embB gene mutations associated with ethambutol resistance in Indian strains of *Mycobacterium tuberculosis*

Ankita Garg[1], Shashikant Srivastava[1], Archana Ayyagari[1,*], Vishwa M. Katoch[2] and Tapan N. Dhole[1]

[1]Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226 014, India
[2]Department of Microbiology and Molecular Biology, Central JALMA Institute of Leprosy, Tajganj, Agra 282 001, India

Ethambutol (EMB) mono-resistance does not occur frequently and scanty data are available regarding the resistance mechanism of EMB. The present study analyses mutations in the *embB* gene in EMB-resistant Indian isolates of *Mycobacterium tuberculosis*. Thirty-seven isolates were collected from different parts of India and the 364 bp of *embB* gene involved in resistance to EMB was amplified by PCR and subjected to single-stranded conformational polymorphism (SSCP). Mutations associated with EMB resistance were found to be present mostly with isoniazid and rifampicin. SSCP showed altered mobility in 30/37 isolates. The most common mutation was observed at codon 306 in 9/22 isolates followed by 299 in 6/22 isolates. We have found novel mutations not reported so far at codon 239, 257, 282, 296, 299, 304 and 311. SSCP revealed 22 (73.3%) isolates had mutation in the 364 bp fragment of *embB* gene. Our results suggest that alterations in amino acid sequence could be present from position 239 to 311 and functional analysis of the genetic variants of embB protein may provide a possible mechanism for EMB resistance.

Keywords: *embB* gene, ethambutol, mutation, *Mycobacterium tuberculosis*.

ETHAMBUTOL (EMB) is a frontline antituberculous drug with a broad spectrum of antimycobacterial activity. EMB is widely used in the treatment of *Mycobacterium avium*, *M. kansasi*, *M. xenopi*, *M. marinum* and *M. malmoense* infections[1]. In this context it has broader application than isoniazid (INH), which is effective against *M. tuberculosis* only. As a consequence of the increase in multidrug-resistance tuberculosis (MDR-TB) and the relative restricted number of therapeutic agents, there have been efforts during the last several years to define the molecular basis of drug resistance in *M. tuberculosis*. Resistance to drug develops due to particular genomic mutations in specific genes of *M. tuberculosis*[2]. Genes which are known to be linked to resistance to first-line anti-tubercular drugs are: *katG*, *inhA*, *ahpC* and *kasA* for INH resistance; *rpoB* for rifampicin (RIF) resistance; *rpsL* and *rrs* for streptomycin (STR) resistance; *iembB* for EMB resistance; and *pncA* for pyrazinamide resistance. Resistance to multiple drugs is the consequence of an accumulation of mutations[3,4].

EMB targets the mycobacterial cell wall through interaction with arabinosyl transferases involved in arabinogalacton (AG) and lipoarabinomannan (LAM) biosynthesis[5,6]. It specifically inhibits polymerization of cell-wall arabinan, thereby leading to accumulation of β-D-arabinofuranosyl-1-monophosphoryldecaprenol (DPA)[7]. AG deprivation is also responsible for the accumulation of mycolic acid in *M. smegmatis*, a result consistent with the finding that EMB causes declumping and morphological changes[8]. Genetic and molecular studies suggest arabinosyltransferase enzyme as the putative target for EMB[9,10]. The arabinosyltransferases are encoded by homologous genes belonging to the *emb* operon and have been identified in *M. smegamtis*, *M. tuberculosis* and *M. leprae* as *embC*, *embA* and *embB*[10]. Mutations leading to replacement of amino acid residues are found to be present in EMB-resistant organisms cultured from humans. Most commonly affected amino acid lies at codon 306 of the *embB* gene that results in replacement of wild type Met306 with Ile, Leu or Val. Most studies show that 65% clinical isolates harbour mutation at 306 amino acid position, forming ethambutol resistance determining region (ERDR)[9,11]. However, the precise effect of this mutation on EMB resistance is not known.

