Simultaneous ethambutol & isoniazid resistance in clinical isolates of *Mycobacterium tuberculosis*


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**Background & objectives:** There is a need to understand the nature of drug resistance patterns and predictors of emergence of drug resistance in *Mycobacterium tuberculosis*. There could be common factors/mechanisms for resistance to the drugs, isoniazid and ethambutol, both acting on cell wall. The present study was conducted to analyze the antimycobacterial susceptibility patterns of *M. tuberculosis* isolates to determine the minimum inhibitory concentrations (MICs) of ethambutol for *M. tuberculosis*; and to find out possible association of ethambutol resistance with isoniazid resistance.

**Methods:** A total of 380 *M. tuberculosis* isolates were tested for their susceptibilities to ethambutol at 2, 4, 6 µg/ml, isoniazid at 1 µg/ml and rifampicin at 64 µg/ml using MIC method.

**Results:** 44.21, 24.73 and 14.21 per cent isolates were resistant to ethambutol at 2, 4, 6 µg/ml, isoniazid at 1 µg/ml and rifampicin at 64 µg/ml respectively. At 6 µg/ml of ethambutol concentration, 85.18 per cent ethambutol resistant isolates were resistant to isoniazid also. At the same ethambutol concentration a fraction of 28.75 per cent isoniazid resistant isolates were ethambutol resistant.

**Interpretation & conclusion:** Ethambutol resistance was accompanied with isoniazid resistance in a large percentage of isolates whereas ethambutol resistance was weakly linked with multidrug resistance. On the other hand, association between isoniazid and ethambutol resistance was weak showing one way linkage.

**Key words** Ethambutol - isoniazid - minimum inhibitory concentration - multi drug resistant - rifampicin - tuberculosis
Global prevalence of *Mycobacterium tuberculosis* infection has been estimated to be 32 per cent. Eighty per cent of all incidence cases are found in 22 countries, with more than half the cases occurring in five Southeast Asian countries (India, Indonesia, Bangladesh, Philippines, and Vietnam). According to another estimation, 30 per cent of world’s tuberculosis patients live in India.

Ethambutol [EMB; D-2,2’-(ethylenediimino)-di-1-butanol] is being used successfully in multi drug therapy for the treatment of tuberculosis as well as to treat opportunistic infections of AIDS patients caused by *M. avium* complex. It is well absorbed after oral administration and is widely distributed in the body. Although pre-treatment primary resistance to EMB was not common in the early years, yet global prevalence ranging from 0-4.2 per cent has been reported. In India, initial EMB resistance (0-14.3%) has been reported by various investigators. Few reports are available on acquired EMB resistance and except for one study from Philippines acquired resistance ranging from 0-13.7 per cent to EMB have been reported from different parts of the world (Table I).

Ethambutol susceptibility testing in *M. tuberculosis* is complicated as different workers have used different methodologies and cut-off levels viz., 2 µg/ml by proportion method [Lowenstein Jensen (LJ) medium], 8 µg/ml or more by minimum inhibitory concentration (MIC) method [LJ medium] and resistance ratio method. Susceptibility testing of *M. tuberculosis* isolates against ethambutol is problematic partially due to the narrow range of MICs of clinical isolates and also drug activity is dependent upon exposure time, temperature and concentration conditions. Moreover, microcolonies are also encountered during ethambutol susceptibility testing but the presence of microcolonies alone does not indicate resistance. The susceptibility testing results need to be analyzed in the light of achievable plasma concentrations of antitubercular drugs so as to have a better understanding of the clinical response of drug resistant *M. tuberculosis* isolates.

Concurrent presence of isoniazid (INH) and ethambutol (EMB) resistance has been reported by Madison *et al* who suggested mechanistic or epidemiological reasons for such a phenomenon. It needs to be investigated whether concurrent presence of INH and EMB resistance occurs in other geographical settings like India. Understanding the reasons for such an association may provide information relevant to improve the case management.

