

## ORIGINAL ARTICLE

# embB Gene Association with Ethambutol drug Resistance in Mycobacterium Tuberculosis patients

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## ABSTRACT

**Background:** Tuberculosis (TB) is fatal and life threatening infectious disease. The transmission rate of tuberculosis is very high. Various drugs are used as treatment for TB. Recently it has been observed that one of the most important factor for fast TB spread is development of anti-TB drug resistant mycobacterium tuberculosis (MTB). Various combination of drugs like isoniazid (INH), rifampicin (RIF), Streptomycin (SM), pyrazinamide (PZA) or ethambutol (EMB) are in global use for TB treatment. Improper usage of these drugs makes the person prone to develop anti-TB drug resistant tuberculosis.

**Aim:** To evaluate association of embB gene with ethambutol resistance in Mycobacterium Tuberculosis.

**Methods:** 104 Specimens of sputum from suspected tuberculosis patients were processed for inoculation in *Lowenstein J Medium* after it has been decontaminated properly. Kit method by using QIAamp DNA Mini kit was utilized for extraction of DNA. Then region from base 6953 to 10249 of embB gene was amplified through PCR and then followed by sequencing with the aid of softwares blast2seq and ClustalW2. Three primer sets were utilized to amplify *embB* gene. Ethambutol (EMB) Resistant MTB specimens were processed to study mutation in embB gene.

**Results:** Out of the total 104 sputum specimens, 14 samples were found to have ethambutol resistance. These 14 samples were then processed for mutational analysis. DNA sequence analysis of these 14 samples confirmed embB gene mutation in 10 samples. Mutational analysis revealed that 08 samples showed mutation at codon 306 and two samples showed mutation at 319 codon. The reported mutation Methionine → Isoleucine was seen in 07 samples with ATG codon replaced by ATA codon at codon position 306. One sample showed mutation as Methionine → Isoleucine with ATG codon replaced by ATC codon at codon position 306. Two samples showed mutation as Tyrosine → Serine with TAT codon replaced by TCT at 319 codon position in embB gene.

**Conclusion:** This study concludes that mutation of certain genes particularly point mutation of embB gene at codon 306 and 319 is associated with drug resistance of ethambutol in ethambutol resistant mycobacterium tuberculosis patients.

**Keywords:** Ethambutol, embB gene, Mycobacterium tuberculosis.

## INTRODUCTION

TB is very serious infectious disease and it poses big threat to human life affecting almost one third of world's population leading to significant number of deaths per year globally. The contemporaneous difficulty with the worldwide inception of anti-TB drug-resistant strains and the soaring morbidities of TB has magnified the need for collective effort, and for better understanding of the disease and treatment options<sup>1</sup>.

One of the important factor for brisk TB spread globally is the development of anti-TB drug resistant tuberculosis. Treatment of anti-TB drug resistant tuberculosis has increased economic burden on patients. Early research on anti-TB drug resistant tuberculosis is vital for patients and also to minimize the transmission<sup>2</sup>.

Two or more anti-TB drugs in combination are commonly utilized to reduce the occurrence of anti-TB drug resistant mycobacteria. Among the treatment regimen, four anti-TB medicinal agents are used against TB. The systematic utilization of these agents frequently lasts for around 6 to 9 months. Most frequently utilized medicinal agents are isoniazid (INH), rifampin (RIF), pyrazinamide (PZA) and streptomycin (SM) or ethambutol (EMB). The misuse of these drugs makes a person resistant to disease<sup>3</sup>.

Molecular phenomenon of various anti-TB agents has described the genetic affiliation of drug resistant with Mycobacterium Tuberculosis (MTB) in a lucid way. In MTB, drug resistance is affiliated with mutation within drug target gene<sup>4</sup>.

Among various types of Mycobacterium tuberculosis, emb genes have remained unchanged and highly conserved<sup>5</sup>.

Many research works have described that there are surface membrane- attached enzymes such as arabinosyltransferases.

These are potential targets of anti-TB drug Ethambutol (EMB). Arabinosyl transferases are highly preserved and conserved within mycobacterium and are involved in arabinan biosynthesis in these bacteria. Arabinan is chemically an 'arabinogalactan' which is found in cell wall of Mycobacterium Tuberculosis. When formation of arabinan is obstructed by EMB, it leads to accumulation of mycolic acids which causes bacterial cell death<sup>6</sup>.