Although India alone contributes to one-third of global TB, no data are available from Indian isolates[12,13]. It is important to study the molecular basis of drug resistance in *M. tuberculosis* in a region with high disease burden and to compare the data from other parts of the world. Therefore, the aim of the present study was to analyse mutations in the *embB* gene in *M. tuberculosis* clinical isolates of Indian origin. Also, the possibility of structural alteration as a result of these mutations in the *embB* gene was explored.

For this study, 37 *M. tuberculosis* isolates were recovered from various parts of India. Apart from nineteen strains from our centre, thirteen ethambutol-resistant *M. tuberculosis* isolates were obtained from the main repository of North India (Central JALMA Institute for Leprosy, Agra) and five from a geographically distinct region in South India (National Tuberculosis Institute, Bangalore). Species identification of the isolates was based on standard microbiological tests: colony morphology, acid fast staining, and biochemical tests. Lowenstein–Jenssen (LJ) medium was used for cultivation of the isolates, and susceptibility testing was performed by proportion method for EMB, INH and RIF at cut-off value of 2, 0.1 and 40 µg/ml respectively, on L–J medium according to Tuberculosis Research Centre, Chennai, guidelines[14]. Minimum inhibitory concentration (MIC) was determined only for EMB by E-test according to manufacturers protocol on Middlebrook 7H10 agar supplemented with 10% OADC enrichment. EMB was tested at concentration ranging from 0.016 to

*For correspondence. (e-mail: a_ayyagari@yahoo.com)

256 µg/ml. *M. tuberculosis* H37Rv was used as control in each set of experiments.

Genomic DNA was isolated from mycobacterial cultures by the lysozyme/proteinase K, CTAB procedure and precipitated with isopropanol[15]. Purified DNA was dissolved in 20–50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8)). 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′. The primers were designed using Primer Premier (PREMIER Biosoft International, Palo Alto, CA, USA), in such a way that spanned the ERDR, embB306 codon. PCR was performed according to standard reaction mixture with the following cycling parameters: 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. Final PCR product was analysed on 1.5% agarose gel stained with ethidium bromide. In each set of reaction one negative control and one positive control (*M. tuberculosis* H37Rv DNA) was included.

All the 37 *M. tuberculosis* isolates were analysed for the presence of mutations in the 364 bp fragment of *embB* gene by the single-stranded conformation polymorphism method (SSCP). PCR for SSCP was carried out as mentioned earlier with addition of α-P^{32}-CTP for radiolabelling. All the radioactivity experiments were carried out in radio hazard chamber. PCR product was gel-eluted from low melting agarose by the method of Sambrook *et al.*[16] with minor modifications. 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), the 364 bp fragments were snap-cooled and immediately loaded onto the gels. SSCP analysis of the DNA fragments was performed in a 6% polyacrylamide gel containing 5% glycerine at 200 V (gel plates were cooled at 10°C). After 3 h of electrophoresis, 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 used to confirm the results of SSCP. DNA sequence of isolates was determined with ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin Elmer) and the ABI PRISM 310 automatic sequencer (Applied Biosystems). The sequences have been submitted to GenBank with the accession numbers AJ 579703, AJ 579704, AJ 579705, AJ 579706, AJ 579707. Nucleotide sequences were translated to amino acid using the Proteomics tool available at http://www.expasy.ch/. The nucleotide sequence and translated amino acids were aligned with BLAST program and GenBank database to compare with the corresponding sequence of *M. tuberculosis* H37Rv sequence, accession number NC_000962.

For proportion method, an isolate was considered resistant if 1% or more colonies were grown on drug-containing media. EMB resistance was found to be associated either with INH, RIF or multidrug resistance (INH and RIF) and only four isolates were mono-resistant to EMB (Table 1).

