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DRUG DISCOVERY AND RESISTANCE

Tuberculosis ethambutol resistance: Concordance between phenotypic and genotypic test results

Claudia Plinke [a,f], Helen S. Cox [b,f], Stobdan Kalon [c], Daribay Doshetov [d], Sabine Rüsch-Gerdes [e], Stefan Niemann [a,*]

[a] Molecular Mycobacteriology, Research Center Borstel, Borstel, Germany
[b] Macfarlane Burnet Institute for Medical Research and Public Health, Australia
[c] Médecins Sans Frontières, Uzbekistan
[d] Ministry of Health, Karakalpakstan, Uzbekistan
[e] National Reference Center for Mycobacteria, Research Center Borstel, Borstel, Germany

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SUMMARY

*embB*306 mutations are potential markers for detecting ethambutol resistance in clinical *Mycobacterium tuberculosis* isolates. However, more recently, *embB*306 mutations have been found in ethambutol susceptible isolates and an association with broad drug resistance rather than ethambutol resistance has been reported.

To further investigate this question, we analyzed the association between *embB*306 mutations and phenotypic ethambutol resistance among 197 isolates from a drug resistance survey performed in Karakalpakstan, Uzbekistan.

39 strains had an *embB*306 mutation, out of which seven were ethambutol susceptible, thus, displaying discrepant test results. After re-analysis, the seven isolates were tested ethambutol resistant. All of these strains had an increased ethambutol MIC, however, three strains showed no or weak growth on the critical concentration of 2 μg/ml on Löwenstein-Jensen. In three strains we confirmed the presence of heteroresistant mixed populations which might influence conventional ethambutol testing. Final concordance between molecular and phenotypic EMB testing was high with a sensitivity of 78% and a specificity of 100%.

Our results confirm that *emb*B306 mutations are useful markers for predicting ethambutol resistance. Discrepancies between molecular and phenotypic ethambutol resistance test results are most likely caused by problems with conventional susceptibility testing.

1. Introduction

The increasing numbers of drug resistant and multidrug resistant tuberculosis (MDR-TB, resistance at least to isoniazid [INH] and rifampicin [RMP]) strains observed in several parts of the world represent a serious challenge for global tuberculosis control.[1,2] In its recent report, the Global Project on Antituberculosis Drug Resistance documented a prevalence of MDR-TB among new cases of tuberculosis ranging from 0% to 22,3% in 81 countries surveyed from years 2002–2007.[2] However, in some areas of the world such as Karakalpakstan (Uzbekistan) MDR-TB rates have reached much more significant levels of up to 14% among patients never treated and up to 40% among previously treated patients.[2,3]

These data underline the importance of the rapid detection of drug resistance in clinical isolates in order to initiate effective therapy that avoids the creation of further resistance and also prevents the spread of drug resistant isolates. In addition to more rapid methods for conventional drug susceptibility testing such as the BACTEC MGIT 960 system,[4] molecular assays (e.g. based on DNA sequencing, Real Time PCR, or stripe technologies) for the detection of mutations conferring resistance have been increasingly used and have the potential to shorten the time to detection of resistance to one working day.[5–7]

However, while molecular mechanisms involved in resistance to INH and RMP have been identified with clear cut associations

* Corresponding author. Molecular Mycobacteriology, National Reference Center for Mycobacteria, Forschungszentrum Borstel, Parkallee 18, D-23845 Borstel, Germany. Tel.: +49 4537 188762; fax: +49 4537 188311.
E-mail address: sniemann@fz-borstel.de (S. Niemann).
[f] These authors have contributed equally to this study.

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between mutations in particular genes such as *katG* and *rpoB* with phenotypic resistance to INH and RMP, respectively,[8] associations between mutations and phenotypic ethambutol (EMB) resistance are less clear. Ethambutol is a key component of the first-line antituberculosis treatment regimen, and is also an important drug to be included in second-line regimens for MDR-TB, where susceptibility can be demonstrated.

