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Ethambutol Resistance as Determined by Broth Dilution Method Correlates Better than Sequencing Results with *embB* Mutations in Multidrug-Resistant *Mycobacterium tuberculosis* Isolates

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We evaluated the correlation of phenotypic ethambutol (EMB) susceptibility as determined by two drug susceptibility methods with *embB* mutations in multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains. The concordance rate for EMB resistance between broth dilution method and sequencing results (83.6%) was significantly higher than between the proportion method and sequencing results (61.7%) ($P = 0.004$). Of the *embB* mutants, 75.4% (46/61) possessed a mutation at *embB306*. Our results demonstrated that ethambutol resistance determined by broth dilution method reveals better correlation with *embB* mutations than the proportion method in MDR isolates.

The emergence of multidrug-resistant (MDR) tuberculosis (TB), defined as tuberculosis that is resistant to at least isoniazid (INH) and rifampin (RFP), is a serious threat to global tuberculosis control (1). China is considered one of the global hotspots of MDR-TB, with an estimated 110,000 MDR-TB cases each year (2). There is an urgent demand for accurate and rapid drug susceptibility testing (DST) to help develop efficient anti-TB drug regimens for appropriate treatment of individual cases (3).

Ethambutol (EMB), one of the key first-line antimicrobial agents for the treatment of tuberculosis (4), plays an important role in the chemotherapy of drug-resistant TB, including MDR-TB (5). EMB inhibits arabinosyl transferases, encoded by the *embCAB* operon, thereby inhibiting biosynthesis of the cell well components arabinogalactan and lipoarabinomannan (6). Previous studies showed that mutations in the *embB* gene are mainly observed in clinical EMB-resistant isolates (7, 8). Of the various mutations of *embB*, mutations at codon 306 are the most commonly detected point mutations conferring EMB resistance, which have been suggested as important molecular indicators for rapid detection of EMB-resistant *Mycobacterium tuberculosis* isolates (9). In contrast, mutations in *emb306* had also been observed among EMB-susceptible *M. tuberculosis* isolates (9). Previous studies have shown that a lack of consistency in conventional DST results for EMB is an inevitable problem in the tuberculosis laboratories (3, 10). It is therefore meaningful to investigate the concordance between phenotypic and genotypic EMB resistance testing. In this study, we screened for *embB* mutations among MDR strains isolated in China in order to assess and compare the correlation of phenotypic EMB susceptibilities determined by two DST methods with *embB* gene mutations among MDR strains.

A total of 158 MDR isolates were collected from a national drug resistance survey of China. Drug susceptibility testing for EMB was performed by both the conventional proportion method recommended by World Health Organization and the broth dilution method according to previous reports (11, 12). For the proportion method, the concentration of EMB in Lowenstein-Jensen (LJ) medium was 2 μg/ml. For the broth dilution method, EMB concentrations were doubling dilutions from 0.125 to 32 μg/ml, and the breakpoint MIC for EMB was taken as 5 μg/ml (12). In addition, a 800-bp fragment of *embB* containing the EMB resistance-determining region was amplified by PCR using the primers *embB*-forward (5′-GGTGA TATTCGGCTTCCT-3′) and *embB*-reverse (5′-ATAGCGCGGTGA TCAAAAAG-3′) as previously reported (4).

Of 158 MDR isolates, 81(51.3%) and 61(38.6%) were identified as EMB resistant by the conventional proportion and MIC methods, respectively. For the 81 strains determined to be EMB resistant by the conventional proportion method, 61.7% (50/81) showed a potential relationship between phenotypic EMB resistance and *embB* mutation. On the other hand, 11 (14.3%) of 77 phenotypically EMB-susceptible *M. tuberculosis* strains harbored *embB* mutations. For the MIC method, 51 (83.6%) of 61 EMB-resistant *M. tuberculosis* strains revealed various *embB* mutations, and *embB* mutations were observed in 10 of 97 (10.3%) EMB-susceptible strains. When the two phenotypic resistance detection methods were compared with genotypic resistance detection, the concordance rate for EMB resistance between the MIC method and sequencing results was significantly higher than that for the proportion method ($P = 0.004$). For EMB-susceptible strains, the concordance rates did not show a significant difference between these groups ($P = 0.424$). When results of the two phenotypic detection methods, MIC and proportion methods, were compared with *embB* sequencing results, the overall concordance rates were 87.3% and 76.6%, respectively, and this difference was statistically highly significant ($P = 0.002$) (Table 1).

