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Characterization of *Emb CB* Genes Associated with Ethambutol Resistance in Human Isolates of *Mycobacterium tuberculosis*

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Abstract: The worldwide emergence and spread of multidrug - resistant strains of *Mycobacterium tuberculosis* has adverse effects on tuberculosis control programs. The goal of this paper is to describe the advances made in the understanding of the molecular basis of *M. tuberculosis* resistance to ethambutol drugs and to discuss the potential of molecular methods in early diagnosis of drug-resistant strains. In this study, PCR mediated automated DNA sequencing was used to check for prevalence of ethambutol resistance among treatment failure cases of pulmonary tuberculosis. The identified mutations were dispersed along the *emb CB* genes, but some degrees of clustering of mutations were found at the following regions: 280, 282 Val→ Leu: 284,286 Gly→Val: 290,292Val→Phe: 295,297Ser→Thr: 303,05Gly→Thr: 308, 310Ala→Ser: 309,311 Asp→Glu: 315,317Ser→Ala: 316,320Phe→Tyr. Molecular methods to detect the most frequent mutations in the gene encoding functions that are targets for first-line anti-TB drugs have provided encouraging results for early diagnosis of multidrug resistant tuberculosis. If we are to prevent an epidemic of drug-resistant tuberculosis we must take steps to ensure that all treatment failure patients are diagnosed early and effectively treated. This will reduce treatment cost and potentially limit the spread of ethambutol drug-resistance.

Key words: EMB resistance · Bioanalyzer · Arabinosyl transferases · PCR · DNA sequencing

INTRODUCTION

Mycobacteria remain the causative agents of devastating infections. The increasing appearance of multiple and extremely drug resistant strains poses further threats and underscores the need for novel therapeutic agents. The mycobacterial cell wall contains a number of carbohydrate residues or glycans in the form of unique species-specific glycolipids and lipoglycans, several of which play important roles in the physiology and virulence of these bacteria. Thus, the specific pathways leading to their synthesis are of interest for drug development [1]. The resistance in a great majority of clinical *M. tuberculosis* strains to antimycobacterial agents is due to modification of the drug target brought about by spontaneous mutations (mis-sense or nonsense point mutations and/or small deletions or insertions) in key target genes [2]. Deletion of the entire gene of the drug target or acquisition of new genes is rare. The frequency of spontaneous generation of mutants occurs at the rate of 1×10^{-6} to 1×10^{-8}. The bacterial load in a well-developed cavity in pulmonary TB is quite high (1×10^{9}) ensuring that drug-resistant strains will also be present. Though multiple drug therapy is designed to virtually eliminate the emergence of drug - resistant strains, selective growth of drug - resistant mutants occurs when inappropriate drug regimens (due to inadequate therapy) suppress the growth of susceptible strains but permit multiplication of resistant strains [3,4]. The long duration of the therapy and poor patient compliance add further to the emergence of drug resistance.

The *Mycobacterium tuberculosis emb* operon is a gene cluster of three contiguous genes, namely, *embC*, *embA* and *embB*, which encode mycobacterial arabinosyl transferases [5]. These enzymes are involved in the polymerization of the cell wall arabinan [6]. Inhibition of arabinan synthesis by EMB results in the accumulation of mycolic acids, leading to cell death. Ethambutol (EMB) (a structural analog of arabinose) is a

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first-line anti-TB drug that also inhibits the incorporation of mycolic acids into mycobacterial cell wall [7]. Genetic and biochemical studies have shown that resistance to ethambutol is mediated by mutations in *embB*, one of the three genes encoded by *embCAB* operon. The *embB* gene encodes an arabinosyl transferase, an integral membrane protein with 12 transmembrane domains that is inhibited by the drug. DNA sequence analyses of *embB* genes from ethambutol-resistant strains have shown that EMB-resistance determining region (ERDR) in *embB* is located in the cytoplasmic loop of the membrane spanning domain. Nearly 50-70% of ethambutol-resistant strains contain mis-sense mutations within ERDR of *embB* gene with majority (47-60%) of strains carrying alterations at codon 306. However, the molecular basis of EMB resistance involves other targets as well since nearly 30% of ethambutol-resistant strains lack mutations in the ERDR of embB [8, 9].

Drug-resistant tuberculosis, multidrug-resistant (MDR) tuberculosis and extensively drug resistant tuberculosis are among the greatest threats to the success of tuberculosis control in the world [10, 11]. Treatment of drug-resistant tuberculosis is costly and the outcomes, including survivorship, can be poor [12, 13]. Therefore, early and rapid detection of drug resistance is very important. The aim of this present work was to screen the *embBC* genes with mutations in order to assess the contribution of mutations within these genes to EMB resistance in *M. tuberculosis* clinical isolates from Puducherry state of India.

MATERIALS AND METHODS

Sampling: Two sputum samples[Strain no. 18256/2008(1) and 870/2008(2)] were collected in sterile rigid and wide mouthed container from treatment failure cases hospitalized at State TB Training and Demonstration Centre (Intermediate Reference Laboratory) Government Hospital for Chest Diseases, Pondicherry.

