Studies on mutations in *embB* locus in Indian clinical isolates of *Mycobacterium tuberculosis* having high degree of ethambutol resistance

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Ethambutol [EMB: dextro-2.2′-(ethylenediimino)-di-l-butanol], a frontline antituberculosis drug, targets the mycobacterial cell wall. Genetic events resulting in structural mutations at the *embB* locus have been proposed as a major mechanism for EMB resistance in *Mycobacterium tuberculosis*. In this study, mutations in the *embB* locus of Indian isolates and their correlation with the MIC levels in *M. tuberculosis* have been investigated. We have sequenced and analysed 260 bp of the *embB* gene region containing the hotspot association with EMB-resistance isolates of *M. tuberculosis* in 39 EMB-resistant and 13 susceptible clinical isolates. Out of 39 resistant isolates, 17 (44%) had mutation in the *embB* locus at codon 306, whereas the remaining 22 (56%) resistant and 13 sensitive isolates did not have any mutation in this region. Five different substitution mutations of ATG(Met) in the resistant strains were: GTG (Val) 41%, ATA (Ile) 29%, ATC (Ile) 18%, TTG (Leu) 6% and CTG (Leu) 6%. All these mutations at codon 306 were associated with a high degree of ethambutol-resistant phenotype. Results suggest that the *embB306* mutations might prove to be one of useful markers for detection of ethambutol resistance in isolates of *M. tuberculosis* in India. Lack of mutation, however, does not rule out ethambutol resistance.

Keywords: Ethambutol resistance, *embB* mutation, Indian isolates, *Mycobacterium tuberculosis*.

ETHAMBUTOL (EMB) is an important first line of antituberculosis drug and is used for treatment of disease caused by *Mycobacterium tuberculosis* as well as infection caused by *M. avium* complex[1,2]. The primary target of EMB on mycobacterial cell wall is an outer layer of mycolic acids covalentaly bound to peptidoglycon via arabinogalactan[3]. EMB inhibits polymerization of cell-wall arabinon, which causes an accumulation of the lipid carrier decaprenol phosphoarabinose. It has been suggested that the drug interferes with the transfer of arabinose to the cell-wall acceptor[2,4]. The *embCAB* genetic locus of *M. tuberculosis* for resistance has been proposed to encode for mycobacterial transferases[5]. Earlier studies have shown that genetic alteration at codon 306 of the *embB* resulting in amino acid substitution, is associated with ethambutol resistance[6–8]. As novel mutations may be responsible for drug resistance in isolates from different countries[9], it becomes important to gather such information for EMB resistance from India. In the present study, the sequence polymorphism of the *embB* region containing codon 306 has been investigated in clinical isolates of *M. tuberculosis*.

We have randomly selected 52 isolates from previously treated patients of pulmonary tuberculosis, which were deposited in the Mycobacterial Repository Centre at the National JALMA Institute for Leprosy and other Mycobacterial Diseases, from various parts of India (Jaipur 29, Varanasi 6, Agra 6, Haridwar 4, Port Blair 3, Kanpur 2, Delhi 1, Lucknow 1) and identified as *M. tuberculosis* according to standard criteria[10]. These isolates along with *M. tuberculosis* $H_{37}R_v$ were tested for drug sensitivity using Lowenstein–Jensen (LJ) medium containing MIC cut-off levels for resistance–rifampicin 64 μg/ml, isoniazid 1 μg/ml (refs 11–13) and ethambutol 2, 4, 6, 8 μg/ml (ref. 10). The peak plasma concentration achieved[14,15] with a single adult dose is 3.5–5 μg/ml. Therefore we have selected 8 μg/ml as MIC cut-off level for screening of EMB resistance. All these drugs were obtained from Sigma Chemical Co, USA. A standard bacilli suspension (4 mg/ml) prepared according to the procedure described by Canetti *et al.*[11] was used for inoculation onto LJ medium slants with loop of 3 mm internal diameter. All the inoculated bottles were incubated at 37°C. Growth was observed on the 28th day. MIC was determined using standard criteria of counting the colony-forming units (CFUs) and comparing with culture controls[11,12,16].

