Correlation of the phenotypic ethambutol susceptibility of *Mycobacterium tuberculosis* with *embB* gene mutations in Korea

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The phenotypic resistance to ethambutol (EMB) in *Mycobacterium tuberculosis* with *embB* gene mutations is still unclear. This study was designed to better understand EMB resistance due to *embB* gene mutation. Sequencing analysis of the *embB* gene was performed for 124 EMB-susceptible and 93 EMB-resistant *M. tuberculosis* strains isolated from South Korea. The MIC was determined for EMB-susceptible *M. tuberculosis* strains with the *embB* mutation and wild-type on Löwenstein–Jensen (LJ) solid medium in duplicate. Two (2.8%) of 72 pan-susceptible, two (9.1%) of 22 any-drug-resistant but EMB-susceptible, nine (30.0%) of 30 multidrug-resistant (MDR) but EMB-susceptible and 84 (90.3%) of 93 EMB-resistant *M. tuberculosis* strains possessed *embB* mutations at various codons including 306, 319, 354, 360, 399, 405, 406, 459 and 497. Strains with *embB* mutations at codons 306, 354, 399, 405 and 497 had highly pronounced EMB resistance, while strains with mutations at codons 319 and 406 mutations were moderately resistant and those with an *embB*459 mutation were EMB-susceptible at the critical concentration ($2.0 \mu\text{g ml}^{-1}$) on LJ solid medium. However, the mean MIC for strains with *embB* mutations ($1.42 \mu\text{g ml}^{-1}$) was higher than that for strains without the *embB* mutation ($1.0 \mu\text{g ml}^{-1}$) in EMB-susceptible *M. tuberculosis* isolates ($P=0.0052$). Three novel *embB* mutations at codons 399, 405 and 459 were identified in this study. These results support the hypothesis that *embB* mutation except for a few specific mutation types may be the main cause of EMB resistance.

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INTRODUCTION

Tuberculosis is a major global health problem (WHO, 2009). The emergence of multidrug-resistant (MDR) tuberculosis and, more recently, of extensively drug-resistant tuberculosis, is widely considered to be a serious threat to global tuberculosis control (CDC, 2006; Migliori *et al.*, 2007; Raviglione & Smith, 2007). The control of tuberculosis remains one of the more elusive goals in medicine. Rapid detection of drug resistance is essential to design appropriate treatment regimens, prevent treatment failure and thus reduce the further spread of drug-resistant isolates.

Ethambutol (EMB) [dextro-2,2'-(ethylenediimino)-di-1-butanol] is a narrow-spectrum antimycobacterial agent that is used for the treatment of tuberculosis. EMB is a first-line anti-tuberculosis agent that is especially important when used in drug combinations to prevent the emergence of drug resistance or to treat single drug-resistant tuberculosis (WHO, 1997). Further, streptomycin

has been replaced by EMB as a key drug in the intensive phase of tuberculosis chemotherapy as it is less expensive and patient compliance is better with this drug (Rabarijaona *et al.*, 1999). This agent has been proposed to be an arabinose analogue; the specific target is likely to be an arabinosyltransferase, presumably a functionally important site. A two-gene locus (*embAB*) that encodes arabinosyltransferase has been studied to elucidate a potential mechanism of EMB resistance (Alcaide *et al.*, 1997; Telenti *et al.*, 1997).

Amino acid replacements at position 306 of *embB* have been shown in many studies to be present in EMB-resistant, but not EMB-susceptible, organisms (Alcaide *et al.*, 1997; Ramaswamy & Musser, 1998; Sreevatsan *et al.*, 1997). However, Mokrousov *et al.* (2002) detected mutations of *embB*306 in both EMB-susceptible and EMB-resistant strains. EMB is an important antimycobacterial drug as it enhances the effect of other companion drugs including aminoglycosides, rifamycins and quinolones. Moreover, this drug has also been shown to significantly decrease the levels of bacteraemia in patients with AIDS (Kemper *et al.*, 1994).

Abbreviations: EMB, ethambutol; MDR, multidrug-resistant.

A supplementary table is available with the online version of this paper.

Consequently, mutations in codon 306 have been suggested as molecular markers for the rapid detection of EMB resistance (Lee *et al.*, 2004; Plinke *et al.*, 2006; Van Rie *et al.*, 2001). Furthermore, two recent studies demonstrated a clear causative relationship between *embB306* point mutations and *in vitro* EMB resistance by allelic-exchange experiments (Safi *et al.*, 2008; Starks *et al.*, 2009). Identification of additional mutations occurring in EMB-resistant organisms will be useful in further understanding the mechanisms of resistance to this primary anti-tuberculosis agent. In our study, we tried to understand EMB resistance in detail by studying its relationship with mutations in the *embB* gene.