Of the mutants with detectable *embB* mutations, 75.4% (46/

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TABLE 1 Correlation of phenotypic ethambutol susceptibility with *embB* mutations in MDR TB strains

DST method and result[a]	No. of isolates with sequencing result[b]		Concordance rate (%)[c]		
	R	S	For EMB-resistant strains	For EMB-susceptible strains	Overall
Proportional			61.7	85.7	76.6
R	50	31			
S	11	66			
MIC			83.6	89.7	87.3
R	51	10			
S	10	87			

[a] R, resistant; S, susceptible.
[b] R, having a mutation in the EMB resistance-determining region of *embB*; S, having a wild-type locus.
[c] Concordance rates were determined on the basis of using phenotypic DST as the standard.

61) possessed a mutation at *embB306*. The most common *embB* mutations were ATG→GTG (Met306Val; 52.2% [24/46]) and ATG→ATA (Met306Ile; 43.5% [20/46]). Among 46 MDR isolates with *embB306* mutations, 7 (15.2%) showed MICs lower than 5 μg/ml, i.e., ranging from 1 μg/ml to 4 μg/ml. The other 8 mutations detected were at codons 328 (4/61; 6.6%), 406 (4/61; 6.6%), and 497 (6/61; 9.8%) of *embB*. In addition, we identified novel *embB* mutations at codon 246. However, this new mutant type was found in only one EMB-susceptible *M. tuberculosis* isolate with a MIC of 0.5 μg/ml, indicating that it might be a single nucleotide polymorphism that did not confer EMB resistance (Table 2).

We also analyzed the isolates that showed discordant phenotypic DST results. As shown in Table 3, a total of 30 isolates had type I discordancy (resistant by the proportion method but susceptible by the MIC method), including 8 (26.7%) with *embB* mutations and 22 (73.3%) without detectable *embB* mutations. Among these type I isolates, 36.7% (11/30) showed an EMB MIC of 4 μg/ml, which was close to the breakpoint determined by the MIC method. In contrast, 90% of isolates with type II discordancy (susceptible by the proportion method but resistant by the MIC method) harbored *embB* mutations. It is noteworthy that 70.0% (7/10) of type II isolates had an EMB MIC of 8 μg/ml, which is close to the breakpoint MIC definition of EMB resistance (Table 3).

Mycobacterial drug susceptibility testing is still widely used as a routine tool for the generation of effective anti-TB regimens, especially in management of MDR-TB patients (3). However, due to the lack of consistency in conventional phenotypic DST results for EMB, it is presently not recommended that EMB DST results be used in the design of individualized treatment for MDR-TB (10). Consequently, there is an urgent need to establish a reliable method for detecting drug susceptibility of *M. tuberculosis* isolates. At the genetic level, *embB* mutations, especially *embB306* mutations, are believed to be a major cause of EMB resistance in *M. tuberculosis*, while several studies revealed that *embB* mutations had been observed in both EMB-resistant and EMB-susceptible *M. tuberculosis* strains (5, 9). Because conventional culture-based phenotypic methods of EMB susceptibility testing are notoriously problematic, the presumably inaccurate conventional drug susceptibility results may be the major cause of the "EMB-susceptible" isolates that harbored *embB* mutations (13, 14). Similarly, the problems of conventional proportion susceptibility were most likely responsible for the discrepancies between molecular and phenotypic EMB resistance test results (15). Here, our results demonstrate that ethambutol resistance determined by the broth microdilution MIC method revealed better correlation with