DNA from the growth of each isolate on LJ slant was extracted using the method of van Embden *et al.*[17] using lysozyme, proteinase-K, deproteinization and precipitation with isopropanol. A 260 bp region of *embB* locus from codon 284 to codon 350 of published sequence (U68480) was amplified using the PCR reaction set up in a 50 μl volume containing 200 ng of genomic DNA, 1X reaction buffer (50 mM KCl, Tris-HCl, pH-9.0), 0.2 mM each of dNTPs, 1 unit of Taq DNA polymerase, 1.5 mM $MgCl_2$ and 0.1 μM each of the primers: forward, 5′-CGG CAT GCG CCG GCT GAT TC-3′ and reverse 5′-CCA CAG ACT GGC GTC GCTG-3′ (ref. 18). The reaction mixture was then processed in a 'Gene Amp PCR System 9700' thermal cycler (Applied Biosystems, USA), using the following parameters: initial denaturation 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extension step at 72°C for 2 min. Final extension was done at 72°C for 10 min.

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The amplicon was resolved on 1% agarose gel and 260 bp of the band was excised. DNA was extracted from the gel slices using QIAEX^{R} II Gel Extraction kit (QIAGEN, USA). Each sequencing reaction of 20 µl volume consisted of 200 ng template, 3.5 pmol primer, 8 µl sequencing mix (Big Dye Terminator Cycle Sequencing kit V3.1, Applied Biosystems) and adjusted to a final volume of 20 µl by adding distilled water. The cycling parameters were as follows: 30 cycles of 96°C for 10 s and 65°C for 4 min. PCR product was purified to remove unincorporated labelled ddNTPs. This was done by adding 0.1 volume of 3M sodium acetate (pH 4.5) and 2.5 volume of absolute ethanol. Sequencing of the amplicon was carried out using the ABI PRISM 310 Genetic Analyzer. Analysis 3.3^{TM} was used for analysing gel information. Sequences generated using the program were compared to their respective wild type ($H_{37}R_v$) sequences using MegAlign program (DNASTAR, Madison, WI, USA).

Out of 52 isolates studied, 39 EMB-resistant (8 µg/ml) isolates were further evaluated using EMB concentration of 10, 20 and 40 µg/ml. Eight out of 39 (20.5%) isolates exhibited MIC level ≥ 20–40 µg/ml and showed mutation Met306Ile. Nine out of 39 (23%) isolates exhibited MIC levels ≥ 40 µg/ml and showed mutation Met306Leu,Val. Twenty-two out of 39 (56%) resistant (8 µg/ml) isolates were found sensitive at 10, 20 µg/ml and showed no mutation in the *embB* locus. The mutations identified included ATG → GTG (Met306Val) 7/17(41%), ATG → ATA (Met306Ile) 5/17 (29%), ATG → ATC (Met306Ile) 3/17 (18%), ATG → TTG (Met306Leu) 1/17 (6%) and ATG → CTG (Met 306 Leu) 1/17 (6%) substitutions. All of these mutations were found only at codon 306 in the *embB* locus. None of the mutations at codon 306 had any associations with EMB resistance with others drugs like RFM + INH (MDR), EMB and RFM resistance or EMB and INH resistance alone. These results are summarized in Table 1.

Area-wise, distribution of the nature of mutations did not vary significantly. Twelve (44%) out of a total of 27 resistant isolates from Jaipur 7 (26%) had Met306Ile and five (18%) had Met306Val substitution. Considering two (50%) out of total four resistant isolates from Varanasi, one (25%) had Met306Val and the other (25%) had Met306Leu substitution. One (25%) out of total four resistant isolates from Haridwar region had Met306Val substitution. Both the isolates from Kanpur region were resistant, out of which one (50%) had Met306Ile and other (50%) had Met306Leu substitution. Among 13 resistant isolates from Agra (6), Port Blair (3), Lucknow (1) and New Delhi (1), none had any mutation in this *embB* gene region.

