

## Novel mutations in *emb B* gene of ethambutol resistant isolates of *Mycobacterium tuberculosis*: A preliminary report

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**Background & objectives:** Ethambutol (EMB) resistance, thought to be occurring due to mutations in *embB* gene of *Mycobacterium tuberculosis* on the rise is a cause of grave concern. The present study was planned to investigate the presence of EMB resistance in *M. tuberculosis* isolates and to look for prevalent mutations in *embB* gene.

**Methods:** A total of 591(283 from new and 308 from previously treated cases) sputum samples from the same number of pulmonary tuberculosis cases were cultured. Isolates were tested by 1 per cent proportion method for resistance to isoniazid, rifampicin streptomycin and ethambutol. Minimum inhibitory concentration (MIC) of EMB was measured by absolute concentration method. Ten randomly selected isolates were subjected to single strand conformational polymorphism (SSCP) and direct DNA sequencing to look for mutation in 364 bp segments of *embB* gene.

**Results:** Of 353 isolates of *M. tuberculosis* from 591 sputum samples, 62 (17.58%) were resistant to EMB, of which, 16 (25.8%) showed initial resistance and 46 (74.2%) acquired. Mono resistance to EMB was rare. Only two isolates showed resistance to EMB alone. From 62 EMB resistant isolates, 88.7 per cent (55) were resistant to INH, 82.2 per cent (51) to rifampicin and 61.2 per cent (38) were resistant to streptomycin. Co-resistance to isoniazid and rifampicin (multidrug resistant, MDR-TB) with EMB resistance was seen in 41(66.1%) isolates. High level of EMB resistance was seen in 16.5 per cent isolates. SSCP showed altered mobility in 8 of 10 isolates tested. Among the 8 mutants, 4 had known mutations at codon Met 306 being replaced by Val/ Leu. The second most frequent mutation encountered was at codon Phe 287 being replaced by Val, Cys or Leu (novel mutations). Sequence analysis revealed 10 novel mutations in codon 221, 225, 227, 271, 272, 281, 282, 287, 293 and 294 within *embB* gene.

**Interpretation & conclusions:** Presence of high frequency of EMB resistance, occurrence of high level EMB resistance, co-existence of MDR-TB with EMB resistance and novel mutations in *emb B* gene of *M. tuberculosis* clinical isolates reported highlight the need to work on larger samples to identify the diagnostic marker of EMB resistance in mycobacteria.

**Key words** Ethambutol resistance - *embB* gene - novel mutation - *Mycobacterium tuberculosis*

Ethambutol (EMB) has been used as a primary drug for the treatment of tuberculosis since 1966 in combination with other drugs<sup>1</sup>. It is also advocated for use in treating *Mycobacterium avium* infections in HIV/TB patients, infections due to mycobacteria other than tuberculosis (MOTT) and even multidrug resistant tuberculosis<sup>2</sup>. Further, streptomycin has been replaced by ethambutol as a key drug in the intensive phase of tuberculosis chemotherapy as it is less expensive and patient's compliance is better with this drug<sup>3</sup>. Although initial resistance to EMB was not commonly reported in the early years of its discovery, global initial prevalence was reported to be between 0-4.2 per cent<sup>4</sup>.

EMB targets the mycobacterial cell wall through interaction with arabinosyl transferases involved in arabinogalactan (AG) and lipoarabinomannan (LAM) biosynthesis<sup>5,6</sup>. It specifically inhibits polymerization of cell wall arabinan, thereby leading to accumulation of  $\beta$ -D-arabinofuranosyl-1-monophosphoryldecaprenol (DPA)<sup>7</sup>. AG deprivation is also responsible for accumulation of mycolic acid in *M. smegmatis*, a result consistent with the finding that EMB causes declumping and morphological changes<sup>8</sup>. Molecular studies suggest that arabinosyl transferases are encoded by homologous genes belonging to the *emb* operon and have been identified in *M. smegmatis*, *M. tuberculosis* and *M. leprae* as *embC*, *embA* and *embB*<sup>9,10</sup>. Mutations leading to replacement of amino acid residues are found to be present in EMB-resistant organisms. Most studies have shown that 65 per cent of EMB resistant clinical isolates harbour mutations at the 306 amino acid positions in *emb B* gene, making it the ethambutol resistance determining region (ERDR)<sup>9,11</sup>. Identification of additional mutations occurring in EMB-resistant organisms will be useful in further understanding of the mechanisms of resistance to this primary antituberculosis agent.