Mutations in the *embCAB* operon (mainly the *emb*B gene) have been most commonly associated with EMB resistance with variations of codon *embB*306 as a hot spot for mutations. Furthermore, mutations have been described e.g. in *embB*406 and *embB*497. In a recent study, Safi *et al.* demonstrated that transferring *embB*306 point mutations into EMB susceptible clinical *Mycobacterium tuberculosis* strain 210 increased the EMB MIC.[9] However, if clinical isolates are considered, the percentage of EMB resistant strains which show mutations in *embB306* ranges from 30 to 68%.[10,11] Additionally, a number of recent investigations described the occurrence of *embB*306 mutations in EMB susceptible strains,[12–14] and several studies reporting an association with broad drug resistance rather than with EMB resistance alone.[15,16] These contradictory results cast doubt on the potential use of *embB*306 mutations as molecular markers for predicting EMB resistance in clinical isolates.

To address this important question, we investigated the correlation between *embB*306 mutations and phenotypic EMB resistance in a population based study. Therefore, strains from a cross sectional survey performed in Karakalpakstan, Uzbekistan, a setting with high TB incidence and a high level of drug resistance,[3] were investigated for sequence variations at *embB* codon 306 and phenotypic EMB resistance. Special attention was drawn to the analysis of strains with discrepant results that were re-tested and screened for mixed populations of mutants and wild type.

2. Material and methods

2.1. Study setting

The Autonomous Republic of Karakalpakstan is located in the west of Uzbekistan, south of the Aral Sea. Karakalpakstan has a high TB burden with a total case notification rate of 482/100,000 in 2001.[3] A cross-sectional drug resistance survey conducted in 2001/2002 found MDR-TB infection among 13% of new cases and 40% of previously treated patients.[3] TB isolates from this survey were used in the current study, with recruitment as previously described.[3]

2.2. Strain cultivation and drug susceptibility testing

Primary isolation and cultivation of mycobacterial isolates were performed at the Supranational Reference Laboratory (SRL) Borstel, Germany. For all strains, resistance to the antimycobacterial drugs (INH, RMP; EMB, streptomycin [SM]) was determined by using the proportion method on Löwenstein-Jensen (LJ) medium (critical concentration used for EMB was 2 μg/ml). If growth was insufficient, drug-susceptibility testing was performed by using the modified proportion method in BACTEC 460TB (Becton-Dickinson). Re-testing of strains with discordant results was performed both on LJ medium (as before), and the Bactec MGIT 960 system (5.0 μg/ml for EMB) as described previously.[4]

For MIC determination, suspensions were made from fresh cultures grown on LJ. The suspensions were adjusted to the optical density of McFarland 1. An inoculum of 0.1 ml of a 10^{-2} dilution was used for the EMB containing medium. The following drug concentrations were investigated on LJ slants: 0.125, 0.250, 0.500, 1, 2 and 4 μg/ml EMB. Duplicates were inoculated for each concentration. Two drug-free controls were included, one with 0.1 ml of a 10^{-2} dilution (representing 100% growth) and the other one with 0.1 ml of a 10^{-4} dilution (representing 1% growth). Incubation was at 37 °C for three weeks. The lowest concentration of EMB that inhibited 100% of the bacterial population was considered the MIC.

2.3. DNA techniques

Extraction of genomic DNA from the mycobacterial strains and DNA fingerprinting using IS*6110* as a probe were performed according to a standardized protocol as described elsewhere.[17] Additionally, all isolates were analyzed by the spoligotyping technique as described previously by Kamerbeek et al.[18] The molecular typing data were analyzed with the Bionumerics software (version 4.5; Applied Maths, Sint-Martens-Latem, Belgium) as instructed by the manufacturer. The spoligotyping data were used to additionally confirm strain relationships and for the identification of Beijing genotype isolates (no hybridization to spacers 1–34, hybridization to spacers 35–43).[19]

A 334 bp PCR fragment of the *embB* gene was amplified by using the primers embF-new (5'-TGGACGGGCGGGGCTCAAT-3', nucleotide position 725-743) and embR-new (5'-GGCAGGCGCATCACAGACT-3', nucleotide position 1058–1039). Mutations in codon 306 were investigated by direct sequencing of the entire PCR products using an ABI Prism 3100 capillary sequencer (Applied Biosystems, CA, USA) and the ABI Prism BigDye Terminator kit v.1.1. according to the manufacturer's instructions.