The region of ethambutol targetting *embB* locus contains sequences which are conserved in *M. tuberculosis*, *M. avium, M. leprae* and *M. smegmatis*[2,5]. Among these four species, the Met residue is present at position 306. Biochemical and sequence homology suggests that *embB* is glycosyltransferase[2]. In the present study the aim was to identify the presence or absence of mutations at the *embB* locus in resistant as well as sensitive isolates of *M. tuberculosis* in India. In our study 44% of EMB-resistant isolates of *M. tuberculosis* exhibited mutations at codon 306 in the *embB* locus. Other studies have also reported similar trend, e.g. Russian study – 48.28% (ref. 19), German study – 50% (ref. 7) and American study – 29% (ref. 6).

MIC levels of EMB were higher for Met306Leu or Met306Val substitution (≥ 40 µg/ml) than Met306Ile (≥ 20–40 µg/ml) substitution. Our observations are similar to those reported by Sreevatsan *et al.*[8]. These *embB306* mutations were found only in EMB-resistant and not EMB-susceptible isolates. One study had reported mutations at Ser297Ala, Asp328Gly, Phe330Val Gly406Asp, Asp959Ala, Met1000Arg and Asp1024Asn[6], while another study had reported mutations at Gly497Arg in EMB-resistant isolates of *M. tuberculosis*[20]. However, in our

Table 1. Results of relationship between drug susceptibility profiles and sequencing results of *embB* locus in *Mycobacterium tuberculosis* isolates

Isolates investigated	No. of isolates	EMB sensitivity	Codon alteration	Amino acid change
Resistant to EMB + RFM + INH	6	R	ATG → GTG	Met306Val
	4	R	ATG → ATA	Met306Ile
	3	R	ATG → ATC	Met306Ile
	1	R	ATG → CTG	Met306Leu
	1	R	ATG → TTG	Met306Leu
Resistant to EMB + RFM	1	R	ATG → GTG	Met306Val
Resistant to EMB + INH	1	R	ATG → ATA	Met306Ile
Resistant to EMB + RFM + INH	16	R	None	None
Resistant to EMB + RFM	3	R	None	None
Resistant to EMB	3	R	None	None
Resistant to RFM + INH	6	S	None	None
Resistant to RFM	3	S	None	None
Resistant to INH	1	S	None	None
Sensitive to EMB	3	S	None	None
Total no. isolates	52			

EMB, Ethambutol; RFM, Rifampicin; INH, Isoniazid; R, Resistant; S, Sensitive.

study no other known/novel mutation was found at the *embB* locus between codon 284 and 350 in resistant isolates of *M. tuberculosis*. The presence of *embB306* mutation did not correlate with MDR (Table 1) and similar findings have been reported by others as well[19]. Unlike the situation in the case of rifampicin resistance in our preliminary study, no novel mutation was found at the *embB* locus in Indian strains. Similar mutations in *embB306* have also been reported in other studies[6,8].

In the isolates studied Met replacement with Val (41% (7/17)) or Ile (47% (8/17)) in the 306th codon was observed in a significant proportion of EMB-resistant isolates. These mutations have been found to be less frequent in other studies. Met306Val substitution has been reported in 9% (ref. 6) and 34% (ref. 19) of EMB-resistant isolates from USA and Russia respectively. Likewise, Met306Ile substitution has been reported in 5% (ref. 6), 14% (ref. 19) and 27% (ref. 21) of isolates from other countries. Frequently observed mutations in EMB-resistant isolates in these studies were Met306Val and Met306Ile. For early detection of EMB resistance the associated *embB306* mutations could be of help in developing new techniques/probes for detection of EMB-resistant clinical isolates of *M. tuberculosis*. Presence of such mutations would be a definitive evidence of ethambutol; lack of such mutations will not be meaningful.

Our results also provide the information that 56% (22/39) of EMB-resistant isolates have no mutation at the *embB* locus. Thus the possibility of involvement of other mutation(s) in the region of *embB* loci, like embCA or other mechanisms like permeability and efflux-pumps also associated with EMB resistance cannot be ruled out. In-depth studies will be required to understand other alternate mechanisms of action of EMB responsible for resistance in nearly half of the EMB-resistant isolates of *M. tuberculosis* in India and other parts of the world.

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