The present study was therefore planned to find the presence of EMB resistance in *M. tuberculosis* isolates obtained from cases of pulmonary tuberculosis. Mutations in *embB* gene of EMB resistant isolates of *M. tuberculosis* were also studied in a few randomly selected isolates.

### Material & Methods

*Sample collection, history taking and sample processing:* A total of 591 (283 from new and 308 from previously treated cases) sputum samples collected from the same number of patients with pulmonary

tuberculosis who were referred to the Department of Microbiology, Chhatrapati Sahuji Medical University, Lucknow, were enrolled consecutively during 2002 and 2004. Patients were interviewed to determine their prior history of anti-tuberculosis drug intake. The study protocol was approved by the institutional ethical committee. Sputum samples were checked for presence of acid fast bacilli (AFB) by smear microscopy according to Revised National Tuberculosis Control Programme (RNTCP) guidelines<sup>12</sup> and cultured on Lowenstein-Jenson (L-J) media after decontamination by Petroff's method<sup>13</sup>. The culture bottles were incubated at 37°C and read weekly for eight weeks. No growth after eight week of incubation was treated as negative. Growth of *M. tuberculosis* was confirmed by colony appearance, niacin production, catalase activity at 68°C, pH 7 and susceptibility to p-nitrobenzoic acid<sup>14</sup>. Drug susceptibility testing (DST) for all *M. tuberculosis* isolates was performed by 1 per cent proportion sensitivity testing methods<sup>15</sup> against streptomycin (4 µg/ml), isoniazid (INH) (0.2 µg/ml), rifampicin (40µg/ml) and ethambutol (4µg/ml). External quality control was provided by Tuberculosis Research Center, Chennai. Resistance noted in isolates from patients who gave positive history of taking antitubercular treatment in the past was defined as acquired resistance, while the rest were labelled as initial resistance.

Minimum inhibitory concentration (MIC) of EMB was determined by absolute concentration method, using different drug concentrations *i.e.*, 2, 4, 8, 10 and 20 µg/ml on L-J medium. Interpretation of results was done after 28 days of incubation at 37°C. *M. tuberculosis* H37Rv was used as control in each set of experiment. MIC of EMB >20 µg/ml was defined as high level resistance to ethambutol.

*Molecular procedures:* Genomic DNA was extracted from mycobacterial cultures by the method described by Jain *et al*<sup>16</sup>. DNA isolated from *M. tuberculosis* H37Rv strain was used for control. The 364 bp region of the *M. tuberculosis embB* gene was amplified by PCR using primers – 5' - CCGACCACGCTGAAACTG - 3' and 5' GTAATACCAGCCGAAGGGATCCT-3' in such a way that spanned the ERDR, *emb B* gene, codon 189-310<sup>17</sup>. PCR was performed according to standard reaction mixture with the following cycling parameters<sup>17</sup>: denaturation at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 1 min. Each reaction was preceded by an initial denaturation at 94°C for 1 min and terminated with a final extension at

