354	gac→gCc	Asp→Ala	3 (2.2)
*embB*406	ggc→Agc	Gly→Ser	8 (5.8)
	ggc→gAc	Gly→Asp	
	ggc→gCc	Gly→Ala	
*embB*406 and *embB*534 and *embB*539	ggc→gAc and gac→gaT and cgg→cgA	Gly→Asp and Asp→Asp and Arg→Arg	1 (0.7)
*embB*439	gca→Aca	Ala→Thr	1 (0.7)
*embB*469	cgt→cAt	Arg→His	1 (0.7)
*embB*497	cag→cCg	Gln→Pro	15 (10.9)
	cag→cGg	Gln→Arg	
*embB*497 and *embB*627	cag→cGg and T inserts between CG	Gln→Arg and frameshift	1 (0.7)
*embB*497 and *embB*1024	cag→cCg and gac→Aac	Gln→Pro and Asp→Asn	1 (0.7)
*embB*508	gtt→Ttt	Val→Phe	1 (0.7)
*embB*534	gac→gaT	Asp→Asp	2 (1.4)
*embB*534 and *embB*1000	gac→gaT and atg→aGg	Met→Arg	1 (0.7)
*embB*534 and *embB*1002	gac→gaT and cac→cGc	His→Arg	1 (0.7)
*embB*627	T inserts between CG	frameshift	2 (1.4)
*embB*651	agc→aCc	Ser→Thr	1 (0.7)
*embB*667	aca→aAa	Thr→Lys	1 (0.7)
*embB*1024	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 *embB*306 mutation and ethambutol resistance is statistically significant (odds ratio=5.1, $\chi^2=16.7$, $P<0.0001$), our data suggest that *embB*306 is not the sole causative mutation of ethambutol resistance, but is a sensitive candidate marker for ethambutol resistance analysis. Our finding that *embB*306 mutations exist in both ethambutol-resistant and -susceptible clinical *M. tuberculosis* isolates differs from the work of Plinke *et al.*,[9] in which they showed no *embB*306 mutation in ethambutol-susceptible MDR strains, but agrees with the observations of others.[12,15] One possible explanation for this inconsistency between phenotypic and genotypic testing results is that *embB*306 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.[6,16,17,30] The occurrence of the *embB*306 mutations has been compared in different lineages and genotypes to identify the association between mutations and genetic structures.[12,14] However, in MDR strains, it is not well addressed whether the genetic background contributes to the levels of ethambutol resistance in *embB*306 mutants. In this study, after locating the ethambutol-susceptible isolates with *embB*306 mutations into the phylogenetic map, we found that

although 80% (8/10) of ethambutol-susceptible *embB*306 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 *embB*306 mutants (Figure 1a). Interestingly, two pairs of *embB*306 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 *embB*306 mutation have been reported to affect the ethambutol MICs for *M. tuberculosis* and the results of ethambutol susceptibility testing.[6,10,17] However, the pair 1 isolates (isolate 55 versus isolate 92) both carried a single *embB*306 mutation (ATG→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 *embB*306 mutation (ATG→ATA), of which isolate 99 was ethambutol resistant while isolate 100 was ethambutol susceptible (Figure 1b). Thus, the pheno-genotype discordance of *embB*306 mutation and ethambutol resistance is not likely related to different types of *embB*306 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,[15] 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 *embB*306 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.[5,16] 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→CTG mutation at *embB*306, which was associated with a higher ethambutol MIC and conferred a growth advantage under sub-MICs of isoniazid or rifampicin,[6,10] 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 *embB*306, but *embB*497 and *embB*406 as well. Mutations in *embB*497 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 *embB*406 mutation. However, a low frequency of mutations *embB*497 and *embB*406 (≤6%) has been reported in ethambutol-resistant strains by a limited number of studies.[8,10,31] A recent study, which analysed all variations in the entire *embCAB* operon of ethambutol-resistant isolates without an *embB*306 mutation, showed that 10/34 (29.4%) non-*embB*306-mutated ethambutol-resistant isolates carried *embB*497 mutations and 8/34 (23.5%) carried *embB*406 mutations,[29] while our results showed 14/39 (35.9%) and 6/39 (15.4%), respectively. The high proportion of *embB*497 and *embB*406 mutations that occurred in ethambutol-resistant isolates lacking an *embB*306 mutation in our study indicates the importance of *embB*497 and *embB*406 mutations as additional hotspots to *embB*306 for the rapid detection of ethambutol resistance using molecular assays, especially in Henan, China. Although *embB*406 mutations had been detected in ethambutol-susceptible isolates in two previous studies,[13,31] *embB*497 mutations have not been found in ethambutol-susceptible strains until now.[8,10,13,29,32] The reason may be that *embB*497 mutations are located out of the common region for detecting *embB*306 mutations and such regions were not examined by many existing studies.

Similar to the finding in *embB*306 mutants, discordant ethambutol susceptibility of *embB*497 mutants with identical genetic background was also observed. Pair 3 isolates (isolate 21 versus isolate 108) sharing an identical VNTR-16 genotype and sole *embB*497 mutation (CAG→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 *embB*497 mutation testing results is probably related to other mutations occurring outside the *embB* gene in the genome of *embB*497 mutants, and provides more evidence that the development of full ethambutol resistance may require certain mutations occurring at multiple genes and *embB*497 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 *embB* coding region analysis. Among 22 pan-susceptible clinical MDR-TB isolates, a single mutation at *embB*534 was detected in one strain, which indicates that *embB*534 is not a specific mutation in drug-resistant *M. tuberculosis*. Nonetheless, among the 12 *embB*534 MDR mutants, only 2 carried a single *embB*534 mutation; 10 of them had additional *embB* mutations (Table 2). Regardless of the *embB*534 mutation, the percentage of *embB* mutants among MDR-TB isolates is 71.0% (98/138), of which 58.2% (57/98) harboured *embB*306 mutations. Our results suggest that excluding *embB*534 mutations, all other *embB*-specific mutations in our patient population (and not simply limited to the most frequently documented *embB*306 mutation) may be candidate markers for the prediction of multi-drug resistance and broad first-line polydrug resistance.

Existing commercial line probe assays are directed at detecting rifampicin or rifampicin and isoniazid mutations that are associated with phenotypic resistance. A rapid molecular assay that could predict polyresistance beyond MDR would be of significant utility to the diagnostic laboratory. Several studies show that the combination of a limited number of mutation sites can be applied to predict the drug resistance of *M.*

tuberculosis.[24,33] 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 (χ^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 *embB*306 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.

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Transparency declarations

None to declare.

Supplementary data

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

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