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Detection of *embB306* Mutations in Ethambutol-Susceptible Clinical Isolates of *Mycobacterium tuberculosis* from Northwestern Russia: Implications for Genotypic Resistance Testing

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A total of 183 epidemiologically unlinked *Mycobacterium tuberculosis* isolates collected in the St. Petersburg area of Russia from 1996 to 2001 were screened for alterations in codon 306 of the *embB* gene; mutations in this codon are reported to confer resistance to ethambutol (EMB). The *embB306* mutations were detected in 14 (48.3%) of 29 EMB-resistant strains and, quite surprisingly, in 48 (31.2%) of 154 EMB-susceptible strains. A discrepancy between the results of phenotypic and genotypic EMB resistance tests was restricted to the strains already resistant to other antitubercular (anti-TB) drugs. In particular, 40 (60%) of the 69 EMB-susceptible strains resistant to rifampin, isoniazid, and streptomycin but none of the 43 pansusceptible strains harbored an *embB306* mutation. We hypothesize that the phenomenon observed could reflect the presence of a target other than EmbB for the drug in tubercle bacilli; this unknown target could be sensitized and affected, sensu lato, by EMB during treatment with other first-line anti-TB drugs. Comparison with DNA fingerprinting data showed that, irrespectively of the phenotypic susceptibility profiles, 46 (50.6%) of 91 Beijing family strains and 16 (17.4%) of 92 strains of other genotypes had a mutation in *embB306*.

Ethambutol (EMB) [dextro-2,2′-(ethylenediimino)di-1-butanol] is one of the first-line drugs included in the directly observed therapy short-course antitubercular regimen recommended by the World Health Organization (25) and the standard treatment protocol officially adopted by the Russian Ministry of Health (13). The major mechanism of acquisition of resistance to EMB in *Mycobacterium tuberculosis* has been shown to be associated with the point mutations in the *embCAB* operon encoding different arabinosyl transferases (1, 17). In particular, 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 (1, 15, 16). Five different mutations were detected in this codon that alter its first or third base (ATG → GTG, CTG, ATA, ATC, or ATT) and result in three amino acid shifts (Met → Val, Leu, and Ile) (16). Several additional loci encoding proteins that may participate in the response of *M. tuberculosis* to EMB treatment have been identified (2), and a recent extensive study investigated more genes in relation to the development of EMB resistance in tubercle bacilli (14). However, one-fourth of EMB-resistant strains still lacked any known mutation linked to the EMB resistance (14), implying multiple molecular pathways to its development, and some of them have yet to be discovered.

A variation in the prevalence of particular mutations linked to EMB resistance is likely to be observed, depending on the geographic area under study. Therefore, a preliminary analysis of a representative sample from a survey area is necessary, and our aim was to screen for *embB306* mutations among *M. tuberculosis* strains isolated in the northwestern region of the Russian Federation in order to assess whether they are reliable markers for the detection of EMB resistance in this region.

***M. tuberculosis* culture and susceptibility testing.** The 183 *M. tuberculosis* isolates tested were recovered from 183 different adult patients (15 to 63 years old) with recently and previously diagnosed pulmonary tuberculosis (TB). These patients were randomly selected for this study; they were from St. Petersburg and three neighboring provinces of northwestern Russia (Leningrad Oblast, Novgorod, and Pskov) and were admitted to the hospitals of the St. Petersburg Research Institute of Phthisiopulmonology and the City Anti-Tuberculosis Dispensary of St. Petersburg between 1996 and 2001. For each patient, only the first available isolate was included in the study. Löwenstein-Jensen medium was used for cultivation of isolates. Susceptibility testing for anti-TB drugs was done by the method of absolute concentration, as recommended by the Russian Ministry of Health (order no. 558 of 28 June 1978) and has been described previously (23). The drug concentrations for susceptibility testing were as follows: isoniazid (INH), 1 μg/ml; streptomycin (STR), 5 and 50 μg/ml; rifampin (RIF), 20 and 50 μg/ml; EMB, 2 and 5 μg/ml (23). Strain H37Rv was included as a control in each susceptibility test. The absolute-concentration method used was previously shown in our setting to give results concordant with those generated by the proportion method in a comparative study conducted with the National Mycobacterial Reference Laboratory in Turku, Finland (23). In that study, a 100% interlaboratory concordance was ob-

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served for culture-based EMB susceptibility detection in a total of 135 isolates.

DNA used for PCR analysis was extracted as described by van Embden et al. (19). Strain differentiation was performed by spoligotyping as described by Kamerbeek et al. (6) and IS*6110* restriction fragment length polymorphism (RFLP) typing as described by van Embden et al. (19, 20).

Monitoring for contamination during microbiological and genetic experiments was performed as recommended in references 24 and 4, respectively. In particular, monitoring for possible contamination with previously amplified amplicons was preformed by including a negative control sample (distilled water) in each PCR run. No contamination was detected.