METHODS

Strains. The strains were randomly selected from among *M. tuberculosis* isolates from pulmonary tuberculosis patients registered in public health centres of South Korea from 2005 to 2007 that were stored at the Korean Mycobacterial Resource Center (KMRC). We included 72 pan-susceptible, 22 EMB-susceptible with any drug resistance but non-MDR, 30 EMB-susceptible and MDR and 93 EMB-resistant with or without other drug resistances (Supplementary Table S1 in JMM Online). All strains had different IS6110 RFLP types, as performed by a standardized procedure described previously (Park *et al.*, 2000).

Drug susceptibility testing. Phenotypic drug susceptibility testing was performed against isoniazid ($0.2 \mu\text{g ml}^{-1}$), rifampicin ($40 \mu\text{g ml}^{-1}$), streptomycin ($10 \mu\text{g ml}^{-1}$) and EMB ($2 \mu\text{g ml}^{-1}$) in LJ medium using the absolute concentration method (Canetti *et al.*, 1969). The MICs of EMB were compared between pan-susceptible strains and EMB-susceptible but other-drug-resistant *embB* mutant strains.

Selected isolates were inoculated onto LJ medium containing various concentrations of EMB ($0\text{--}32 \mu\text{g ml}^{-1}$). These strains were retested twice to obtain a mean EMB MIC for each strain.

PCR and sequencing. Primers were designed to target an 800 bp region of the *embB* gene encompassing codons 297–497 for analysis. A pair of primers, *embB*-F (5'-GGT GAT ATT CGG CTT CCT-3') and *embB*-R (5'-ATA GCG CGG TGA TCA AAA AG-3'), was designed for the PCR. The *embB306*, -406 and -497 mutations have been found to be a main cause of EMB resistance. This study was focused on 800 bp of the hot spot region and excluded the possible mutations in the remaining region of the 3.3 kb *embB* gene.

In a total volume of 20 μl , PCR mixtures contained 500 mM KCl, 100 mM Tris/HCl (pH 9.0), 1 % Triton X-100, 200 μM deoxynucleoside triphosphates (dATP, dGTP, dTTP and dCTP), 10 pmol of each of the members of the primer pair, 1.5 mM MgCl_2 , 1 U *Taq* DNA polymerase (Qiagen) and 1 μl template DNA. After heating the mixture to 95°C for 7 min, amplification was carried out in 35 sequential cycles with denaturation at 94°C for 30 s, primer annealing at 56°C for 30 s and primer extension at 72°C for 1 min. The last extension step was performed by incubation at 72°C for 7 min. The DNA fragments were routinely examined on agarose gels stained with ethidium bromide. The direct sequencing of the *embB* gene with PCR products was performed using a BigDye Terminator v3.1 Cycle Sequencing kit and analysed with an ABI 3730XL genetic analyser.

Statistical analysis. Statistical analyses were performed using SAS version 9.1.3. We performed the trend analysis using Bartholomew's test, which is a more powerful test than the chi-square test for ordered

data, and also employed the Wilcoxon rank sum test to analyse MIC differences by the *embB* mutation.

RESULTS

Analysis of the *embB* mutation in EMB-susceptible *M. tuberculosis* strains

A total of 124 EMB-susceptible and 93 EMB-resistant *M. tuberculosis* strains were analysed to determine a potential relationship between EMB resistance and *embB* mutation. Two (2.8 %) of 72 pan-susceptible *M. tuberculosis* strains possessed *embB* mutations at codons 360 (silent mutation, Val to Val) and 459 (Gly to Asp) (Table 1). These two strains revealed MICs of $1.25 \mu\text{g ml}^{-1}$ and $0.75 \mu\text{g ml}^{-1}$, which were not higher than the MICs for pan-susceptible *M. tuberculosis* strains without *embB* mutation (Table 2). Two strains (9.1 %) out of 22 any-drug-resistant EMB-susceptible *M. tuberculosis* strains had *embB* mutations (Table 1). These strains were rifampicin mono-resistant with an *embB406* mutation. MICs for these strains were higher than those for pan-susceptible strains (Table 2).

