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Significance of Mutations in *embB* Codon 306 for Prediction of Ethambutol Resistance in Clinical *Mycobacterium tuberculosis* Isolates

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We analyzed 159 *Mycobacterium tuberculosis* isolates (101 ethambutol [EMB]-resistant strains, 33 multidrug-resistant but not EMB-resistant strains, and 25 fully susceptible strains) for the presence of mutations in *embB* codon 306 (*embB*306). Mutations were detected only in EMB-resistant strains ($n = 69$; 68%), thus confirming the significance of *embB*306 mutations for the prediction of resistance to EMB.

Drug-resistant tuberculosis (TB) has become a major public health problem in several regions around the world (14). The third report of the WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance confirmed the serious magnitude and widespread occurrence of drug resistance. Documented rates of drug-resistant TB have reached tremendous levels of up to 57% among new cases (14).

The rapid determination of drug resistance is the prerequisite for the initiation of effective chemotherapy to ensure successful treatment of the patient and to prevent the further spread of drug-resistant isolates (7). Based on the knowledge that the development of drug resistance in *Mycobacterium tuberculosis* complex isolates is the result of random genetic mutations in particular genes conferring resistance (15), molecular assays which allow the prediction of drug resistance in clinical isolates within 1 working day have been established. Thus, these assays are potentially the most rapid methods for the detection of drug resistance (6).

In the case of ethambutol (EMB), which, in combination with isoniazid, rifampin, and pyrazinamide, is a key component of the first-line anti-TB treatment regimen, resistance was most frequently associated with mutations in the *embCAB* operon and particularly with mutations in *embB* codon 306 (*embB*306) (15). Overall, approximately 60% of EMB-resistant *M. tuberculosis* isolates carry a mutation in *embB*306 (15). The determination of alterations of this codon was suggested as a rapid screening method for the detection of EMB resistance in clinical isolates (3, 8, 13).

More recently, however, discrepancies between the results of genotypic and phenotypic EMB resistance testing have raised concerns about the accuracy of molecular assays based on the detection of point mutations in *embB*306 for the prediction of EMB resistance (2, 4, 5, 11). Discordant results were especially reported for multidrug-resistant (MDR) strains phenotypically susceptible to EMB (5), and in a recent paper, Hazbón and colleagues described for *embB* codon 306 mutations "a novel association with broad drug resistance and IS*6110* clustering rather than ethambutol resistance" (2).

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To further investigate this question, we analyzed a large collection of 159 *M. tuberculosis* strains isolated in Germany in the year 2001 for the presence of mutations in *embB*306 and the association with phenotypic resistance to EMB. Resistance to the key antimycobacterial drugs was determined at the Supranational Reference Laboratory (SRL) in Borstel, Germany, by using the proportion method on Löwenstein-Jensen medium (critical concentration, 2.0 μg/ml for EMB) (1) and/or the modified proportion method in the BACTEC 460TB system (Becton Dickinson Microbiology Systems, Cockeysville, MD), according to the manufacturer's instructions (critical concentration, 3.75 μg/ml for EMB). As a member of the WHO SRL network, the laboratory participates in an ongoing quality assurance and proficiency testing program.

Among the 159 strains investigated, 101 were resistant to EMB. Seventy-two of these 101 strains showed additional resistance to rifampin. As controls, 33 MDR but not EMB-resistant strains and 25 fully susceptible strains were included (Table 1). This strain collection comprises all available EMB-resistant strains (including EMB-resistant MDR strains) and, in addition, all MDR strains without EMB resistance sent to the SRL for susceptibility testing in 2001. The population structure of the strains was determined by IS*6110* DNA fingerprinting, as described elsewhere (12). The most prominent genotype was the Beijing family, which accounted for 60% of the EMB-resistant strains and 49% of the MDR but not EMB-resistant strains (data not shown).