PCR-RFLP analysis of *embB306*. Allelic variation in *embB306* was analyzed by a PCR-RFLP assay by using restriction endonucleases *Nla*III and *Hae*III as previously described (10). In short, any mutation in *embB* codon 306ATG changes the *Nla*III site (CATG ↑). At the same time, the third base of this codon (G) is associated with an *Hae*III recognition site (GG ↑ CC) and hence the *Hae*III site can be altered only if a mutation occurs in the third base of *embB306*. To summarize, *Nla*III distinguishes between the wild-type allele and any mutant allele while *Hae*III permits further discrimination of the mutants, depending on the base mutated (first [A] or third [G]) (10). It should be noted that this PCR-RFLP assay design allows monitoring for possible strain contamination during isolation and culture. Since the PCR-RFLP profiles are clearly different for the wild-type and mutant alleles (10), a mixed culture would result in a complex PCR-RFLP profile combining two different profiles in a single lane. However, no mixed (contaminated) profiles were observed for any of the strains included in the present study. In addition, 20 strains with wild-type and different mutant *embB306* alleles were sent to the Interlaboratory Group of Molecular Systematics "Taxon" (Zoological Institute, Russian Academy of Sciences, St. Petersburg) for repetition of the DNA isolation and PCR-RFLP analysis in a blinded fashion. The *embB306* allelic variation results obtained were identical to those produced in our laboratory.

Results and discussion. Twenty-nine of the strains studied were identified phenotypically to be resistant to EMB. Twenty-five of them were also resistant to STR, RIF, and INH; one strain was resistant to STR and INH; one strain was resistant to STR and RIF; one strain was resistant to INH and RIF; and one strain was resistant to INH.

The distribution of *embB306* allelic variants among 29 EMB-resistant strains was as follows: ATG (wild type), 15 strains; BTG, 10 strains; ATH, 4 strains (according to the degenerated-base code, B is G, C, or T and H is A, C, or T). On the basis of the reported distribution of the *embB306* mutations (15, 16), we consider ATH most likely to be the ATA allele (Ile) and BTG most likely to be the GTG (Val) or CTG (Leu) allele. Our findings on the distribution of the *embB306* alleles, wild type versus mutated, among EMB-resistant strains correspond to previously published results (14, 15, 16).

A limited selection of 17 strains isolated in 2001 and susceptible to EMB but resistant to other drugs was initially included in the study as a control group, and quite unexpectedly, we found 10 strains to harbor *embB306* mutations that should have invariably conferred the EMB resistance phenotype. It should be noted that the substitutions in EmbB306 were reported to confer high levels of resistance to EMB (MICs of 20 to 40 μg/ml) (16). We therefore extended the *embB306* mutational analysis to a larger sample of epidemiologically unlinked EMB-susceptible strains isolated in a wider time frame. Thus, a retrospective study was carried out with DNA preparations from a total of 154 EMB-susceptible strains isolated in 1996 to 2001. The results are summarized in Table 1. A discrepancy between the results of phenotypic and genotypic drug resistance tests was found for 48 (31.2%) of 154 strains that were phenotypically susceptible to EMB but had an *embB306* mutation. All 48 discrepant cases were retested by the PCR-RFLP assay, and the prior results were confirmed. The phenotypic susceptibility testing was repeated for all available cultures (35 of 48), and the initial results were confirmed. Positive growth (>100 colonies) was observed on the control medium without EMB, but not a single colony was detected on the media with concentrations of 2 and 5 μg of EMB per ml. A general concern arising when encountering discrepant susceptibility testing results is that of possible drug instability in the test medium (5). However, had this been the case in our experiments, it would have resulted in growth of an EMB-susceptible isolate on the EMB-containing medium and such an isolate would have misleadingly been considered resistant. This, however, was not the case. As has been mentioned above, no growth occurred in the presence of EMB despite an *embB306* mutation while clearly positive growth of an isolate was observed on a control medium without EMB.

TABLE 1. Distribution of *embB306* alleles among EMB-susceptible *M. tuberculosis* strains with different resistance profiles[a]

embB306 allele[b]	No. of strains (no. in Beijng family)							
	Susceptible	S	H	R	SH	SR	SHR	Total
ATG-Met (wt)	43 (11)	14 (4)	1	3 (2)	11 (4)	5 (1)	29 (19)	104 (41)
BTG-Val/Leu					1 (1)		23 (21)	24 (22)
ATH-Ile		1 (1)	1		5 (5)		17 (6)	24 (12)
Total	43 (11)	15 (5)	2	3 (2)	17 (10)	5 (1)	69 (46)	154 (75)

[a] Drug resistance abbreviations: S, STR; H, INH; R, RIF; e.g., SHR means resistance to STR, INH, and RIF.

[b] *embB306* alleles: wt, wild type; BTG, any mutation in the first base of *embB306;* ATH, any mutation in the third base of *embB306* (according to the degenerated-base code, B represents G, C, or T and H represents A, C, or T).

