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Short communication

Role of *embCAB* gene mutations in ethambutol resistance in *Mycobacterium tuberculosis* isolates from India

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ABSTRACT

In the present study, ethambutol (EMB) resistance-associated mutations were characterised in the *embCAB* genes of clinical isolates of *Mycobacterium tuberculosis* (MTB) collected in India. Thirty MTB isolates were tested for their susceptibility to first-line antitubercular drugs using the Löwenstein–Jensen proportion method, and EMB minimum inhibitory concentrations of MTB isolates were determined by the resazurin microtitre assay. Sequencing of various regions of the *embCAB* genes was performed to identify EMB resistance-associated mutations. Mutations of *embB*306 were detected in 15 of 23 EMB-resistant MTB isolates. Three EMB-resistant isolates had mutations at codon 270 of the *embC* gene, two of which also harboured *embB*306 mutations. No mutation was identified in the *embA* gene. All seven EMB-sensitive MTB isolates had the wild-type *embCAB* sequence. In summary, *embB*306 mutations were associated with EMB resistance, and mutation at codon 270 of the *embC* gene may contribute to high-level EMB resistance in some MTB isolates.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is a major cause of human mortality worldwide, claiming 2–3 million human lives annually. Directly observed therapy short-course (DOTS) has been recommended as the standard treatment strategy for TB. DOTS therapy comprises rifampicin (RIF), isoniazid (INH), pyrazinamide and ethambutol (EMB) as well as streptomycin (STR). Proper prescription and patient compliance almost always cures new/untreated TB patients; however, improper prescription and/or non-compliance may lead to the emergence of a drug-resistant MTB population and ultimately to treatment failures.

EMB [2,2′-(ethylenediimino)-di-1-butanol] is an important primary antitubercular drug used during the intensive phase of DOTS therapy. It acts against MTB by targeting the membrane-associated arabinosyl transferases, which are encoded by *embCAB* genes [1,2]. Mutations in these genes have previously been mapped in EMB-resistant MTB isolates [2]. The majority of EMB-resistant isolates characterised from different geographical settings have mutations in their *embB* gene [3–7]. Several Indian studies have also reported EMB resistance-associated mutations in MTB isolates [8–10]. However, there are still ca. 25–50% of EMB-resistant MTB isolates that lack mutations in the genes sequenced in various studies, including *embCAB*, *iniBAC*, *rmlD*, *rmlA2* and *wbbL* [2,8,10]. As limited information is available regarding the role of all the *embCAB* genes in EMB resistance, there is a need for more in-depth studies to decipher the mechanisms of EMB resistance in MTB isolates. The present study was undertaken with the objective of characterising mutations in *embCAB* genes of MTB isolates collected in India as well as their association with the level of EMB resistance.

2. Material and methods

2.1. *Mycobacterium tuberculosis* isolates

A total of 7 EMB-sensitive and 23 EMB-resistant MTB isolates from the Mycobacterial Repository Centre of the National JALMA Institute for Leprosy and Other Mycobacterial Diseases (Agra, India) were included in the present study for characterisation of their *embCAB* genes. Random sampling was used to select both EMB-sensitive and -resistant isolates separately from the EMB-sensitive and -resistant isolates deposited in the repository during the period September 2004 to August 2005. The included isolates were obtained both from treated and untreated patients. Geographically, the isolates were from North Indian districts, including isolates from Agra ($n = 18$), Kanpur ($n = 9$) and Jaipur ($n = 3$). MTB isolates were freshly subcultured on Löwenstein–Jensen (LJ) medium before being used for further microbiological assays.

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Table 1
Summary of the primer pairs used for polymerase chain reaction amplification of *embCAB* genes.

Gene	Primers	Sequence (5′ → 3′)	Annealing temperature (°C)	Amplicon size (bp)
embC	embC(F)	GATACCCGCTACAGCAGCA	63.5	334
	embC(R)	GGTCGTAGTACCAGCCGAAA		
embA	embA(F)	GCCGGCTATGTAGCCAACTA	63.0	338
	embA(R)	GACCGTTCCACCAACACC		
embB	embB1(F)	CTGAAACTGCTGGCGATCAT	65.0	415
	embB1(R)	GGTCTGGCAGGCGCATCC		
	embB2(F)	TGGAGGCCAGCAAACCCG	65.5	451
	embB2(R)	TAGTAGTAACGCAGGTTCTC		
	embB3(F)	TTCGCCCGAGCAAAGATG	65.5	368
	embB3(R)	TCGCGGGACAGGTAGGTG		

2.2. Drug susceptibility testing

Drug susceptibility testing of MTB isolates to the antitubercular drugs RIF, INH, EMB and STR (Sigma Chemical Co., St Louis, MO) was done on LJ medium according to the standard proportion method [11]. An isolate showing ≥1% growth on drug-containing slopes in comparison with drug-free slopes was considered resistant, otherwise it was considered as sensitive.