Nine (30.0 %) of 30 MDR but EMB-susceptible *M. tuberculosis* strains revealed various *embB* mutations such as Met306Ile, Met306Val, Tyr319Ser, Gly406Asp, Gly406Ala and Gln497Lys (Table 2). Only one strain (3.8 %) was EMB-susceptible out of 26 strains with Met306Ile mutation (Table 1). Two (6.3 %) of 32 strains with Met306Val mutation were MDR but EMB-susceptible. One (50 %) of two strains with Tyr319Ser mutation was EMB-susceptible and had a higher MIC ($1.5 \mu\text{g ml}^{-1}$) than the MICs for pan-susceptible strains.

Four (66.7 %) of six Gly406Asp mutants (excluding *embB405*, 406 simultaneous mutant) and two (66.7 %) of three Gly406Ala mutants were EMB-susceptible. *embB406* mutation seems to be less associated with EMB resistance at the LJ medium critical concentration ($2.0 \mu\text{g ml}^{-1}$) in this study. One (50.0 %) of two strains with a Gln497Lys mutation was EMB-susceptible. However, all other Gln497Pro and Gln497Arg mutants revealed EMB resistance (Table 1).

embB mutation rate according to drug resistance

The *embB* mutation rate for 93 EMB-resistant *M. tuberculosis* strains was 90.3 %, but 2.8 % in pan-susceptible strains, 9.1 % in any-drug-resistant but non-MDR strains and 30.0 % in MDR but EMB-susceptible *M. tuberculosis* strains (Table 1). The proportion of strains with *embB* mutations increased with increasing anti-tuberculous drug resistance (i.e. in the order of pan-susceptible, any-drug-resistant EMB-susceptible, MDR EMB-susceptible and EMB-resistant *M. tuberculosis* strains) as shown by Bartholomew's test (a statistical value of 141.65, higher than the critical value, which is in the range 3.805–4.354).

In the 93 EMB-resistant strains, the *embB306* mutation was most prevalent (67.9 % of strains), followed by the *embB497*

Table 1. Distribution of the *embB* mutations

S, Susceptible; R, resistant; MDR, multidrug-resistant.

Amino acid change	Nucleotide change	EMB-S			EMB-R (93)
		Pan-S (72)	Any-drug-R but non-MDR (22)	MDR (30)	
Met306Ile	ATG→ATC				6
Met306Ile	ATG→ATA			1	18
Met306Ile	ATG→ATT				1
Met306Leu	ATG→CTG				2
Met306Val	ATG→GTG			2	30
Tyr319Asp	TAT→GAT				1
Tyr319Ser	TAT→TCT			1	1
Asp354Ala	GAC→GCC				3
Val360Val	GTG→GTA	1*			
Asn399Thr†	AAC→ACC				2
Glu405Asp†	GAG→GAC				1
Glu405Asp, Gly406Asp	GAG→GAC, GGC→GAC				1
Gly406Asp	GGC→GAC		2	2	2
Gly406Ala	GGC→GCC			2	1
Gly406Ser	GGC→AGC				2
Gly406Gly	GGC→GGA				1*
Gly459Asp†	GGC→GAC	1			
Gln497Lys	CAG→AAG			1	1
Gln497Pro	CAG→CCG				2
Gln497Arg	CAG→CGG				9
Mutation total		2 (2.8 %)	2 (9.1 %)	9 (30.0 %)	84 (90.3 %)
None		70 (97.2 %)	20 (90.9 %)	21 (70.0 %)	9 (9.7 %)

*Synonymous mutation.

†Novel mutation.

mutation (14.3 %) and the *embB406* mutation (7.1 %) (Table 3). The rate of *embB* mutation in EMB-resistant strains also increased in proportion to the number of drugs that the strains were resistant to (a statistical value of 76.21, higher than the critical value of 3.82 by Bartholomew's test).

Comparison of MICs

The mean MICs for six pan-susceptible strains versus 11 EMB-susceptible strains but with other drug resistances and *embB* mutations at codons 306, 319, 406 or 497 were 1.0 µg ml⁻¹ and 1.42 µg ml⁻¹, respectively. This result shows that the *embB* mutation in the drug-resistant *M. tuberculosis* strains is associated with increased MICs of EMB (Wilcoxon statistic=29.0, *P*-value=0.0052), even though the critical concentration for EMB-susceptible strains was 2.0 µg ml⁻¹.