Ethambutol plays its role by obstructing mycobacterium cell wall formation. Mycolic acids get attached to the 5'-OH groups of aldopentose 'D-arabinose' moieties of biopolymer arabinogalactan and produces a complex (mAGP) known as mycolyl-arabinogalactan-peptidoglycan in mycobacterial cell wall which hampers arabinogalactan synthesis by inhibiting enzyme arabinosyl transferase. Thus arabinogalactan synthesis inhibition by ethambutol prevents the manufacture of mycobacterial cell wall complex which ultimately leads to enhanced bacterial cell wall permeability<sup>7</sup>.

In Mycobacterium tuberculosis and *Mycobacterium smegmatis*, *embB* genes are associated to operon comprising of three genes, which are *embA*, *embC* and *embB*. It is observed that the gene target of drug ethambutol is *embB* protein by inhibiting the production of arabinan. For the purpose of elaborating the functional correlation of *embB* protein with arabinan production, *emb A, B, C* genes are inactivated separately by the process of homologous recombination. The *embB* mutant gene is most slowly growing mutant strain among the three<sup>8</sup>.

EMB-resistant bacterial strains possess point mutation at codon 306 (Met) in *embB* gene and its frequency is reported to be significant in many molecular studies<sup>9</sup>.

Some other reported point mutation in emb gene are described at codon 287, in which Phenylalanine is replaced by amino acid valine, leucine or cysteine and are labelled as novel mutation. Various other novel mutations at different codons are

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also described and labelled under the umbrella of novel mutations of *embB* gene leading to ethambutol resistance<sup>10</sup>.

## MATERIAL AND METHODS

**Selection Criteria for cases:** After approval from Institutional Ethical Committee, suspected TB samples were collected from outdoor and indoor of Mayo Hospital Lahore and submitted to Institute of Public Health (IPH) at TB reference lab Lahore (Punjab Provincial Reference Lab for Tuberculosis control). Our Study cases included cases with history of cough for 03 or more weeks, giving no response to treatment with anti TB antibiotics and the cases showing signs of cough with blood tinged sputum, significant loss of weight and history of rise of temperature in evening. All Cases giving response to antibiotics were excluded from study.

**Sample Collection:** 104 Sputum specimens from MTB cases were collected in wide-mouthed and transparent jar and submitted to IPH Lahore after taking informed consent from TB patients from June to December 2020. Sputum was then processed for decontamination.

After proper inoculation, the slopes were processed for incubation at 37°C for a period of 08 weeks. Para-nitro benzoic acid (PNB) was then utilized to restrict growth of MTB colonies in Lowenstein J medium. The bacterial growth was inhibited with the help of PNB (500µg/ml). The drug sensitivity test was processed on Lowenstein J medium carrying anti-TB antibiotic ethambutol and then processed for incubation at 37°C for three weeks. After 03 weeks incubation, the growth of *Mycobacterium TB* colonies was evaluated. It was labelled as Ethambutol (EMB) resistant if it still showed resistance against ethambutol after 03 weeks' time. The bacterial DNA extraction was achieved by using QIAamp DNA mini-kit (Qiagen). The DNA extracted was utilized or stored as per requirement. The quantification of bacterial DNA was achieved by agarose 0.8% gel. The designed primers and reported Primers for *embB* gene of *Mycobacterium tuberculosis* (GeneBank Accession no. NC\_000962.2) were utilized<sup>11</sup>.

Primers were then processed for PCR optimization for their annealing temperatures (50°C to 60°C). Total 14 EMB resistant DNA samples were processed for PCR amplification using three amplification and sequencing primers (Table 2). Amplification was processed by using Electrophoresis (Agarose Gel method). After the bacterial DNA amplification of the desired fragments of the DNA, PCR products were subjected to purification and sequencing.

Analysis of the DNA sequences was done by Alignment of sequences. This alignment was done with the utilization of softwares blast2seq and ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2>). The aligned DNA sequences were analyzed and compared with sequence of wild type strain H37RV and gene mutations were identified and these gene mutations were analyzed with the available existing data on NCBI<sup>12</sup>.

## RESULTS

Out of these 104 MTB cases, 14 patients were Ethambutol (EMB) resistant. These 14 EMB Resistant MTB cases were analyzed for presence of *embB* gene mutation. The mutation in *embB* gene was detected in 10 samples of ethambutol resistant MTB patients (9.6% of total MTB cases and 71.4% of ethambutol resistant MTB cases (Table 1).