2.3. Minimum inhibitory concentration (MIC) determination by the resazurin microtitre assay (REMA)

All the isolates were tested for determination of their MICs to EMB by the REMA plate method exactly as described previously [11]. EMB concentrations prepared directly in Middlebrook 7H9 broth (Difco, Franklin Lakes, NJ) were 1.25, 2.5, 3.75, 5.0, 6.25, 7.5, 8.75 and 10.0 mg/L. A standard bacterial suspension of No. 1 McFarland standard was prepared and diluted 1:20 in 7H9 broth. Then, 100 μL of inoculum was used to inoculate each well of the REMA plate. Plates were sealed and incubated at 37 °C for 1 week. Twenty-five microlitres of 0.02% resazurin (Sigma Chemical Co.) solution were added to each well and plates were re-incubated for an additional 2 days. Each isolate was tested in triplicate and actual MICs were read as described previously [11].

2.4. DNA isolation and polymerase chain reaction (PCR) sequencing

Mycobacterial DNA was extracted as per the standard physicochemical procedure standardised in our laboratory. PCR was performed for various gene loci of *embC*, *embA* and *embB* using the primer sets described in Table 1. Amplified gene products were extracted from agarose gel using QIAEX® II Gel Extraction Kit (QIAGEN, Hilden, Germany). Sequencing reactions of the amplified products were performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) followed by DNA sequencing in an ABI 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Sequences

Table 2
Results of susceptibility testing and DNA sequence analysis of *Mycobacterium tuberculosis* isolates.

Isolate code	Resistance	Ethambutol MIC (mg/L)	Nucleotide polymorphisms and amino acid changes in:	
			embC gene	*embB* gene
DKU-324	–	0.625	wild-type	wild-type
DKU-291	H	1.875	wild-type	wild-type
DKU-276	HS	1.25	wild-type	wild-type
DKR-190	HS	1.25	wild-type	wild-type
JAL-548	RH	2.5	wild-type	wild-type
JAL-254	RHS	2.5	wild-type	wild-type
JAL-634	RHS	2.5	wild-type	wild-type
JAL-595	RHE	>5	wild-type	ATG → GTG (Met306Val)
DKR-111	HES	3.125	wild-type	ATG → ATA (Met306Ile)
JAL-427	RHES	4.375	wild-type	wild-type
JAL-313	RHES	3.125	wild-type	wild-type
JAL-335	RHES	3.125	wild-type	wild-type
DRF-107	RHES	>5	wild-type	wild-type
BC-752	RHES	3.75	wild-type	wild-type
JAL-384	RHES	>5	wild-type	wild-type
JAL-267	RHES	>5	ACC → ATC (Thr270Ile)	wild-type
JAL-250	RHES	4.375	wild-type	wild-type
JAL-419	RHES	3.125	wild-type	ATG → GTG (Met306Val)
BC-788	RHES	5	wild-type	ATG → GTG (Met306Val)
BC-754	RHES	5	wild-type	ATG → GTG (Met306Val)
DKU-311	RHES	>5	ACC → ATC (Thr270Ile)	ATG → GTG (Met306Val)
DRF-110	RHES	5	wild-type	ATG → GTG (Met306Val)
JAL-588	RHES	>5	wild-type	ATG → GTG (Met306Val)
DAR-122	RHES	4.375	wild-type	ATG → GTG (Met306Val)
JAL-577	RHES	>5	wild-type	ATG → GTG (Met306Val)
DRF-139	RHES	>5	wild-type	ATG → GTG (Met306Val)
JAL-249	RHES	5	ACC → ATC (Thr270Ile)	ATG → ATA (Met306Ile)
JAL-584	RHES	>5	wild-type	ATG → ATA (Met306Ile)
JAL-276	RHES	3.125	wild-type	ATG → ATA (Met306Ile)
JAL-434	RHES	3.125	wild-type	ATG → ATA (Met306Ile)

MIC, minimum inhibitory concentration; H, isoniazid; S, streptomycin; R, rifampicin; E, ethambutol.