Novel *embB* mutations and EMB susceptibility

We identified novel *embB* mutations at codons 399, 405 and 459. We found two Asn399Thr and two Glu405Asp *embB* mutations in EMB-resistant *M. tuberculosis* strains. One Gly459Asp *embB* mutation was found in pan-susceptible *M. tuberculosis* strains.

DISCUSSION

The ever-increasing burden of drug resistance is a serious concern in the world, particularly for patients with *M. tuberculosis* infection. This mycobacterium uses various mechanisms to evade killing by therapeutic drugs, including mutations in genes that encode drug target proteins (Cole *et al.*, 1998; Morris *et al.*, 1995; Musser, 1995; Shi *et al.*, 2007). The objective of this study was to identify mutations in a drug target gene in strains of *M. tuberculosis* prevalent among the Korean population. *embB* gene mutation was believed to be the main cause of EMB resistance in *M. tuberculosis*. However, the relationship between *embB* gene mutation and EMB resistance is controversial because of *embB* mutation in EMB-susceptible *M. tuberculosis* strains (Mokrousov *et al.*, 2002; Lee *et al.*, 2004; Van Rie *et al.*, 2001; Shi *et al.*, 2007; Hazbón *et al.*, 2005).

Hazbón *et al.* (2005) reported that 46 of 100 *embB306* mutants were EMB-susceptible while 54 were EMB-resistant. After retesting, a few of the mutants were reclassified from susceptible to resistant. The EMB MICs for the *embB306* mutants ranged from <1 to >32 µg ml⁻¹ (Hazbón *et al.*, 2005). *embB306* mutation was considered a common polymorphism with no relation to EMB resistance by Srivastava *et al.* (2009).

Table 2. EMB MIC for *M. tuberculosis* strains retested in duplicate on LJ medium after 5 weeks culture

ID	Phenotype of DST*	<i>embB</i> mutation codon and type	Mean MIC of duplicate retest ($\mu\text{g ml}^{-1}$)
KMRC 00119-90955	Pan-S	None	1.25
KMRC 00119-90995	Pan-S	None	0.75
KMRC 00119-90807	Pan-S	None	1.0
KMRC 00119-90808	Pan-S	None	1.0
KMRC 00119-90999	Pan-S	Val360Val (GTG-GTA)	1.25
KMRC 00119-90806	Pan-S	Gly459Asp (GGC-GAC)	0.75
KMRC 00119-89635	R	Gly406Asp (GGC-GAC)	1.75
KMRC 00119-90870	R	Gly406Asp (GGC-GAC)	1.63
KMRC 00119-84132	IR (MDR)	Met306Ile (ATG-ATA)	1.5
KMRC 00119-87595	IR (MDR)	Met306Val (ATG-GTG)	1.5
KMRC 00119-89732	IR (MDR)	Met306Val (ATG-GTG)	0.88
KMRC 00119-88533	IR (MDR)	Tyr319Ser (TAT-TCT)	1.5
KMRC 00119-83962	IR (MDR)	Gly406Asp (GGC-GAC)	0.88
KMRC 00119-84671	IR (MDR)	Gly406Asp (GGC-GAC)	1.38
KMRC 00119-86964	IR (MDR)	Gly406Ala (GGC-GCC)	1.75
KMRC 00119-89589	IR (MDR)	Gly406Ala (GGC-GCC)	1.5
KMRC 00119-86862	IRS (MDR)	Gln497Lys (CAG-AAG)	1.38
KMRC 00119-84185	IE	None	8
KMRC 00119-89348	IRE	Met306Ile (ATG-ATC)	4
KMRC 00119-90333	IRE	Met306Ile (ATG-ATA)	6
KMRC 00119-87849	ISE	Met306Leu (ATG-CTG)	6
KMRC 00119-90084	IRE	Met306Val (ATG-GTG)	6
KMRC 00119-83615	IRSE	Asn399Thr (AAC-ACC)	12
KMRC 00119-88939	IRSE	Asn399Thr (AAC-ACC)	6
KMRC 00119-88791	IE	Gly406Ala (GGC-GCC)	4
KMRC 00119-84589	IRE	Gln497Lys (CAG-AAG)	6
KMRC 00119-89319	IRSE	Gln497Pro (CAG-CGG)	12

*DST, Drug-susceptibility testing; Pan-S, pan-susceptible; I, isoniazid-resistant; R, rifampicin-resistant; S, streptomycin-resistant; E, ethambutol-resistant.