72°C for 5 min. Amplified PCR product was analyzed on 1.5 per cent agarose gel stained with ethidium bromide. PCR for SSCP was carried out with addition of  $\alpha$ -P<sup>32</sup>-CTP for radiolabelling. All the radioactivity experiments were carried out in radio hazard chamber. For single strand conformational polymorphism (SSCP)-PCR same cycling parameters were used as described above. Final PCR product was analyzed on 1.5 per cent agarose gel stained with ethidium bromide. In each set of reaction one negative control (sample without template DNA) and one positive control (*M. tuberculosis* H37Rv DNA) was included. PCR product was gel-eluted from low melting agarose by previously described methods<sup>18</sup>. After heating for 5 min at 96°C with an equal volume of loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol and 95% formamide), PCR product was snap-cooled and immediately loaded onto the gels (6% polyacrylamide gel containing 5% glycerine). Electrophoresis was done at 200 V for 3 h (gel plates were cooled at 10°C). The gels were exposed on Kodak X-ray film. After 4 h of exposure, the film was developed in an automated developer. The experiment was performed twice to confirm the polymorphism profile.

Automatic nucleotide sequencing was done to identify single nucleotide polymorphism with ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin Elmer, Massachusetts, USA) and the ABI PRISM 310 automatic sequencer (Applied Biosystems, California, USA). Nucleotide sequences were translated to amino acids using Bio Edit software, version 7.0.5.2. ([www.bioedit.ncsu.edu](http://www.bioedit.ncsu.edu)). The nucleotide sequence and translated amino acids were aligned with the Clustal W function of the Bio Edit Sequence Alignment Tool and compared with the corresponding sequence of *M. tuberculosis* H37Rv sequence (accession number NP-218312).

**Reproducibility:** All experiments were performed twice to confirm the polymorphism profile and sequence data with reverse primers including drug susceptibility test.

## Results

Of 368 mycobacterial isolates (166 from new cases, 202 from treated cases), 353 were *M. tuberculosis* and 15 were MOTT (mycobacteria other than tuberculosis). MOTT were not further typed/tested. Of the 353 *M. tuberculosis* isolates 62 (17.5%) were found to be EMB resistant, of which 16 had initial and 46 had acquired resistance. Mono resistance to EMB was rare. Only two isolates showed resistance to EMB alone. Co-resistance with INH, rifampicin and streptomycin

was common. Of the 62 EMB-resistant isolates, 88.7 per cent (55) were resistant to INH, 82.2 per cent (51) were resistant to rifampicin and 61.2 per cent (38) were resistant to streptomycin. Fifty (80.6%) EMB resistant isolates were resistant to INH and rifampicin (MDR-TB) both (Table I).

MIC range of EMB resistant isolates were in between 4 to >20 µg/ml. Only 10 of 62 (16.1%) EMB-resistant isolates showed high level EMB resistance (MIC >20 µg/ml). Of the 41 EMB resistant MDR isolates, 6 (14.6%) had high level of EMB resistance (Table II).

Ten randomly selected EMB resistant isolates were subjected to SSCP and DNA sequencing to look for mutations in 364 bp fragments of *embB* gene. Altered mobility was seen in only 8 isolates when compared with *M. tuberculosis* H37RV genome (Fig.). Two isolates did not