For molecular analyses, chromosomal DNA was extracted as described previously (12). PCR primers OG240 (5′-CGTTCC GGCCTGCAT-3′) and OG243 (5′-CACCTCACGCGACAG CA-3′) were used to amplify a 344-bp fragment of the *embB* gene (nucleotide positions 764 to 1107, codons 255 to 369) (3). Alterations in codon 306 were investigated by direct sequencing of the entire PCR products by using an ABI Prism 3100 capillary sequencer (Applied Biosystems) and the ABI Prism BigDye Terminator kit (version 1.1), according to the manufacturer's instructions.

Overall, 69 of the 101 EMB-resistant strains investigated (68%) carried a mutation in codon 306 of *embB* (Table 1). Mutations in other codons were detected in 7 strains (7%), and 25 strains (25%) had the wild-type sequence in the region of *embB* investigated (Table 1). The percentage of strains with an alteration in codon 306 did not differ signif-

TABLE 1. Distribution of mutations in *embB* stratified for phenotypically determined drug resistance

Drug resistance[a]	Total no. of isolates	No. (%) of strains with *embB* genotype:						
		ATG (wild type)	Mutation in *embB*306				Other
			GTG	ATA	ATC	ATT	Total	
I and E but not R	27	7 (26)	14 (52)	5 (18)		1 (4)	20 (74)	
I, E, and R	73	19 (26)	28 (38)	12 (16)	5 (7)	2 (3)	48 (64)	7 (10)[b]
E but not R or I	1					1	1 (100)	
E only	101	26 (26)	42 (41)	17 (17)	5 (5)	4 (4)	69 (68)	7 (7)[b]
I and R but not E	33	33 (100)						
Susceptible	25	25 (100)						

[a] Abbreviations: I, isoniazid; E, ethambutol; R, rifampin.
[b] Mutations outside *embB*306 were verified in codons 297 (TCG to TTG; $n = 1$), 319 (TAT to TCT; $n = 1$), 328 (GAT to GTG; $n = 3$), 332 (TGG to CTG; $n = 1$), and 334 (TAC to CAC; $n = 1$).

icantly if the EMB-resistant strains were stratified according to MDR and non-MDR isolates. However, mutations outside of codon 306 were found in the MDR group only (Table 1). There was no significant difference in the presence of *embB*306 mutations among non-Beijing and Beijing strains (data not shown). In the control groups of 33 MDR strains without phenotypic EMB resistance and 25 fully susceptible isolates, no mutations in codon 306 or the entire 344-bp region of *embB* were detected.

The results obtained here are in line with earlier findings that correlated mutations in *embB*306 with EMB resistance (9, 10) and suggest that these mutations predict EMB resistance in the population of *M. tuberculosis* strains included in this investigation. They are in clear contradiction with the findings presented in recent papers that reported on discrepancies between genotypic and phenotypic EMB resistance (18 and 60% of EMB susceptible MDR strains carrying a mutation in *embB*306 [5, 11]) or that even proposed that mutations in *embB*306 are more likely to be a marker for broad drug resistance and IS*6110* clustering rather than EMB resistance (2).

How can these different findings be explained? Hazbón and colleagues (2) suggested that the clear association between mutations in *embB*306 and EMB resistance found in several older studies might be due to the use of pansusceptible strains as control groups. However, that does not apply to our study, as we included 33 EMB-susceptible but MDR strains as controls and did not detect any alteration in *embB*306 in these strains. A number of further explanations might be conceivable: in the case of EMB there is a small difference between the critical concentration used for drug susceptibility testing and the MIC, making susceptibility testing more problematic; heteroresistant bacterial populations might lead to discordant results; and Mokrousov et al. (5) hypothesize an unknown mechanism in MDR *M. tuberculosis* strains that leads to susceptibility to EMB. The most important question is now, what gives us the right answer for the treatment of the patient? Must a strain with a mutation in *embB*306 in any case be considered resistant to EMB, irrespective of the drug susceptibility testing result, or are these mutations really not associated with resistance to EMB? Due to the importance of EMB for the actual applied anti-TB therapy, these questions must be urgently addressed in further well-controlled studies combining molecular as well as conventional drug susceptibility testing procedures.

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