The analysed 364 bp fragment included codon M306 known to undergo nucleotide changes. Results of SSCP analysis were compared with those from nucleotide sequencing of the same fragments. SSCP analysis showed altered mobility in 30/37 EMB-resistant clinical isolates when compared with *M. tuberculosis* H37Rv (Figure 1). Seven isolates did not show the altered mobility pattern, explaining presence of wild type nucleotide in the *embB* region. Automated sequencing of the isolates that differed in SSCP pattern from *M. tuberculosis* H37Rv showed presence of mutations in 22/30 isolates. Most of the isolates had mutation only at one locus; five were mutated at more than one loci, but all of these had one synonymous polymorphism, i.e. alteration in the nucleotide had no effect on the amino acid sequence. The most common mutation encountered in the isolates was at codon 306. Among the 22 mutants, nine had mutation at codon Met306 being replaced by Val, Ile or Leu. The second most frequent mutation encountered was at codon Asp299Glu (6/22) followed by Asn296Lys (2/22). All the other mutations

Table 1. Resistance profile of *Mycobacterium tuberculosis* clinical isolates

Antibiotics	No. of isolates resistant (n = 37)	Isolates with nucleotide polymorphism (n = 22)	Isolates with mutation at codon 306 (n = 9)
E	4	3	0
I + E	10	5	2
R + E	1	0	0
I + R + E (MDR)	22	14	7
Total	37	22	9

E, Ethambutol; I, Isoniazid; R, Rifampicin; MDR, Multi-drug resistant.

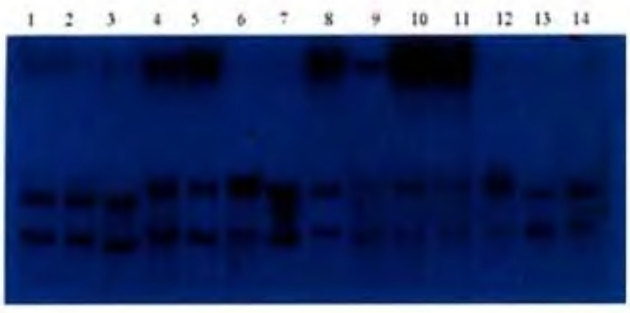

Figure 1. Representative SSCP autoradiograph of *embB* gene. Mobility compared with *Mycobacterium tuberculosis* H37Rv. Lane 1, *M. tuberculosis* H37Rv; lanes 2–14, EMB-resistant *M. tuberculosis* isolates (Lanes 3–14, White arrows showing altered mobility compared to *M. tuberculosis* H37Rv).

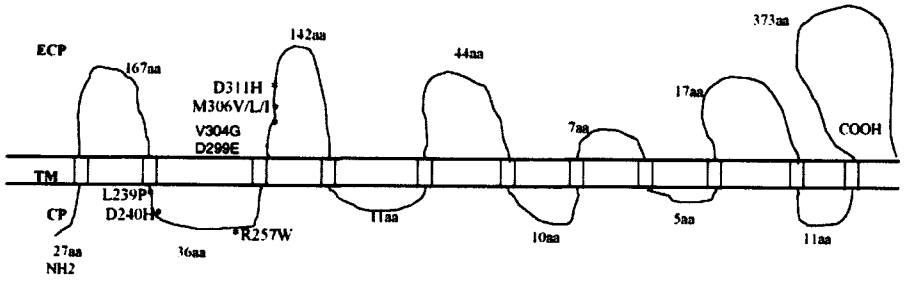

Figure 2. Mapping of mutations observed in *embB* gene based on HMM of EmbB protein structure prediction.

Table 2. Genetic characteristics of EMB-resistant *M. tuberculosis* isolates with mutations at *embB* locus (n = 22)

EMB MIC (μg/ml)	Base changes	Amino acid changes
16	AAT → AAA	Asn296Lys
4	GAC → GAA	Asp299Glu
	CTG → TGG	Leu304Trp
	GCA → GCC	259 Synonymous*
2	GAC → GAA	Asp299Glu
2	GAC → GAA	Asp299Glu
2	GAC → GAA	Asp299Glu
	ATC → ATT	303 Synonymous
32	ATG → ATC	Met306Ile
16	ATG → TTG	Met306Leu
256	CTC → CCC	Leu239Pro
12	GAC → CAC	Asp311His
256	ATG → TTG	Met306Leu
5	GTG → GGG	Val282Glu
4	GCA → GCC	259 Synonymous
	GAC → GAA	Asp299Glu
6	ATG → TTG	Met306Leu
4	GCA → GCC	259Synonymous
12	ATG → ATA	Met306Ile
4	GAC → CAC	Asp240His
96	ATG → GTG	Met306Val
6	ATG → ATA	Met306Ile
2	CGG → TGG	Arg257Trp
	GCA → GCC	259Synonymous
48	ATG → GTG	Met306Val
2	AAT → AAA	Asn296Lys
	GAC → GAA	Asp299Glu
	ATC → ATT	303 Synonymous
8	ATG → GTG	Met306Val