<table>
<thead>
<tr>
<th>Country</th>
<th>Initial resistance (%)</th>
<th>Acquired resistance (%)</th>
<th>Combined resistance (%)</th>
<th>EMB conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td>0-4.2</td>
<td>0-14.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Philippines</td>
<td>32.0</td>
<td>38.0</td>
<td>70.0</td>
<td>10</td>
</tr>
<tr>
<td>Pakistan</td>
<td>NS</td>
<td>8.7</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>India</td>
<td>0.5</td>
<td>0.0</td>
<td>0.5</td>
<td>≥ 8</td>
</tr>
<tr>
<td>India</td>
<td>4.0</td>
<td>NS</td>
<td>-</td>
<td>NM</td>
</tr>
<tr>
<td>India</td>
<td>0.5</td>
<td>NS</td>
<td>-</td>
<td>&gt; 8</td>
</tr>
<tr>
<td>India</td>
<td>1.7</td>
<td>NS</td>
<td>-</td>
<td>&gt; 8</td>
</tr>
<tr>
<td>India</td>
<td>2.6</td>
<td>NS</td>
<td>-</td>
<td>NM</td>
</tr>
<tr>
<td>India</td>
<td>0.2</td>
<td>NS</td>
<td>-</td>
<td>&gt; 8</td>
</tr>
<tr>
<td>India</td>
<td>NM</td>
<td>NM</td>
<td>4.72</td>
<td>8</td>
</tr>
<tr>
<td>India</td>
<td>14.3</td>
<td>16.6</td>
<td>15.0</td>
<td>2</td>
</tr>
<tr>
<td>Present study</td>
<td>–</td>
<td>–</td>
<td>14.2</td>
<td>6</td>
</tr>
</tbody>
</table>

NM, not mentioned; NS, not studied
Superscript numerals denote reference numbers
Hence in the present study, an attempt was made to analyze the antimycobacterial susceptibility patterns of *M. tuberculosis* isolates, determine the MICs of EMB against these isolates, and to specifically investigate the extent of association between EMB and INH resistance.

**Material & Methods**

*M. tuberculosis* isolates: This study was conducted at National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India. A total of 380 isolates of *M. tuberculosis* from hospitals of different regions of the country - 332 isolates from north India including Jammu and Kashmir (4), Chandigarh (3), Shimla (4), Delhi (100), Meerut (3), Hardwar (3), Jaipur (106), Agra (77), Etah (5), Ghatampur (3), Varanasi (24); 19 isolates from south India (Cochin), and 29 isolates from Andman and Nicobar (Port Blair) were collected during August 2001 to July 2003, identified according to standard criteria\(^\text{19}\), and were deposited in Mycobacterial Repository Centre of the Institute. These isolates (360 pulmonary and 20 extrapulmonary) were included in this study along with control strain *M. tuberculosis* H37Rv (TMC 102) [Tredeau Mycobacterial Collection (TMC), National Institute of Health (NIH), Bethesda, Maryland, USA] and were tested for susceptibility to ethambutol, rifampicin and isoniazid.

Treatment history of patients was provided by the concerned medical officer/laboratory sending the isolates. As we were not directly involved in the questioning of patients and this history could not be re-verified, we calculated the combined resistance. Combined resistance was defined as the prevalence of drug resistance among all cases of tuberculosis, regardless of prior treatment\(^\text{20}\).

**Drug concentrations:** Stock solutions of ethambutol (Sigma Chemical Co., St. Louis, USA) and isoniazid (Novartis India Pvt. Ltd., Mumbai) were made in distilled water and solution of rifampicin (Sigma Chemical Co., St. Louis, USA) was made in dimethyl sulphoxide. These solutions were sterilized by membrane filter (Pore size 0.22 µm; Millipore Corporation, USA). These drugs were incorporated in LJ medium to final pre-inspissation concentrations of 2, 4, 6 µg/ml for ethambutol\(^\text{19}\), and 1 and 64 µg/ml for isoniazid and rifampicin (RIF) respectively\(^\text{21}\).