Depending upon mutation site or type, EMB resistance was found to be different. We found two *embB* mutations among 72 pan-susceptible *M. tuberculosis* strains. One of

the two strains had a synonymous mutation at codon 360, which did not affect EMB resistance. The second pan-susceptible strain had a Gly459Asp mutation, which is a novel mutation. However, this mutation also did not affect EMB resistance. *embB* mutation in pan-susceptible strains in particular may not affect EMB resistance. Similar to our results, Hazbón *et al.* (2005) also could not find *embB306* mutation in 582 pan-susceptible *M. tuberculosis* strains. Our findings suggest that certain mutations of the *embB* gene may not affect EMB resistance as had been postulated in previous reports (Hazbón *et al.*, 2005; Sekiguchi *et al.*, 2007).

Only three MDR isolates (5.0 %) out of 60 *embB306* mutants were EMB-susceptible in this study. Even though we also detected the *embB306* mutation in EMB-susceptible clinical isolates, an *embB306* substitution experiment from wild-type to mutation type proved that *embB306* mutation increased the EMB MIC (Safi *et al.*, 2008; Starks *et al.*, 2009). Safi *et al.* (2008) found that the substitution at *embB306* did not affect rifampicin or isoniazid MICs. Further, we found that six (54.5 %) out of 11 *embB406* mutant strains (excluding two strains, one with a synonymous mutation

Table 3. Distribution of mutation codons in 93 EMB-resistant strains

Mutation codon	Drug resistance			Total
	EMB mono	Non-MDR	MDR	
306	2	23	32	57 (67.9 %)
319	1		1	2 (2.4 %)
354		1	2	3 (3.6 %)
399			2	2 (2.4 %)
405			1	1 (1.2 %)
405, 406			1	1 (1.2 %)
406		1	5	6 (7.1 %)
497			12	12 (14.3 %)
Total	3 (3.6 %)	25 (29.8 %)	56 (66.7 %)	84 (100.0 %)
Wild-type		4	5	9

and another with an additional mutation at *embB405*) were EMB-susceptible (Table 1). *embB406* mutations have also been detected previously in EMB-susceptible isolates (Lee *et al.*, 2004; Shi *et al.*, 2011; Ramaswamy *et al.*, 2004). We found only one EMB-susceptible (7.7%) strain out of 13 *embB497* mutants. In a recent study, *embB406* and allelic *embB497* exchange increased the EMB MIC 3–3.5-fold and 6-fold, respectively, over that for the wild-type *embB* gene and these allelic exchanges did not affect the MIC of other anti-tuberculous drugs such as isoniazid or rifampicin (Safi *et al.*, 2010).

For the minor mutations, two of three *embB319* mutants were EMB-resistant and another one was EMB-susceptible with MDR as shown in Table 2. Likewise, the Tyr319Ser and Tyr319Asp mutations were found in EMB-resistant *M. tuberculosis* strains in previous reports (Plinke *et al.*, 2006; Sugawara *et al.*, 2005). The Asp354Ala mutation revealed EMB resistance, which has been reported previously (Sekiguchi *et al.*, 2007).

In this study, three novel *embB* mutations (Asn399Thr, Glu405Asp and Gly459Asp) were detected. Though all three of the codon sites were also found in another study, those mutations produced different amino acids (Srivastava *et al.*, 2009). Among the three novel mutations, two (Asn399Thr and Glu405Asp) were associated with EMB resistance.

Our study further suggested that the proportion of strains with *embB* mutations increased with the increasing number of drug resistances in both EMB-susceptible and EMB-resistant strains. This finding also agreed with those in other studies (Lee *et al.*, 2004; Shi *et al.*, 2011; Shen *et al.*, 2007).

Along with the above results, we found that the mean EMB MIC for *embB*-mutated EMB-susceptible strains was higher than that for pan-susceptible strains. This may be caused by the limitation of conventional drug susceptibility testing methods for EMB (Kim, 2005). Kim (2005) reported that 90% of probably EMB-susceptible strains and 30–50% of probably EMB-resistant strains were inhibited at the critical concentration of 2.0 µg ml⁻¹ on LJ solid medium.

However, we compared only the MICs for EMB-susceptible strains with those for pan-susceptible and other-drug-resistant strains with the *embB* mutation, so we need further studies to compare the MICs of EMB in EMB-susceptible strains with and without the *embB* mutations.

In conclusion, the exact mechanism of EMB resistance is not yet clearly understood. Our data support that the *embB* mutation, except for a few specific mutations such as Gly459Asp, may be the main cause of EMB resistance.

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