*Synonymous; No change in amino acid.

observed were present in single isolate each at codons Leu239Pro, Asp240His, Asp311His, Leu304Trp and Arg257Trp respectively. The nonsynonymous mutations other than at codon306 encountered in our isolates were novel, and not reported so far. Details of mutations have been described in Table 2 and Figure 2. On sequencing eight isolates which showed polymorphisms by SSCP were found to have wild-type nucleotide sequence. SSCP method revealed that 22 (73.3%) of the isolates had mutations in 364 bp fragment of *embB* gene. Good correlation (81%) was found between SSCP and sequencing results.

The MICs for the 37 EMB-resistant isolates were correlated with the mutations. Of the nine isolates with mutation at codon 306, six had MIC > 10 μg/ml, one had 8 μg/ml and two were inhibited at 6 μg/ml of EMB concentration (Table 2). However, isolates with mutation other than Met306 had an MIC < 10 μg/ml, except three isolates (Table 2). No correlation could be observed between MICs for strains with single or multiple mutations. Mutation associated EMB resistance was found to be present mostly with multidrug resistance, i.e. along with resistance to INH and RIF. The resistance profile of isolates with nucleotide polymorphism, either at ERDR or other loci, is given in Table 1.

The present study provides additional molecular evidence on EMB-resistant clinical isolates from the Indian subcontinent. The target for EMB is the EmbB protein encoded by the *embB* gene of operon *embCAB* in *M. tuberculosis*[5,10]. Mutations in the operon *embCAB* of *M. tuberculosis* and *M. smegmatis* have been shown to be associated with EMB resistance[10]. Certain mutations, particularly at the amino acid position 306 of *embB* were found to be associated exclusively with EMB-resistant *M. tuberculosis*[9,10,17,18]. Thus, *embB* is regarded as ERDR.

Worldwide studies[10,19] report that percentage of EMB-resistant strains with mutation in *embB* gene varies from 50 to 60%; no such report has been documented from the Indian subcontinent, which contributes one-third of global TB. Besides the documented mutation at amino acid position 306 of the *embB* gene, we observed few novel mutations in our study not reported so far. The most frequent mutation observed was at ERDR, i.e. codon Met306 being replaced by Leu, Ile or Val. The other most frequent mutation encountered was Asp299Glu (6/22) followed by Asn296Lys (2/22). Mutation at codon Leu304Trp and Val282Gly was present in a single isolate each. However, we did not find any isolate with mutation at amino acid Trp as observed by Lee *et al.*[19] in EMB-resistant *M. tuberculosis* isolates from Korea. A wide range of mutations other than Met306 have been reported, previously by Sreevatsan *et al.*[9] and later by Ramaswamy *et al.*[11]. These strains included either of the three (gr I *katG463* CTG plus *gyrA95* ACC; gr II *katG463* CGG plus *gyrA95* ACC; gr III *katG463* CGG plus *gyrA95* AGC) principle genetic