The *embB306* mutations in EMB-susceptible strains were previously described in South African *M. tuberculosis* strains (8 of 51 strains included in a population-based study), with phenotypic susceptibility to EMB reconfirmed (21). However, the authors of that report attributed the discordance found to an unknown systemic error of the traditional culture-based susceptibility testing. On the contrary, we believe that phenotypic susceptibility results, when reconfirmed, cannot simply be ruled out even if they contradict the genotypic data.

One striking observation from Table 1 is that discrepant results were restricted to the drug-resistant, and especially to the multidrug-resistant (MDR), strains: 40 (60.0%) of the 69 EMB-susceptible strains resistant to STR, RIF, and INH had a mutation in *embB306*. In contrast, no *embB306* mutations were detected in any of the 43 pansusceptible isolates. The same was found in the study cited above (21), in which both pansuscep-

tible isolates had the *embB306* wild-type allele while all eight discrepant cases were phenotypically EMB susceptible but resistant to other drugs (one strain was resistant to INH, one was resistant to INH and STR, three were resistant to INH and RIF, and three were resistant to INH, STR, and RIF) and harbored an *embB306* mutation. We speculate that the phenomenon observed could have the following explanation. Generally, not only different mechanisms of resistance but also the mechanisms of susceptibility can exist because of multiple targets for the drug in a cell, either constitutive or putative. Some of the latter are manifested in response to the combined action of different drugs, and the combination of STR, INH, and RIF could trigger such an additional EMB susceptibility mechanism. This supposition would also explain the low percentage of (phenotypically) EMB-resistant strains, averaging 4 to 9% worldwide, with initial resistance (15).

We also investigated the distribution of *embB306* alleles among different strains (genotypes) of *M. tuberculosis* in particular, comparing the Beijing genotype strains with other strains of other genotypes (designated non-Beijing). The Beijing family, initially found to be endemic to the countries of East Asia (22), is marked by high transmissibility and currently shows a worldwide distribution (3). Previously, we found its high prevalence in the northwestern region of Russia by IS*6110* RFLP typing and the direct-repeat-based spoligotyping technique (11, 12). In the present study, 91 (49.7%) of the 183 strains tested showed characteristic IS*6110* RFLP patterns and a spoligotyping profile consisting of signals 35 to 43, which are typical of the Beijing family. Among both EMB-resistant and -susceptible isolates, irrespectively of their phenotypic susceptibility profiles, 46 (50.6%) of 91 Beijing strains and 16 (17.4%) of 92 non-Beijing strains had a mutation in *embB306* (odds ratio, 4.60; 95% confidence interval, 2.33 to 9.08). Similarly, all 15 Beijing strains and only 4 of 36 non-Beijing strains in the above-cited study (21) had an *embB306* mutation. The Beijing genotype thus seems to acquire the most frequently arising (resistance) mutations more readily than do other genotypes, not only in *embB306* but also in *rpoB531* (8, 9, 21) and *katG315* (11). On the other hand, 27 (58.7%) of 46 Beijing and 13 (56.5%) of 23 non-Beijing EMB-susceptible MDR strains included in the present study had an *embB306* mutation. We therefore hypothesize that a "wrong" emergence of *embB306* mutations in EMB-susceptible strains may be predetermined not by the intrinsic genome structure (IS*6110* RFLP profile) but rather by the previously acquired multidrug resistance. Since MDR TB in Russia seems to be associated with circulation of the Beijing strains (7, 11, 18), this situation may be the driving force behind the high prevalence of the *embB306* mutant variants among these strains.

To summarize, the *embB306* mutations were found in 48.3% of the EMB-resistant and 32.5% of the EMB-susceptible strains studied, and this implies that the *embB306* alterations have limited value as a reliable genetic marker of EMB resistance in *M. tuberculosis* clinical strains in northwestern Russia. The *embB306* alteration is not necessarily indicative of EMB resistance, especially in MDR strains. We suggest caution in the acceptance of *embB306* mutations as a marker of the EMB resistance phenotype; a preliminary evaluation study is necessary for each particular geographic area. We suggest that our results provide indirect evidence of an unknown mechanism of susceptibility to EMB based on the existence of a target other than EmbB for this drug in tubercle bacilli. This supposed target could be activated, sensu lato, during treatment of a patient with a combination of the first-line anti-TB drugs. A hypothetical target molecule could become sensitized and consequently affected by EMB, or alternatively, an unknown pathway could be inhibited by EMB, leading to accumulation of toxic intermediates, both of which courses would result in cell death. In this light, EMB appears to play a more crucial role in the directly observed therapy short-course regimen when all of the major anti-TB drugs are applied together. Even if the *rpoB* and *katG* mutations are selected, the combined action of RIF, INH, and STR will likely result in additional sensitization of the cell to EMB, regardless of an eventual *embB306* mutation. Undoubtedly, further study is necessary to clarify the basis of this phenomenon and a comprehensive understanding of the mechanism of action of this drug is still required.

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