Staining:

Ziehl-Neelsen Method: Take large yellow purulent portion of the sputum samples and spread evenly onto central portion of the microscopic slide and fix the smear to the slide. Cover Cover the slides with freshly filtered 1 % carbol fuchsin and heat underneath until steam rises from the stain and allow the hot carbol fuchsin to react for at least 5 minutes. Wash Add 25% sulphuric acid to react for 2-4 minutes after water washing. Counter stain with 0.1% Methylene blue for 30 seconds and wash as before with water and slope the slides to air dry. Examine the slides under the Microscope to observe for the presence of tubercle bacilli [14].

Fluorescence Method: Smear the specimen onto the centre portion of the slide and allow smears to air-dry for 15 minutes and fix the smear to the slide. Flood the slides with freshly filtered auramine-phenol. Let stand for 7-10 minutes. Decolorize by covering completely with acid-alcohol for 2 minutes, twice. Wash well with running water, as before to wash away the acid alcohol. Counter stain with 0.1% potassium permanganate for 30 seconds. Wash as before with water and slope the slides to air dry. Positive smears were graded into four degrees of positivity using the 20x, 25 x objectives along with 10 x eyepieces [15].

Sputum Processing for Acid Fast Bacilli Culture: To each volume of sputum, 2 volumes of 4% NaOH were added and the caps were screwed firmly on. The bottles were shaken by hand for 1 minute and then they were shaked gently for 20 minutes in the shaking machine. The sputum bottles were centrifuged for 15 minutes at 4000 rpm and the supernatant was carefully poured off into the disinfectant bath and the rim of each bottle was wiped with sterile filter paper. The bottles were filled with 20 ml sterile distilled water, shaken by hand to mix the deposit and were centrifuged for 15 minutes at 4000 rpm. The supernatant was poured off as before and again the neck of the bottle was wiped with sterile filter paper. Finally the sediment was inoculated with a 5 mm diameter loop onto the pre-sterilized and numbered Lowenstein-Jensen's slopes and incubated at 37°C [16].

Isolation and Identification of *Mycobacterium Tuberculosis* Isolates: The isolated strains were identified using various biochemical tests according to Venkataraman, *et al*. [17], Kubica, *et al*. [18] and Tsukamura, *et al*. [19]. The identification process is based on the combination of observation of colony morphology, inability to grow on a culture medium containing p-nitrobenzoate and results of two biochemical tests specific for thermo labile catalase and nitrate reductase. The strains were processed immediately for the further study and aliquots of the decontaminated specimens were kept at -20°C.

Drug Susceptibility Test-proportion Method (Stand and Economic Variant): With a loop, a representative sample of approximately 4-5mg is taken from the primary culture and placed in a McCartney bottle containing 1 ml sterile distilled water and 3mm diameter of 6 glass beads. The bottle was vortexed for 20-30 seconds and the opacity of the bacterial suspension was then adjusted by the addition of distilled water to obtain a concentration of 1mg /ml of tubercle bacilli by matching with McFarland standard No.1.After preparing the standard neat suspension, the dilution 10^{-2} dilution 10^{-4} were produced by discharging two loopfuls (24 SWG-3mm Nichrome wire) of the bacterial suspension. Mix the contents by shaking. Two slopes of medium without drug and one slope of medium with ethambutol drug (2µg/ml) are inoculated with a loopful of each dilution. Incubate the slopes at 37° C and read the proportion tests at 28 days and again at 42 days [20].

Mycobacterium DNA Extraction: One loopful of culture was taken in 100 µl of sterile distilled water and was homogenized. DNA was extracted from the homogenized samples by the phenol: chloroform method according to Mani, *et al*. [21]. The DNA pellet was air-dried and dissolved in 20µl of 1x TE buffer.

PCR Amplification of Template DNA: The isolated template DNA was amplified using IS6110 primer in an authorized thermal cycler (Eppendorf Gradient Cycler). This confirms the template DNA as *Mycobacterium tuberculosis*. The PCR reaction was set up as follows using the primer for *Mycobacterium IS6110* amplification F 5'GTGAGGGCATCGAGGTGG 3' (10 pmol/µl) R 5'CGTAGGCGTCGGTCA CAAA 3' (10 pmol/µl) [22]. The PCR cycling parameters were 94°C for 5 minutes; followed by 40 cycles of 94°C for 1 minute, 57°C for 1 minute and 72 °C for 1 minute; and a final extension of 72°C for 10 minutes. The amplified PCR product was run on a 2% Agarose gel in TAE buffer. The ethidium bromide stained gels were observed in a UV Trans illuminator and photographed using a Geldoc.

PCR Amplification of Drug Target Genes: The isolated template DNA was amplified using *emb C* and *embB* primers; Extr1 5' CGGAGGTAGATGGTAGC CGG 3', Extr2 5' GTTCGACAAGCG C GCCACAC 3' and Extr1 5' GCCAGCA GGTCGTAGTACCA 3'Extr1 5' TGAAGATGGCC GCCATGATC 3' respectively in an authorized thermal cycler (Eppendorf Gradient Cycler) [23]. The PCR cycling parameters were 94°C for 5 minutes; followed by 40 cycles of 94°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute; and a final extension of 74°C for 10 minutes. The amplified PCR product was run on a 2% Agarose gel in TAE buffer. The ethidium bromide stained gels were observed in a UV Trans illuminator and photographed using a Geldoc.