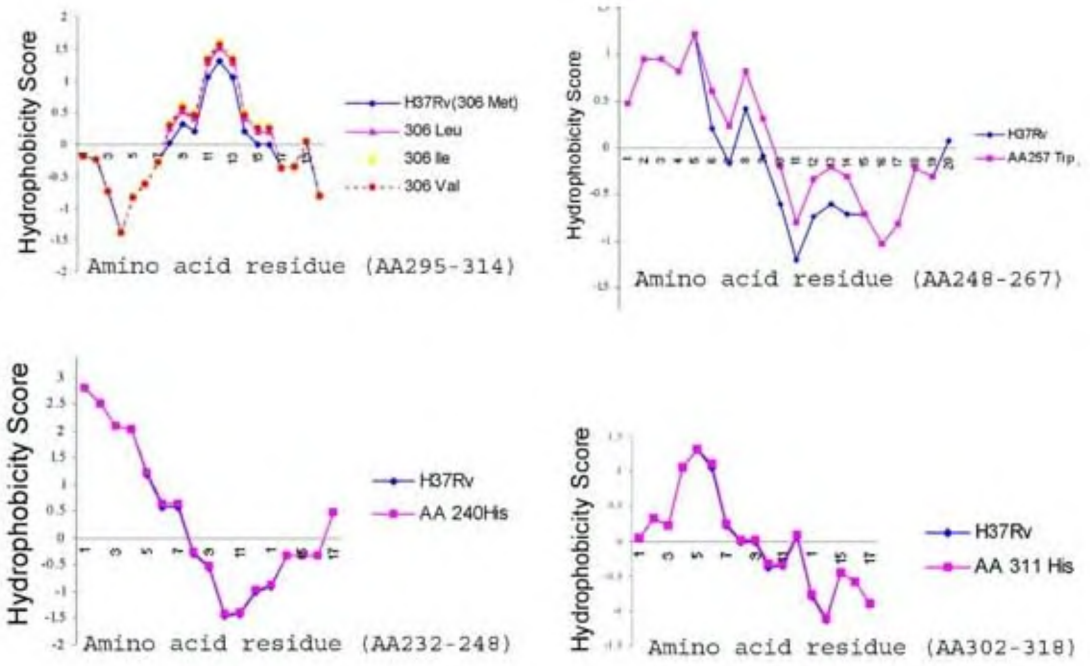

Figure 3. Hydrophobicity plots of EMB-resistant *M. tuberculosis* with mutations at respective codons. Regions mapped are mentioned in each increased hydrophobicity compared to 22/30 *M. tuberculosis* H37Rv.

groups. However, none of the mutations was common in both studies. Mutations other than Met306, observed in our EMB-resistant population of *M. tuberculosis*, have also not been reported so far and give important information regarding the prevalence of any particular type of codon replacement in Indian strains that are known to be genetically distinct; these were Leu239Pro, Arg257Trp, Val282Gly, Asn296Lys, Asp299Glu, Leu304Trp and Asp311His. Ramaswamy *et al.*[11] identified up to seven nucleotide polymorphisms of synonymous type, i.e. nucleotide polymorphism not altering the amino acid. We observed two such types of alteration at codon 259 (GCA → GCC) and codon 303 (ATC → ATT). EMB-resistant *M. tuberculosis* isolates with resistance-associated mutations in other genes along with *embB* were found to be associated with putative regulatory mutation in the *embC–embA* intergenic region upstream of the start codon[11]. We observed 5/22 *embB* mutant isolates with amino acid replacement at more than one codon in the *embB* gene. However, at least one of these mutations was synonymous and only two isolates had two nonsynonymous mutations. Ramaswamy *et al.*[11] suggest targetting amino acids 300 to 500 of the *embB* gene for mutation studies. Our result of 22 mutant isolates indicates that alteration could be present from amino acid 239 to 311.

Occurrence of mutation at Met306 has been found to be associated with moderate to high level of EMB resistance (MIC ranging from 10 to 50 μg/ml). This is consistent with previous studies[17]. Two of our isolates with Met → Val, Leu had high level of EMB resistance (MIC 96 μg/ml). A total of 15 isolates did not show any mutation at the nucleotide level, of which nine had an MIC < 8 μg/ml and two had MIC of 8 μg/ml. This is consistent with the earlier observation suggesting that low level of EMB resistance may not be associated with Met306 mutation[17,19]. However, no correlation has been established between MICs for strains with a particular type of mutation or multiple-resistance associated mutations.