**Drug susceptibility testing:** The standard bacterial suspension of 4 mg/ml prepared as per the method of Canetti *et al.*\(^\text{16}\) was used as inoculum with the help of a loop (3 mm internal diameter) and inoculated on to LJ slants. The culture bottles were incubated at 37°C and readings were taken after 4 wk of incubation. The MIC was determined by counting the colony forming units (cfu) and comparing with control cultures. To assess the quality of inoculum, culture control was read as growth of ++ (more than 100 colonies, usually 150-200 colonies) and considered essential for reading the results\(^\text{16,21,22}\). An isolate was considered resistant if it yielded a growth of 20 colonies or more at a given concentration of drug and sensitive when there was no growth.

**Results**

**Drug susceptibility testing of *M. tuberculosis* for ethambutol:** The standard strain H37Rv of *M. tuberculosis* used in the study was found to be sensitive at ≥2 µg/ml levels for EMB, 1 µg/ml for INH and 64 µg/ml for RIF. Of the 380 isolates tested, 168 (44.21%), 94 (24.73%), and 54 (14.21%) isolates were resistant at EMB concentrations of 2, 4, and 6 µg/ml respectively (Table II).

The MICs of EMB for *M. tuberculosis* isolates were analyzed in a range of 2 to 6 µg/ml on LJ medium and were: 2 µg/ml for 212 isolates, 4 µg/ml for 74 isolates, and 6 µg/ml for 40 isolates.

**Occurrence of EMB resistance and multi drug resistance:** 67 of 168 (39.88%) and 29 of 54 (53.70%) isolates of *M. tuberculosis* with EMB resistance were resistant to both INH and RIF. 67 of 168 (39.88%) and 29 of 54 (53.70%) isolates of *M. tuberculosis* with EMB resistance were resistant to both INH and RIF.
at 2, 4 and 6 µg/ml concentrations respectively were found to be MDR, and these figures were 67 (72.04%), 43 (46.23%) and 29 (31.18%) of the total 93 MDR isolates (Table III).

Discussion

Ethambutol is one of the important components of current antituberculosis regimen and is being used extensively. Over the years, a number of techniques have been used to determine the drug susceptibility for *M. tuberculosis*. These data should be based on comparable value of techniques if wider importance is to be realized. Some of the conventional techniques are drug incorporation methods (on LJ as well as other media) such as proportion, MIC and resistance ratio\(^{11,12,16,18,22}\). In the present study, there was a sharp decline in percentage of resistance from 2 µg/ml to 4 and 6 µg/ml levels of EMB and at 6 µg/ml EMB concentration only 14.21 per cent isolates were found to be resistant. Our results showed slightly higher proportion of EMB resistance (Table I) as compared with the results of Cohn et al\(^{11}\) who have reported global initial and acquired EMB resistance in *M. tuberculosis* isolates from Phillipines. Higher prevalence of EMB resistance in the present study may be partly due to the inclusion of relatively more isolates from treatment failure and relapse cases from various parts of the country referred to our centre, some of which may not have given correct history. Therefore, the results need to be interpreted with caution especially about their epidemiological importance.

In the present study, the MIC of 55.79 per cent isolates was 2 µg/ml of EMB, a concentration which is lower than the usual peak level achievable in the blood of patients. At the same time, 19.47 per cent of isolates had an MIC of 4 µg/ml which is in the reported range of 3.2-5.6 µg/ml of plasma levels after therapeutic doses of EMB in TB patients and healthy volunteers\(^{23,24}\). About ten per cent isolates showed an MIC of 6 µg/ml which is higher than plasma level after therapeutic dose, hence there seems to be no therapeutic relevance for drug susceptibility testing for EMB at higher concentrations. These results were in conformity with those of others who have reported the MIC values of 2-6\(^{19}\) and 1-5 µg/ml\(^{25}\), respectively.