Table 1: Frequency of ethambutol resistant MTB and *embB* gene mutation

Total <i>Mycobacterium Tuberculosis</i> (MTB) patients	104
Ethambutol resistant-MTB cases	14(13.46%)
<i>embB</i> gene mutation found in ethambutol resistant-MTB cases	10(9.6%)
Frequency of <i>embB</i> gene resistance	71.4%

**Mutational Analysis:** From total 14 strains in our study, 10 samples displayed *embB* gene mutations when the query sequences were taken into comparison with the reported reference sequence of *embB* gene available on NCBI website.

All of the observed gene mutations were reported. No novel gene mutation was detected. Genetic analysis revealed that eight samples showed mutation at 306 codon and two showed mutation at 319 codon. While four samples of our study did not have any mutation in *embB* gene.

Variations at *embB* gene codon 306 were reported as being the most prevalent alteration in ethambutol (EMB) resistant MTB strains. ATG is *embB* gene 306 codon and this codon is specific for amino acid Methionine (M). After gene point mutation this codon was substituted by ATA sequence codon which is specific for amino acid Isoleucine (I). This type of gene mutation was observed in seven strains while one showed ATG replacement with ATC rather than sequence ATA at 306 codon position of *embB* gene indicating silent mutation. Out of these 10 mutant samples, two isolates had mutation at *embB* gene 319 codon position in which sequence TAT codon was substituted by TCT codon. The sequence TAT codes for amino acid Tyrosine (Y) and the sequence TCT codes for amino acid Serine (S). So Serine substituted the Tyrosine amino acid at this 319 codon position of *embB* gene (Table 3).

Table 2: *embB* gene Primers

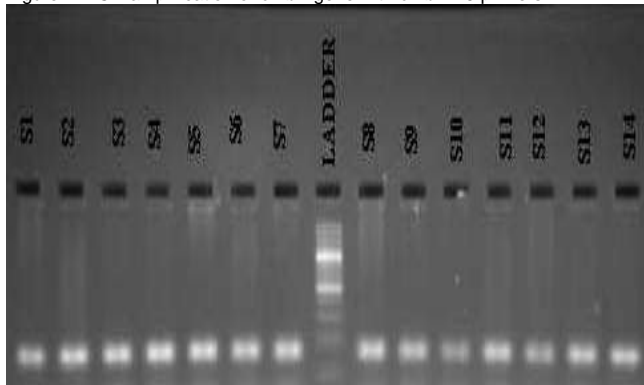
No.	Name of Primer	5'-3' Sequence
1	<i>embB</i> PF5	CATCCTGACCGTGGTGTTC
	<i>embB</i> PR5	GCACACCGTAGCTGGAGAC
2	<i>embB</i> PF2	CTGAAGCTGCTGGCGATCAT
	<i>embB</i> PR2	GGTCTGGCAGGCGCATCC
3	<i>embB</i> PF3	CCGACCACGCTGAAACTG
	<i>embB</i> PR3	GTAATACCAGCCGAAGGATCCT

Table 3: Mutations in *embB* gene in isolates of *Mycobacterium tuberculosis*

Phenotype	Codon	Mutation	Amino acid substitution	No of Isolates
EMB resistant strain	319	TAT→TCT	Tyr→Ser	2
	306	ATG→ATA	Met→Ile	7
	306	ATG→ATC	Met→Ile	1

Amplification by PCR using primers is shown in Figure 1 while sequencing done and mutation at codon 306 and 319 in Sample 1 with highlighted bases showing the position of mutation is shown in Figure 2. Similar results were obtained in other samples.

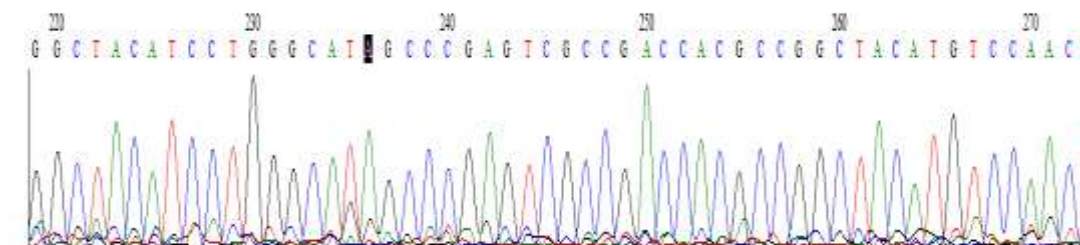
Figure 1: PCR amplification of *embB* gene with *embB* P5 primers