TABLE 2 MIC distribution of *embB* mutants among MDR TB isolates

Locus	Nucleotide substitution	Amino acid substitution	No. of isolates	No. of isolates with MIC (μg/ml) of:								
				0.25	0.5	1	2	4	8	16	32	>32
embB306	ATG→GTG	Met→Val	24				1		14	3	1	5
	ATG→CTG	Met→Leu	1				1					
	ATG→ATA	Met→Ile	20			1	2	1	9	3	1	3
	ATG→ATT	Met→Ile	1				1					
embB328	GAT→CAT	Asp→His	1						1			
	GAT→TAT	Asp→Tyr	2						1			1
	GAT→GGT	Asp→Gly	1							1		
emb406	GGC→AGC	Gly→Ser	1						1			
	GGC→GCC	Gly→Ala	2					1	1			
	GGC→GAC	Gly→Asp	1					1				
emb497	CAG→CCG	Gln→Arg	5							2		3
	CAG→CGG	Gln→Pro	1						1			
embB246	GGC→CGC	Gly→Arg	1		1							
Mutant			61	0	1	1	5	3	28	9	2	12
Wild type			97	2	16	20	23	26	5	0	1	4
Total			158	2	17	21	28	29	33	9	3	16

TABLE 3 Analysis of strains showing discordant DST results between the conventional proportion and broth microdilution MIC methods

Discordancy[a]	Mutation status	No. of isolates (%)	No. of isolates with MIC (μg/ml) of:							
			0.5	1	2	4	8	16	32	>32
Type I (R by proportion method, S by MIC method)	With mutation	8 (26.7)	1		4	3				
	Without mutation	22 (73.3)		5	9	8				
	Total	30 (100.0)	1	5	13	11				
Type II (S by proportion method, R by MIC method)	With mutation	9 (90.0)					6	1	1	1
	Without mutation	1 (10.0)					1			
	Total	10 (100.0)					7	1	1	1

[a] R, resistant; S, susceptible.

embB mutations in MDR-TB isolates. In addition to ethambutol, isoniazid resistance defined by the MIC method has also been shown to have better correlation with the sequencing results than that defined by the proportional method (16). Our data indicate that the microdilution MIC method may be more specific for identifying the EMB resistance than the conventional proportion method in MDR-TB isolates. Considering that the proportional method may still be the gold standard recommended by the WHO (12), our study highlights the requirement for revision of the WHO guideline for the EMB resistance assay. In particular, the present rather low critical concentration for defining EMB resistance should be addressed.

In our study, the Met306Val and Met306Leu amino acid substitutions encoded by *embB306* were associated with high-level resistance to EMB, while the mutants with Met306Ile mutations had MICs that were modestly higher than the critical concentration. These can easily result in false-susceptible EMB testing results (14). We found that 57.1% (4/7) of isolates with MICs lower than 5 μg/ml carried the Met306Ile substitution. Interestingly, mutations within the locus *embB328*, which is a rare codon site conferring EMB resistance, according to previous reports (17, 18), were identified among 4 of our EMB-resistant isolates. Our findings therefore suggest that *embB328* may be more frequently associated with MDR *M. tuberculosis* isolates than non-MDR isolates in China. Further studies are needed to compare the distribution of different *embB* mutant types between MDR and non-MDR isolates. In this study, we also identified 4 isolates lacking the *embB* mutations but showing high MICs (>32 μg/ml) in phenotypic DST, indicating that an undiscovered mechanism may contribute to EMB resistance in *M. tuberculosis*.

In conclusion, our results demonstrated that EMB resistance determined by the broth dilution MIC method showed better correlation with *embB* mutations among MDR-TB isolates than the proportion method. Due to the present limitation of the conventional proportion drug susceptibility testing method, the microbroth dilution MIC method may serve as a better technique than the proportion method for detecting *in vitro* EMB resistance in MDR-TB isolates.

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