**Table II.** Comparison of occurrence of ethambutol (EMB) and isoniazid (INH) resistance

<table>
<thead>
<tr>
<th>EMB conc. (µg/ml)</th>
<th>No. (%) of EMB resistant isolates</th>
<th>No. (%) of EMB resistant isolates with INH resistance</th>
<th>*No. (%) of INH resistant isolates with EMB resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>168 (44.21)</td>
<td>109 (64.88)</td>
<td>109 (68.12)</td>
</tr>
<tr>
<td>4</td>
<td>94 (24.73)</td>
<td>74 (78.72)</td>
<td>74 (46.25)</td>
</tr>
<tr>
<td>6</td>
<td>54 (14.21)</td>
<td>46 (85.18)</td>
<td>46 (28.75)</td>
</tr>
</tbody>
</table>

* fractions were calculated from total number of INH resistant isolates (n=160)
Total numbers of isolates = 380

**Table III.** Occurrence of MDR amongst EMB resistant isolates

<table>
<thead>
<tr>
<th>EMB conc. (µg/ml)</th>
<th>No. (%/ml) of EMB resistant isolates</th>
<th>No. (%) of EMB resistant isolates with MDR</th>
<th>*No. (%) of MDR isolates with EMB resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>168</td>
<td>67 (39.88)</td>
<td>67 (72.04)</td>
</tr>
<tr>
<td>4</td>
<td>94</td>
<td>43 (45.74)</td>
<td>43 (46.23)</td>
</tr>
<tr>
<td>6</td>
<td>54</td>
<td>29 (53.7)</td>
<td>29 (31.18)</td>
</tr>
</tbody>
</table>

* fractions were calculated from total number of MDR isolates (n=93)
Our data suggested that determination of EMB MICs in LJ medium would allow monitoring of chemotherapy with this drug in a more sensitive and reasonable way than with currently used different critical concentrations by various investigators\textsuperscript{5,6,15,22}. We suggest that instead of one concentration as a qualitative criterion of susceptibility, quantitative criteria should be used to evaluate the results. Based on our results, it is suggested that the criteria for susceptibility determination for EMB on LJ medium might be as follows: susceptible, MIC 2 µg/ml; moderately susceptible, MIC 4 µg/ml; slightly resistant, MIC 6 µg/ml, similar to that described elsewhere\textsuperscript{26}. Most of the EMB resistant isolates were INH resistant (1 µg/ml) also, suggesting a high degree of association between EMB and INH resistance especially at 4 and 6 µg/ml respectively. Madison \textit{et al}\textsuperscript{18} have also reported that EMB resistance was accompanied by 96.6 per cent resistance to INH. This association was not merely a matter of chance as the same trend of EMB resistance with INH resistance has also been observed in Agra, Delhi, Jaipur and Varanasi (north Indian cities) individually. Though the number was very small, similar pattern of EMB and INH resistance was observed in the isolates obtained from Cochin (south India) and Port Blair (data not shown). Conversely, a large fraction of INH resistant isolates were not resistant to EMB suggesting that the simultaneous resistance of EMB with INH may have less mechanistic but more epidemiological significance. However, such trends and/or mechanism(s) need to be analyzed in future studies by including statistically significant number of \textit{M. tuberculosis} isolates and using genotyping markers as confirmatory tools.

Further, the association was rather weak between EMB resistance and MDR. It may be inferred that EMB resistance at 6 µg/ml was accompanied with high INH resistance whereas the association of EMB resistance with MDR was relatively weak.

Simultaneous occurrence of INH resistance in a large fraction of EMB resistant isolates can be explained partly on the basis of epidemiological factors of overuse and/or misuse of EMB and INH drugs in the past decades in some countries like India and Philippines, and the selection of resistance, as with other drugs (like RIF and INH), might have led to the emergence of both EMB and INH resistant isoaltes of \textit{M. tuberculosis}. However, the association could be mechanistic as this was observed especially more at higher levels of ethambutol resistance.

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**References**


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