Further, we analysed the 364 bp fragment by designing primers that could amplify AA 220–AA 334 of the translated *embB* gene and secondary structure prediction of the translated sequence using ProtScale tool (ExPASy) and BMERC server bmerc-www.bu.edu/psa/ to study the effect of mutation on putative drug binding, by analysing the mean hydrophobicity index of the mutated amino acid. Computer modelling has predicted Emb proteins to be integral membrane proteins, consisting of multiple α-helical segments spanning the cell membrane. On the basis of Hidden Markov Model of Sonnhammer *et al.*[20], which has an overall accuracy of 77%, EmbB protein has 11 transmembrane helices with ERDR loop located on the extracytoplasmic side of the membrane, and may provide site for protein–drug interaction. Amino acids from 238 to 436 form the segment of protein harbouring the ERDR loop (Figure 2); AA238–274 cytoplasmic, AA 275–293 transmembrane and AA294–436 extracytoplasmic. Hydrophobicity of the sequenced fragment of the *embB* gene was compared with the standard strain *M. tuberculosis* H37Rv, accession no. NC_000962. Based on the hydropathy score of Kyte and Doolittle[21] for individual amino acids, we observed that the alteration of methionine to leucine, isoleucine or valine made the region from amino

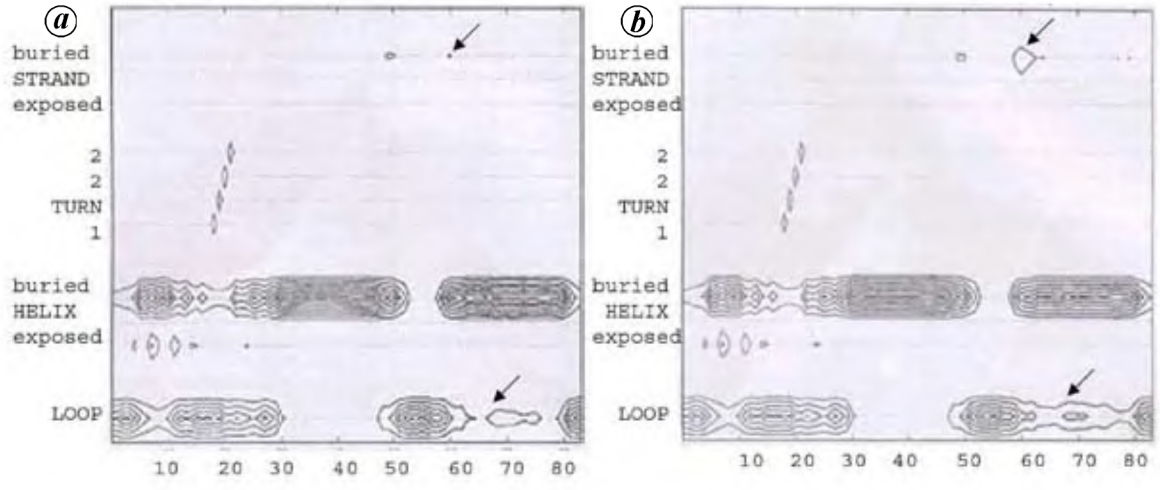

Figure 4. Contour plots for secondary structure determination of EmbB protein. Region spanning AA 243–AA 327. Residue 64 corresponds to Met306 of EmbB. Secondary structure probabilities for *M. tuberculosis* H37Rv with Met306 codon (***a***) and EMB-resistant *M. tuberculosis* with Val306 codon (***b***). Residue 64 shows probability of buried strands and loops. Similar observations also for Ile306 and Leu306.

acid position 302 to 310 more hydrophobic (Figure 3). Similarly, other mutations at codon Asp311His, Asp240His and Arg257Trp (Figure 3) also resulted in increased hydrophobicity with the exception at codon 239, 282, 296 and 304. Mutation at these codons decreased the hydrophobicity index of the surrounding region. There was no change in hydrophobicity due to mutation at codon 299.

