Novel Mutations within the \textit{embB} Gene in Ethambutol-Susceptible Clinical Isolates of \textit{Mycobacterium tuberculosis}

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Ethambutol (EMB) ([\(\text{S,S}'\)]-2,2'-(ethylenediimino)di-1-butanol) is a first-line drug used for antituberculosis therapy. It is often used in combination with isoniazid, rifampin, pyrazinamide, and streptomycin. Membrane-associated arabinosyl transferases have been implicated as the targets for EMB (2, 3, 14, 15). The \textit{Mycobacterium tuberculosis} \textit{emb} operon is a gene cluster of three contiguous genes, namely, \textit{embC}, \textit{embA}, and \textit{embB}, which encode mycobacterial arabinosyl transferases (26). These enzymes are involved in the polymerization of the cell wall arabinan (4, 6, 9, 24, 25, 32). Inhibition of arabinan synthesis by EMB results in the accumulation of mycolic acids, leading to cell death.

Alterations at codon 306 of \textit{embB} have been identified as being the most common alteration in EMB-resistant \textit{M. tuberculosis} clinical isolates (8, 12, 17–20, 23, 29). Initial work on 51 EMB-resistant isolates had shown that 89% of these isolates had alterations at residue 306 of \textit{embB}, but these alterations were not detected in 30 EMB-susceptible isolates (23). A subsequent study confirmed this high frequency of \textit{embB}306 alterations, with 67% of 75 EMB-resistant isolates having mutations not found in EMB-susceptible strains (19). This led to several groups developing targeted strategies for the detection of \textit{embB}306 alterations (7, 16, 21, 30). Amino acids within the EMB resistance-determining region of EMB proteins are well conserved among mycobacterial species, including those from \textit{M. tuberculosis}, \textit{M. leprae}, and \textit{M. smegmatis} (2), and mutations within this region have been detected in EMB-resistant isolates of \textit{M. tuberculosis}.

The aim of this present work was to screen all regions of the \textit{embB} gene with previously reported mutations in order to assess the contribution of mutations within this gene to EMB resistance in \textit{M. tuberculosis} clinical isolates from Singapore.

Drug susceptibility testing was done using the BACTEC 460 radiometric method (Becton Dickinson, Towson, Md.) (2.5 \(\mu\)g/ml). Twenty-five consecutive \textit{M. tuberculosis} isolates resistant to EMB and 20 EMB-susceptible isolates from Singapore were collected as previously described (5, 10).

DNA extracted from the isolates was analyzed by amplifying four fragments, using the PCR primers shown in Table 1. The PCR products were purified (QIAquick PCR purification kit or QIAquick gel extraction kit; QIAGEN) and directly sequenced using the BigDye Terminator sequencing kit and the ABI PRISM 377 automated sequencer (PE Biosystems, Branchburg, N.J.). Confirmation of mutations was done by reamplification and resequencing.

\textit{IS6110} profiling was done according to standard procedures to determine if the isolates were epidemiologically independent (28). All isolates with the same nucleotide substitutions in this study were deemed to be epidemiologically unassociated as they had distinct \textit{IS6110} fingerprints.

Overall, mutations in the \textit{embB} gene were detected in 17 (68%) of the 25 EMB-resistant isolates (Table 2). Mutations at \textit{embB}306 were detected in 12 of these 25 (48%) EMB-resistant isolates. Notably, all of the 12 EMB-resistant isolates with \textit{embB}306 mutations were also resistant to isoniazid. All five EMB-resistant isolates with mutations at codon 497 were resistant to at least three antituberculosis drugs. Three isolates mono-resistant to EMB had no detectable mutations in \textit{embB}.

This is the first report of a double substitution (ATG\textarrow{\rightarrow}\text{ATM}, where M represents the nucleotides A and C), resulting in a Met\textarrow{\rightarrow}Ile alteration at the frequently altered codon 306 of \textit{embB} in an EMB-susceptible isolate (Table 2). This isolate was resistant to both isoniazid and rifampin.

In addition, three other alterations were also detected in EMB-susceptible isolates, G406D and two novel mutations, M423I and A659T. The isolate with the G406D alteration was also resistant to isoniazid and rifampin, while the isolate with the M423I alteration was mono-resistant to isoniazid and the isolate with the A659T alteration was mono-resistant to streptomycin. In total, alterations in the \textit{embB} gene were detected in 4 (20%) of the 20 EMB-susceptible isolates (Table 2).

There is a possibility that these mutations may have occurred in susceptible isolates due to cross-contamination of the PCR product, heteroresistance involving mixed cultures, or errors in the susceptibility testing, though every effort was undertaken to avoid this.

Alterations in \textit{embB} in EMB-susceptible isolates at codons other than codon 306 have been documented in only two isolates with the G406D alteration (20) and one isolate with the...
the predominant alteration in tuberculosis defining the role of the embB gene would be mutated. A similar scenario was observed in studies with polymorphisms, then only a minority of EMB-resistant isolates harboring no mutations (1, 12, 19, 23), and others not including embB306 S347T alteration (8). This paucity of information is due in part to include drug-susceptible isolates as controls.