Molecular analysis of the 334 bp *embB* PCR fragment encompassing codon 306 was carried out blinded without knowledge of the phenotypic drug resistance data obtained during the original drug resistance survey.

2.4. Statistical analyses

Data were analyzed using SPSS 15.0 for Windows (SPSS Inc. Chicago). Odds ratios for univariate associations were calculated using Mantel-Haenszel estimates with exact 95% confidence intervals. Binary logistic regression was used for multivariate analysis of factors associated with *emb*B306 mutations with block entry of combinations of categorical variables. The level of statistical significance was set at 0.05.

3. Results and discussion

A total of 197 strains were investigated for mutations in *embB* codon 306 by direct sequencing of PCR fragments. Details of drug resistance rates and *M. tuberculosis* population structure have been presented in our previous publications.[3,20]

Briefly, 124 of the 197 (62.9%) strains were resistant to at least one first-line antituberculosis drug tested (INH, RMP, EMB, PZA, and SM) and 50 (25%) were MDR-TB. Analysis of *IS6110* DNA fingerprint and spoligotyping data showed that 107 strains (54.3%) belonged to the *M. tuberculosis* Beijing genotype. Seventy nine (40%) strains were grouped in 23 clusters ranging in size from two to 15 strains with identical IS*6110* DNA fingerprint and spoligotype patterns.

Overall, 39 of the 197 (19,8%) strains had a mutation in *embB*306 of which 32 (82,1%) were found to be EMB resistant (Figure 1). Seven strains had *embB*306 mutations without displaying phenotypic EMB resistance (Figure 1), of which one was resistant to multiple drugs but not MDR-TB (poly resistant) and six were MDR-TB. To clarify these discordant findings, all seven strains were re-grown from frozen seeds and both, conventional EMB susceptibility testing (LJ medium and BACTEC MGIT 960) and molecular analysis, were repeated. Out of the originally determined seven EMB susceptible strains with *embB*306 mutation all were found to be EMB resistant in the second analysis and had the same *embB*306