**Table I.** Summary patterns of anti-tuberculosis drug resistance in all isolates

|                            | New cases<br>n (%) | Treated<br>cases<br>n (%) | Total<br>n (%) |
|----------------------------|--------------------|---------------------------|----------------|
| Total tested               | 283                | 308                       | 591            |
| Culture positive           | 166 (58.6)         | 202 (65.5)                | 368 (62.2)     |
| <i>M. tuberculosis</i> *   | 159 (57.2)         | 191 (62.0)                | 353 (59.7)     |
| Any drug R                 | 48 (29.6)          | 84 (43.9)                 | 132 (37.3)     |
| R to STM                   | 13 (8.0)           | 8 (4.1)                   | 21 (5.9)       |
| R to INH                   | 3 (1.8)            | 4 (2.0)                   | 7 (1.9)        |
| R to RMP                   | 1 (0.61)           | 0                         | 1 (0.28)       |
| R to EMB                   | 0                  | 2 (1.0)                   | 2 (0.56)       |
| R to INH + RMP             | 2 (1.2)            | 3 (1.5)                   | 5 (1.4)        |
| R to INH + RMP + EMB       | 9 (5.5)            | 10 (5.2)                  | 19 (5.3)       |
| R to INH + RMP + STM       | 2 (1.2)            | 11 (5.7)                  | 13 (3.6)       |
| R to STM + INH + RMP + EMB | 8 (4.9)            | 23 (12.0)                 | 31 (8.7)       |
| Rest to STM + INH + EMB    | 0                  | 2 (1.0)                   | 2 (0.56)       |
| R to INH + EMB             | 1 (0.61)           | 1 (0.52)                  | 3 (0.84)       |
| R to STM + INH             | 7 (4.3)            | 16 (8.2)                  | 23 (6.5)       |
| R to STM + EMB             | 2 (1.2)            | 3 (1.5)                   | 5 (1.4)        |
| R to RMP + EMB             | 0                  | 1 (0.52)                  | 1 (0.28)       |
| Any R to STM               | 32 (19.7)          | 63 (32.9)                 | 95 (26.9)      |
| Any R to INH               | 32 (19.7)          | 70 (36.6)                 | 102 (28.8)     |
| Any R to RMP               | 22 (13.8)          | 48 (25.1)                 | 70 (19.8)      |
| Any R to EMB               | 20 (12.3)          | 42 (21.4)                 | 62 (17.5)      |

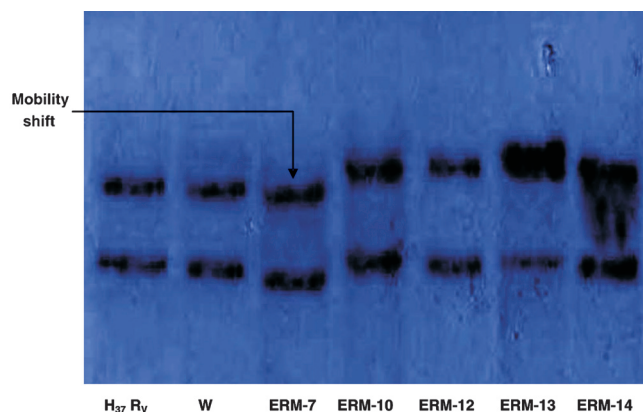
\*Denominator for calculating drug resistance. R, resistant; STM, streptomycin; INH, isoniazid; RMP, rifampicin; EMB, ethambutol

**Table II.** Level of ethambutol resistance in clinical *M. tuberculosis* isolates

| MIC level (µl/ml)         | 2 | 4  | 8  | 10 | 20 | >20 | Total |
|---------------------------|---|----|----|----|----|-----|-------|
| Isolates resistant to EMB | 0 | 13 | 11 | 23 | 5  | 10  | 62    |
| MDR with EMB resistance   | 0 | 6  | 9  | 17 | 3  | 6   | 41    |

EMB, ethambutol; MDR, multi drug resistant

show altered mobility pattern hence were not sequenced. Mutations were seen in all eight isolates. Five isolates had mutations in more than one loci and three had mutations at single locus. Among the 8 mutants, 4 had mutation at



**Fig.** Mobility shift in *emb B* gene as seen by SSCP. Lane 1= *M. tuberculosis* H<sub>37</sub>R<sub>v</sub>, W= wild type strains showing no mutation, strain numbers. ERM 7 - ERM 14 showing mobility shift.

codon Met 306 being replaced by Val/ Leu (previously reported accession numbers AY198118, AY 198119). The second most frequent mutation encountered was at codon Phe 287 being replaced by Val, Cys or Leu (novel mutations) followed by other mutations *viz.*, Ala221Gly, Ala225Ser, Val227Gly Ala271Val, Ser272Cys, Ser272Ile, Ala281Pro, Val282Leu, Ile293Asn, Ile293Asn, Gly294Ser, Gly294Asp, Ala310Thr, Ala310Asp and Asp311Asn (Table III). Seven of the eight isolates had at least one previously reported mutation while one EMB resistant isolate had 3 novel mutations only. The mutations in codon 221, 225, 227, 271, 272, 281, 282, 287, 293 and 294, encountered in our isolates were novel mutations within the *embB* gene of *M. tuberculosis*.