Lane 1-7 Amplified fragments of 500 base pairs of *embB* gene isolated from *Mycobacterium tuberculosis*

Lane M: Marker 1kb SM0313 (Fermentas)

Lane 9-15: Amplified fragments of 500 base pairs of *embB* gene isolated from *Mycobacterium tuberculosis*

Figure 2: Chromatograph showing Transition with *embB* PF5 primer

Product size: 500bp

## DISCUSSION

The genetic evaluation of *embB* gene was done in our study with an aim to find its association anti TB drug ethambutol resistance in MTB patients. This gene is found in *MTB* sequence on Locus Rv3795 tag. The Length of *embB* gene is 3,297 base pairs.

For confirmation of association of *embB* gene with ethambutol resistance, we performed mutational analysis. The objective of this research work was genetic mapping and assessment of the *embB* gene to identify any mutations and to see any association of *embB* gene with ethambutol resistance. This study was done to know about the ethambutol resistance as a result of mutation in *embB* gene. Ethambutol is widely accepted and utilized in combination with primary anti TB drugs since many years for treatment of tuberculosis<sup>13</sup>.

Ethambutol inhibits the synthesis of mycobacterium cell wall by reacting with enzymes arabinosyltransferases which are involved in biosynthesis of glycolipid lipoarabinomannan (LAM) and glycoprotein arabinogalactan (AG) biosynthesis. When synthesis of arabinan is interfered by ethambutol, it eventually leads to accumulations of mycolic acids which results in bacterial cell death<sup>14</sup>.

To understand the underlying mechanism of action of the drug ethambutol, several hypotheses have been proposed. Various studies have shown that very high number of ethambutol drug resistant isolates possess *embB* gene mutations<sup>15,16,17</sup>.

Most studies have shown substitution of amino acid Methionine by Isoleucine at codon 306 position within *embB* gene as was seen in most cases in our study, however in our study some ethambutol resistant strains did not show this type of gene mutation at 306 codon.

Out of the total 104 MTB specimens, 14 ethambutol resistant isolates were detected (13.46% of 104 MTB cases). Out of these 14 EMB resistant strains, 10 samples have shown genetic mutations in *embB* gene region (9.6% of 104 MTB cases). Genetic analysis revealed that eight samples showed gene mutation at 306 codon and two showed mutation at 319 codon, but four samples were devoid of any mutation in *embB* selected gene region. Frequency of *embB* gene mutation in ethambutol resistant 14 isolates was found to be 71.4%. No novel gene mutation was detected in our study. Variation at 306 codon of *embB* gene was found to be the most prevalent alteration in this study. At 306 codon of *embB* gene, seven isolates showed substitution of ATG→ATA and one showed ATG→ATC substitution in ethambutol resistant bacterial strains. At Methionine 306 codon *embB* gene position, Isoleucine 306 substitution was detected. While two strains showed substitution as TAT→TCT variation at *embB* gene 319 codon. In this type of alteration amino acid Tyrosine was replaced by amino acid Serine at 319 codon position. So the hypothesis is endorsed by these statistics that the most common gene mutation causing EMB resistance is on *embB* gene region. All of the observed mutations in this study are already reported mutations.

Presence of 319 codon gene mutation is recently documented in study done by Li et al. (2020). So, our results emphasize gene mutation at these 319 codon position. Although our scope is limited but it strongly indicates that more extensive

studies should be carried out to evaluate *embB* gene mutation at 319 codon and other codon position in ethambutol resistant strains<sup>18</sup>.

## CONCLUSION

It is concluded from the study that point mutation at codon 306 and codon 319 of *embB* gene is associated with development of resistance to the drug ethambutol in ethambutol resistant MTB cases.

**Recommendations:** With this study we came to a conclusion that mutation in *embB* gene is underlying mechanism of ethambutol resistance in mycobacterium tuberculosis patients. So it is strongly recommended that *embB* gene should be evaluated to identify ethambutol resistant MTB cases so that other anti-TB treatment options can be started without any delay.

**Limitations of the study:** Our study has several limitations like number of patients was relatively small. Further large scale studies are needed to elaborate mechanism of ethambutol resistance in MTB cases.

**Conflict of interest:** Nil

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