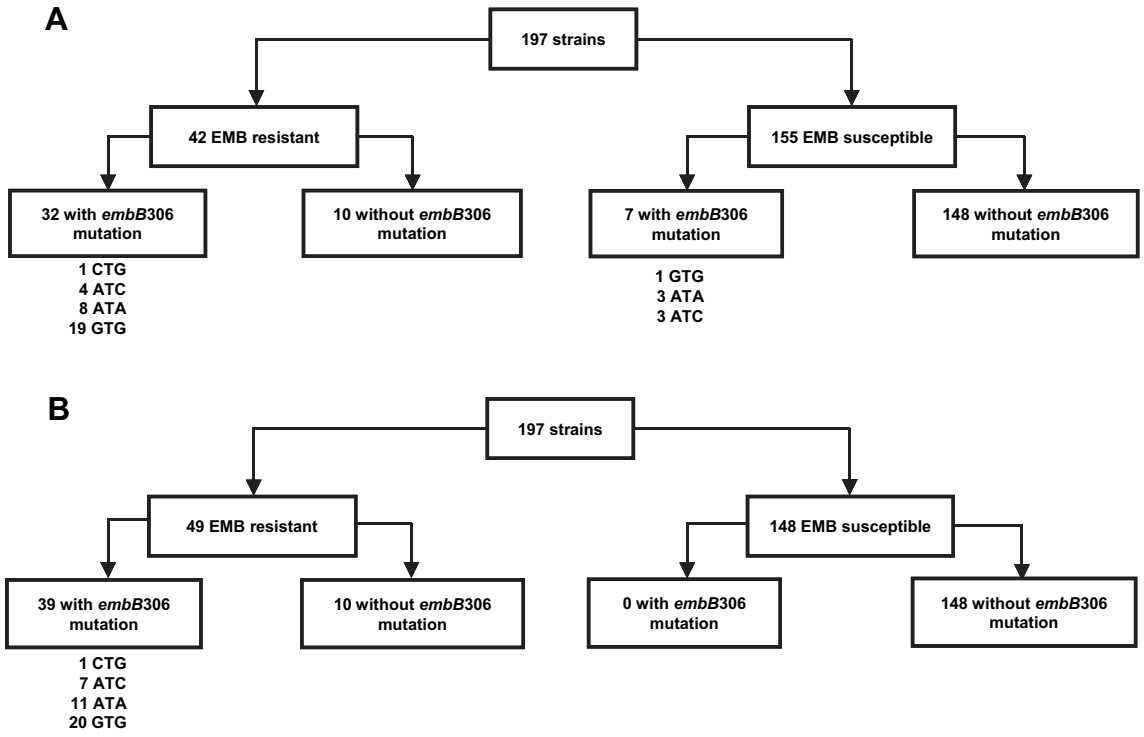

Figure 1. Results of the primary susceptibility testing (A) and results after retesting (B).

mutations (Table 1). However, three of these had intermediate results on LJ medium showing growth only at 1 μg EMB/ml and no or weak growth on 2 μg EMB/ml (compared to the corresponding growth controls). In BACTEC MGIT 960, all three strains clearly tested resistant, however, with a lower growth index when compared to the other four strains (Table 1).

The seven primary discordant strains were further characterised by determination of the MIC for EMB and screened for mixed populations by molecular analysis of isolated single colonies for each strain.

The MIC determination was performed on LJ slants. The reference strain H37Rv was also tested as control (Table 2). All isolates tested EMB resistant in the second analysis showed an increase in MIC compared to H37Rv (0.5 μg/ml EMB). For the MDR strain with *embB*306 GTG mutation the MIC was ≥ 4 μg/ml (Table 2). However, three strains showed no or weak growth on 2 μg/ml EMB reflecting the results of the conventional susceptibility testing on LJ. In general these findings demonstrate that the EMB resistant clinical isolates had an increased MIC compared to the susceptible control, but for three strains the increase of the MIC was below the value of the critical concentration (2 μg/ml EMB). Therefore these strains were tested EMB susceptible by conventional susceptibility testing on LJ.

To investigate, if a mixed bacterial population might be a reason for the primary discordant results of the seven EMB resistant strains, single colony plating was carried out on drug free-medium and single colonies for each strain were isolated. Subsequently the DNA was extracted followed by analysis of the *embB*306 region. Indeed for three of the investigated strains a mixture of *embB*306 mutants and wild type clones was found (Table 3). Interestingly two of these strains with mixed populations belonged to the group of isolates, which would be defined as EMB susceptible according to the susceptibility testing on LJ but were EMB resistant in BACTEC MGIT 960. One of these strains even consisted of 32% wild type and 68% mutant ATA.

After retesting, the overall sensitivity of *embB*306 mutations for detection of EMB resistance in the study population was 78% with a specificity of 100% (39 out of 49 EMB resistant strains showed an *embB*306 mutation but none of the EMB susceptible strains, Figure 1). Statistical analysis revealed a significant association between EMB resistance and *emb*B306 mutations in univariate analysis ($p < 0.0001$), with *emb*B306 mutations the only significant

Table 1
Final susceptibility testing results among the seven strains with EMB susceptibility and *emb*B306 mutations on first analysis.

Strain	LJ EMB [1 μg/ml]	LJ EMB [2 μg/ml]	MGIT960 growth index EMB [5 μg/ml]	*embB* 306	Resistance profile*	Final EMB result
1	Growth	No growth	296	ATC	INH, RMP, SM	res
2	Growth	Weak growth	294	ATC	INH, PZA, SM,	res
3	Growth	Growth	400	ATA	INH, RMP, SM,	res
4	Growth	Growth	400	ATC	INH, RMP, PZA, SM	res
5	Growth	Growth	400	GTG	INH, RMP, SM	res
6	Growth	Growth	383	ATA	INH, RMP, SM	res
7	Growth	No Growth	167	ATA	INH, RMP, SM	res

Abbreviations and symbols: EMB, ethambutol; INH, isoniazid; RMP, rifampcin; PZA, pyrazinamide; SM, streptomycin; LJ, Löwenstein-Jensen medium; WT, wild type; susc, susceptible; res, resistant.

* susceptibility testing results on initial testing.

Table 2
Determination of the EMB minimal inhibitory concentration for the seven strains with discrepant results.