### Discussion

Only a few reports available from India have reported variation in frequency of EMB resistance from different geographic area. A study by Paramasivan *et al*<sup>19</sup> stated that EMB resistance was 3.2-4.6 per cent among new cases of tuberculosis in two districts of Tamilnadu<sup>19</sup>.

**Table III.** Molecular characteristics of eight EMB-resistant *M. tuberculosis* isolates with mutations at *emb B* locus

| Isolate no. | EMB MIC (µg/ml) | Base change  | Amino acid change  | Accession no.** | Resistance to STM | Resistance to RMP | Resistance to INH | Ac/In EMR resistance |
|-------------|-----------------|--|--|-----------------|-------------------|-------------------|-------------------|----------------------|
| ERM-3       | 10              | ATG→GTG  | Met306Val*   |                 | S                 | R                 | R                 | Ac                   |
| ERM-4       | >20             | ATG→GTC  | Met306Val*   |                 | R                 | R                 | R                 | In                   |
| ERM-7       | >20             | GCG→AGC<br>GCG→GTG<br>GCG→CCG<br>ATT→AAC<br>ATG→GTC<br>GCG→ACC | Ala225Ser<br>Ala271Val<br>Ala281Pro<br>Ile293Asn<br>Met306Val*<br>Ala310Thr* | EF376189        | R                 | R                 | R                 | Ac                   |
| ERM-9       | 20              | ATG→TTG  | Met306leu*   |                 | R                 | R                 | R                 | Ac                   |
| ERM-10      | >20             | GCG→GGC<br>GTG→GGC<br>TTT→GTG<br>GCG→GAT                       | Ala221Gly<br>Val227Gly<br>Phe287Val<br>Ala310Asp*                            | EF376190        | R                 | R                 | R                 | Ac                   |
| ERM-12      | >20             | AGC→TGC<br>TTT→TGC<br>GGC→AGC                                  | Ser272Cys<br>Phe287Cys<br>Gly294Ser  | EF376191        | R                 | R                 | R                 | Ac                   |
| ERM-13      | >20             | GCG→AGC<br>GCG→GTG<br>GCG→CCG<br>ATT→AAC<br>ATT→AAC            | Ala225ser<br>Ala271Val<br>Ala281Pro<br>Ile293Asn<br>Ala310Thr*               | EF376192        | R                 | R                 | R                 | Ac                   |
| ERM-14      | 20              | AGC→ATT<br>GTG→CTG<br>TTT→CTG<br>GGC→GAT<br>GTG→AAC            | Ser272Ile<br>Val282Leu<br>Phe281Leu<br>Gly293Asp<br>Asp311Asn*               | EF376193        | R                 | R                 | R                 | In                   |

\*Mutations reported earlier, \*\* only accession numbers of novel mutations reported in present study are listed here. STM, streptomycin; RMP, rifampicin; INH, isoniazid; EMB, ethambutol; R, resistant; S, sensitive; In, initial; Ac, acquired

A recent study reported overall 14.2 per cent EMB resistance in *M. tuberculosis* isolates<sup>20</sup> while previous studies from Jaipur<sup>21</sup> and Gujarat<sup>22</sup> region reported 2.0 and 2.5 per cent EMB resistance respectively. Acquired and initial EMB resistance reported from Jodhpur was 39.4 and 6.6 per cent respectively<sup>23</sup>. Current high level of acquired drug resistance to EMB reflected irregular use of this drug in the area over several years.