Interestingly, all three EMB mono-resistant isolates in this present study did not have any detectable alterations in embB. In contrast, all 58 EMB-susceptible isolates in this and other studies with embB alterations were resistant to other antituberculosis drugs as well (8, 17, 20, 29). These observations support the hypothesis that a target other than EmbB may exist for EMB which may be activated during combination treatment with other first-line antituberculosis drugs, resulting in susceptibility to EMB (17).

Importantly, if all alterations of embB306 are considered polymorphisms, then only a minority of EMB-resistant isolates would be mutated. A similar scenario was observed in studies defining the role of the katG gene in isoniazid resistance in M. tuberculosis. Members of our group and others have shown that the predominant alteration in katG is R463L, which is detected in both isoniazid-resistant and -susceptible isolates and hence is considered a polymorphism and an unreliable indicator of isoniazid resistance (11, 13, 27). Thus, it is imperative for all studies elucidating the molecular mechanisms of drug resistance in M. tuberculosis to include drug-susceptible isolates as controls.

Another interesting finding of this study was the presence of resistance to other antituberculosis drugs when alterations of embB were present. Further investigations are necessary in order to understand the involvement of these drugs in the molecular mechanism of EMB resistance.

In conclusion, alterations at embB306 may not confer resistance to EMB but may be common polymorphisms in clinical isolates of M. tuberculosis. The clinical significance of this alteration is dubious, and further evaluation of EMB-susceptible isolates from other geographic regions is warranted.

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### REFERENCES


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### TABLE 1. Oligonucleotide primer sequences for amplification of the embB gene

<table>
<thead>
<tr>
<th>Primer</th>
<th>Description</th>
<th>Sequence</th>
<th>Nucleotides</th>
<th>Annealing temp (°C)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>embB1(F)</td>
<td>First fragment, sense</td>
<td>5’ CTG AAA CTG CTG GCG ATC AT</td>
<td>7601–7620</td>
<td>58.0</td>
<td>415</td>
</tr>
<tr>
<td>embB1(R)</td>
<td>First fragment, antisense</td>
<td>5’ GGT CTG GCA GGC GCA TCC</td>
<td>8015–7998</td>
<td>58.0</td>
<td>451</td>
</tr>
<tr>
<td>embB2(F)</td>
<td>Second fragment, sense</td>
<td>5’T GG AGG CCA GCA AAC CCG</td>
<td>8082–8099</td>
<td>62.0</td>
<td>528</td>
</tr>
<tr>
<td>embB2(R)</td>
<td>Second fragment, antisense</td>
<td>5’T AGG TAG TAA CGG AGG TTC TC</td>
<td>8532–8513</td>
<td>62.0</td>
<td>528</td>
</tr>
<tr>
<td>embB3(F)</td>
<td>Third fragment, sense</td>
<td>5’ GCT GTT GCG GCG GCG TGT AGG</td>
<td>8743–8760</td>
<td>62.0</td>
<td>528</td>
</tr>
<tr>
<td>embB3(R)</td>
<td>Third fragment, antisense</td>
<td>5’ GAA CCC GAA TCG CCG TCC AG</td>
<td>9270–9251</td>
<td>62.0</td>
<td>528</td>
</tr>
<tr>
<td>embB4(F)</td>
<td>Fourth fragment, sense</td>
<td>5’T TCG GCC CGA GCA AAG ATG</td>
<td>9752–9769</td>
<td>62.0</td>
<td>528</td>
</tr>
<tr>
<td>embB4(R)</td>
<td>Fourth fragment, antisense</td>
<td>5’T CTT GGG TAC GAC TAG GTG</td>
<td>10119–10102</td>
<td>62.0</td>
<td>528</td>
</tr>
</tbody>
</table>

* M represents the nucleotides A and C.

### TABLE 2. Mutations in the embB gene in clinical isolates of M. tuberculosis

<table>
<thead>
<tr>
<th>Phenotype (n*)</th>
<th>Codon</th>
<th>Amino acid change</th>
<th>Mutation</th>
<th>No. (%) of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMB resistant (25)</td>
<td>Met→Ile</td>
<td>ATG→ATC</td>
<td>3 (12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Met→Ile</td>
<td>ATG→ATA</td>
<td>6 (24)</td>
<td></td>
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<tr>
<td></td>
<td>Met→Val</td>
<td>ATG→GTG</td>
<td>2 (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Met→Leu</td>
<td>ATG→CTG</td>
<td>1 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gln→Arg</td>
<td>CAG→CGG</td>
<td>5 (20)</td>
<td></td>
</tr>
<tr>
<td>EMB susceptible (20)</td>
<td>Met→Ile</td>
<td>ATG→ATM</td>
<td>1 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gly→Asp</td>
<td>GGC→GAC</td>
<td>1 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Met→Ile</td>
<td>ATG→ATA</td>
<td>1 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ala→Thr</td>
<td>GCG→ACG</td>
<td>1 (5)</td>
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</tbody>
</table>

* n, no. of isolates.

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