We studied the secondary structure of the putative drug-binding site spanning the Met306 (ERDR) and it was found to be composed of loops and strands. However, the probability of strands being buried particularly at codon position 306 was more in the mutant (Met → Ile, Leu or Val) compared to the wild type *M. tuberculosis* (Figure 4). The probability of short loop structure was more at the mutation spanning region when compared to *M. tuberculosis* H37Rv. Thus, the increased hydrophobicity surrounding the ERDR could be well correlated with the high probability of these residues to get buried instead of being exposed, which may lead to suboptimum interaction of the drug with its putative binding site Met306 of the EmbB protein. We predicted that mutation in Met306 made the region surrounding ERDR, amino acid position 302 to 310, more hydrophobic thus suggesting inaccessibility of the drug to its binding site. Transformation experiments by Lety *et al.*[22] have demonstrated altered drug–protein interaction as a possible mechanism for EMB resistance. Our approach of prediction of secondary structure of mutant isolates also supports this mechanism. Contour plots provide a detailed view of secondary structure probabilities and these showed a high probability, of buried strand as a result of mutation at ERDR. The novel mutations studied, with the exception of Leu239Pro, also made the surrounding region more hydrophobic. Moreover, a limited number of other mutations identified so far also lie in this region of the EmbB protein[9,19]. However, presence of mutation at Met 306 shows the importance of methionine as the key amino acid at position 306 for drug binding and development of resistance and not just a surrogate marker for resistance.

Our results suggest that alterations in amino acid sequence could be present from position 239 to 311, and altered protein conformation of drug-binding site (*embB*) due to mutation a possible mechanism for EMB resistance. Mutations at other loci need to be explored in a large number of EMB-resistant clinical isolates.

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ACKNOWLEDGEMENTS. This work was supported by Indian Council of Medical Research. We thank the Director, Tuberculosis Research Centre, Chennai and the Director National Tuberculosis Institute, Bangalore for providing ethambutol-resistant *M. tuberculosis* strains.

Received 12 September 2005; revised accepted 21 July 2006

Toxicological studies of the water extract of green leafy vegetable Sessile joy weed (*Alternanthera sessilis*)

B. M. Gayathri, K. Balasuriya[1], G. S. Panduka de S. Gunawardena[2], R. P. V. Jayantha Rajapakse[2] and H. Ranjith W. Dharmaratne[1,*]

[1]Natural Products Programme, Institute of Fundamental Studies, Kandy, Sri Lanka
[2]Department of Veterinary Pathobiology, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya, Sri Lanka

***Alternanthera sessilis* (L.) DC. (Sessile joy weed; Amaranthaceae) is a popular leafy vegetable in Sri Lanka and also used as traditional medicine in China, Taiwan, India and Sri Lanka. Histopathological test revealed degenerative and necrotic changes in the liver and kidney in Swiss mice, caused by oral administration of water extract of *A. sessilis* in high doses. The major reason for these changes could be due to the effects of cytotoxic substance/s in *A. sessilis*.**

Keywords: *Alternanthera sessilis*, cytotoxicity, histopathology, leafy vegetables.

GREEN leafy vegetables (greens) play a major role in the Sri Lankan diet, probably due to the influence of traditional herbal medicine, easy accessibility and low cost[1]. Further, green leaves are considered as a main source of vitamins, minerals and fibre for the local consumers. Due to their dietary importance, many scientific studies have been carried out on the nutritive values of green leaves[1–3]. However, there is lack of scientific literature on the toxic effects of green leafy vegetables consumed in Sri Lanka. Due to various reasons, including scientific and other information, certain herbs, e.g. *Sauropus androgynus* (L.) Merr. (Mella) have recently been removed from the diet and the people have been encouraged to consume some other greens such as leaves of *Passiflora edulis* Sims (passion fruit). Information as well as misinformation regarding the true nutritional and/or undesirable effects plays a major role in this selection process. Hence, correct information would be needed to educate the general public on their choice of greens for consumption. This requires a systematic scientific evaluation of the nutritional properties as well as the potential toxic effects of locally available greens for human consumption. Removal of a nutritional herb from the diet based on inadequate or erroneous information will deny the consumers of a readily available cheap source of nutrients. On the other hand, inclusion or potential addition of toxic herbs, when little is known about them can be endangered to human health. There are reports published worldwide about the risk of misidenti-

*For correspondence. (e-mail: hrwd@ifs.ac.lk)