Strain	*embB*306	EMB concentration [μg/ml]						Control 10^{-3}	Control 10^{-5}
		0.125	0.25	0.5	1	2	4		
1	ATC	4+/4+	4+/4+	4+/4+	4+/4+	0/0	0/0	4+/4+	+/+
2	ATC	3+/3+	3+/3+	3+/3+	3+/3+	+/+	0/0	3+/3+	(+)/(+)
3	ATA	4+/4+	4+/4+	4+/4+	4+/4+	3+/3+	0/0	4+/4+	+/+
4	ATC	3+/3+	3+/3+	3+/3+	3+/3+	3+/3+	0/0	3+/3+	±/±
5	GTG	3+/3+	3+/3+	3+/3+	3+/3+	3+/3+	2+/2+	3+/3+	+/+
6	ATA	4+/4+	4+/4+	4+/4+	4+/4+	3+/3+	0/0	4+/4+	+/+
7	ATA	3+/3+	3+/3+	3+/3+	2+/2+	0/0	0/0	3+/3+	+/+
H37Rv	ATG	4+/4+	3+/3+	0/0	0/0	0/0	0/0	4+/4+	±/±

factor in multivariate analysis ($p < 0.0001$). Thus, our study confirms previous investigations that describe a clear correlation between mutations in *emb306* and EMB resistance,[11,21] without any indication of an influence on the resistance to other antituberculous drugs as suggested by other authors.[13,15,16]

This high concordance between both assays was, however, determined only after repeating conventional drug susceptibility testing, pointing to problems with conventional diagnostics of EMB resistance. Indeed, due to the small difference between the critical concentration used for EMB drug susceptibility testing and the MIC, conventional drug susceptibility testing for EMB is less reliable, yields less reproducible results, and has a lower sensitivity when compared with other drugs.[1] Another reason for the difficulties in conventional EMB resistance testing might probably a lower stability of this drug during incubation at 37 °C.[22]

This might be further complicated by the presence of heteroresistant populations that, based on the amount of susceptible and resistant cells, might lead to false susceptible test results. In addition, sub culturing of heteroresistant strains is likely to cause non reproducible test results e.g. if in one case the susceptible population is dominating while in the other the resistant one is dominating. These findings suggest implementation of molecular methods is likely to represent a significant improvement of conventional culture-based EMB resistance testing applied actually.

It is noteworthy that six out of seven strains with mutations that changed from susceptible to resistant on retesting had third base mutations (ATA and ATC) rather than mutations of the first base (GTG). This might be related to differences in the increase of the EMB MIC value conferred by different mutations and, thus, contribute to the difficulties with conventional EMB susceptibility testing. This hypothesis is further supported by the fact that three of the re-tested strains showed an intermediate level of EMB resistance on LJ-medium and a lower growth index in BACTEC MGIT 960 when compared with the other strains (Table 2).

Overall, ten EMB resistant isolates had no mutation in the total region investigated including codon *embB*306. Here, other mutations in the *embCAB* operon might play a role for the development of EMB resistance. For example, Ramaswamy et al. described in their work mutations, which were associated with EMB resistance, in 21 variant codons of the *embCAB* operon.[21] Further investigations of the EMB resistant isolates from Usbekistan, which had no mutation in *embB*306, are planned for future studies.

Table 3
DNA-Analysis of the *embB*306 region extracted from isolated single colonies grown on drug free-medium.

Strain (*embB*306)	Total number of colonies	*embB*306	
		Wild type ATG	Mutant
1 (ATC)	49	–	49
2 (ATC)	50	3	47
3 (ATA)	48	–	48
4 (ATC)	51	2	49
5 (GTG)	15	–	15
6 (ATA)	50	–	50
7 (ATA)	50	16	34

In conclusion, these results demonstrate a highly significant association between *embB*306 mutations and EMB resistance in this study population and indicate a potential role for *embB*306 mutations to be used for the rapid detection of EMB resistance, particularly in the design of individualised treatment regimens for MDR-TB. Discrepancies between molecular and phenotypic EMB resistance data most likely result from problems with conventional EMB susceptibility testing. However, further work is needed to analyse the level of EMB resistance conferred by the different *emb*B306 mutations reported so far. In addition, further studies are necessary to investigate possible resistance mechanisms in strains without mutation in *embB* codon 306.

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