Role of ethambutol in treating MDR-TB can be challenged as co-existence of ethambutol and rifampicin resistance is frequently noted. However, a survey done for more than 40 yr by Tuberculosis Research Center, Chennai reported that addition of ethambutol to treat streptomycin and INH resistant isolates in post rifampicin era, in short course chemotherapy regimen gave excellent result with 91 per cent cure rate among TB patients, when treated with rifampicin and ethambutol in addition to kanamycin<sup>24</sup>. We also noted that high level ethambutol resistance was infrequent. More so, frequency of high level ethambutol resistance in MDR and non-MDR isolates was almost same. This may partially explain the successful use of this drug in MDR-TB cases. Ethambutol is also used in category II and III of Directly Observed Treatment Short course (DOTS) regimens<sup>25</sup>. Continuous monitoring of high-level ethambutol resistance and follow up of response to treatment in category II and III will be helpful to draw guidelines regarding use of EMB in category II and category III subjects. Worldwide studies have reported that 50-60 per cent of EMB resistant strains have mutations in *embB* gene<sup>9,10,11,26,27</sup>. In addition to the documented mutation at amino acid position 306 of the *embB* gene, we observed five novel mutations. These can provide important information regarding prevalence of any particular type of codon replacement in Indian isolates, which are known to be genetically different. The most frequent mutation observed to date was within ERDR, *i.e.*, codon Met 306 being replaced by Val/ Ile/ Leu. Also, 4 of our isolates showed this mutation. This mutation may result in increased hydrophobicity of the surrounding region, suggesting inaccessibility of EMB to its binding site<sup>17</sup>. An other most frequent mutation encountered by us was at codon Phe287 being replaced by Val/ Cys/ Leu. Multiple amino acid replacements in *embB* gene were reported previously<sup>9</sup>. The occurrence of multiple mutations in these strains that affect the same codon and produce different amino acid replacements, can be a hallmark of Darwinian selection by antibiotic pressure<sup>11</sup>. Except mutations seen in codon 306, 310 and 311 others

were not described earlier and are registered as new mutations in the ERDR region of *embB* gene. Several studies have reported an association of high degree EMB resistance and mutation in the gene responsible for ethambutol resistance<sup>9,11</sup>. However, our limited experience is not sufficient to prove any significant association. Earlier reports suggested that targeting amino acids are 239-311<sup>17</sup> and 300- 500<sup>11</sup> of the *embB* gene, but our results suggested that alterations could also be present from 223-311 codon of the *embB* gene. Presence of mutation at Met306 was found to be associated with moderate to high level EMB resistance and this is consistent with previous studies<sup>26-28</sup>. In the present study, we reported 5 novel mutations in *embB* gene, which was also associated with high level of ethambutol resistance like Met 306 mutations. Further analysis of such isolates can explain the mechanism which would trigger these mutations associated with high level of EMB resistance. In the present study 2 of 10 isolates did not show change in mobility as such SSCP is not a very sensitive technique to look for mutations. As SSCP is commonly used for the detection of single point mutation in the genes. Sensitivity of this techniques depends on temperature, pH and fragment lengths of amplified products. Under optimal conditions, approximately 80 to 90 per cent of the potential base exchanges are detectable by SSCP. Ideally these isolates should also be sequenced to rule out presence of mutations in *embB* gene.

A few studies have also reported *embB* mutations in EMB susceptible *M. tuberculosis* isolates<sup>29,30</sup>. Association between *embB* mutations and resistance to increasing number of other antitubercular drugs is highly significant<sup>30</sup>. The exact mechanism of EMB resistance is not yet clearly understood. Some of the mutations reported here may be single nucleotide polymorphism, unrelated to EMB resistance. SSCP is not a very sensitive method and sequencing of the *embB* gene of all phenotypic EMB-resistant *M. tuberculosis* isolates, whether showing mobility shift or not on SSCP along with sequencing of a fair number of EMB sensitive isolates can give an idea about its role and significance.

In conclusion, our results showed presence of high EMB resistance in clinical *M. tuberculosis* isolates, occasional occurrence of high level ethambutol resistance, co-existence of MDR with EMB resistance and novel mutations in *embB* gene. Further studies are required to be done on a larger sample using better molecular techniques to confirm these findings.

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