Agarose Gel Electrophoresis: 5µl aliquot of amplified sample mixed with 1 µl of gel loading dye (0.5mg/ml) was loaded into a well of 2% agarose gel in 0.75x TAE buffer and was subjected to electrophoresis for 30 minutes at 100 volts. The gel was observed under UV Transilluminator for specific DNA bands and was photographed. The DNA bands were identified according to the size by comparing with the molecular weight marker (100 bp DNA ladder) loaded in a separate lane.

Electropherogram Analysis of PCR Products: DNA dye concentration and DNA gel matrix were allowed to equilibrate at room temperature. 25µl of dye concentration was added to DNA gel matrix. Vortexed and transferred to spin filter and centrifuged at 2240 g for 15 minutes. The gel dye was allowed to settle at room temperature for 30 minutes. A new DNA chip was placed on the chip priming station. 9µl of gel dye mix was pipetted into the well marked as G and the chip priming station was closed. The plunger was pressed down until it is held by the chip for 60 seconds. After 5 seconds the plunger was pulled back slowly to 1 ml position. The chip priming station was opened and 9µl of gel dye was pipetted into the well marked G and 1µl of ladder was added to the well labeled ladder.5µl of marker was pipetted into all 12 sample wells and in ladder well. 1µl of sample was added into the well. The chip was placed in the Laser Induced Fluorescent instrument (LIF) and the product was analyzed using the Agilent 2100 bioanalyzer according to the manufacturer's protocol [24]. The "lower" and "upper" markers are internal standards used to align the ladder data with data from the sample wells. This is necessary to compensate for drift effects that may occur during the course of a chip run.

DNA Sequencing: The amplified PCR product *emb CB* gene from clinical isolate strains were run on 2% agarose gel and the PCR product was purified using PCR purification kit (Invitrogen- USA& No. K3100-96). The purified PCR product was directly sequenced in an automated DNA Sequencer at Bioserve in Bangalore.

The nucleotide sequence obtained was analyzed using BLASTn Bioinformatics tool available at National Center for Biotechnology Information [25] to know the specificity of PCR amplification and to identify the nucleotide variation. The sequence was further subjected for BLASTx to know the amino acid changes in comparison with the wild type *Mycobacteruim tuberculosis* (H_{37}Rv).

RESULTS

Mycobacterial DNA isolated from (Strain no. 18256/2008(1) and 870/2008(2) cultured on the L.J. medium was subjected to PCR amplification using species specific primers, targeting the insertion sequence IS6110 (Mtb 5'G TGAGGGCATCGA GGTGG 3') (Mtb 5'CGTAGGCGTCGGTCACAAA 3') specific for *M. tuberculosis*. The PCR product was run on a 2% agarose gel. A clear band was formed at 123 bp region confirming the presence of *M.tuberculosis* in the sputum specimen (Figure 1). The templates of *Mycobacterium tuberculosis* clinical isolates and H37Rv wild type strain were amplified using *emb C* and *emb B* primers Extr1 5' CGGAGGTAGATGGTAGCCGG 3', Extr2 5' GTTCGAC AAGCG C GCCACAC 3' and Extr1 5' GCCAGCAGGTCG TAGTACCA 3'Extr1 5' TGAAGATGGCC GCCATGATC 3, respectively. The amplified PCR products were run on a 2% agarose gel. The clear bands were formed at 318 bp and 355bp region, confirming the amplification of *emb CB* regions of *M. tuberculosis* (Figure 2). The templates were analyzed on Bioanalyzer for purity and specificity of the PCR products and Electropherogram of PCR amplified emb *CB* genes confirmed that the molecular size (318bp and 355bp) of the products (Figures 3, 4).

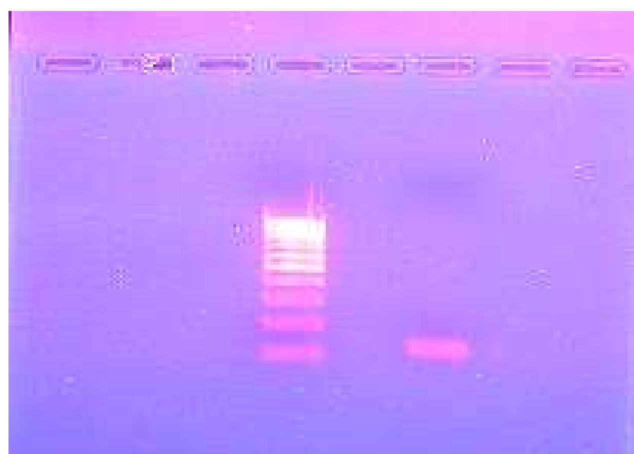

Fig. 1: 123 bp PCR products, Lane 4: 100 bp ladder, Lane 6: 123 bp PCR products

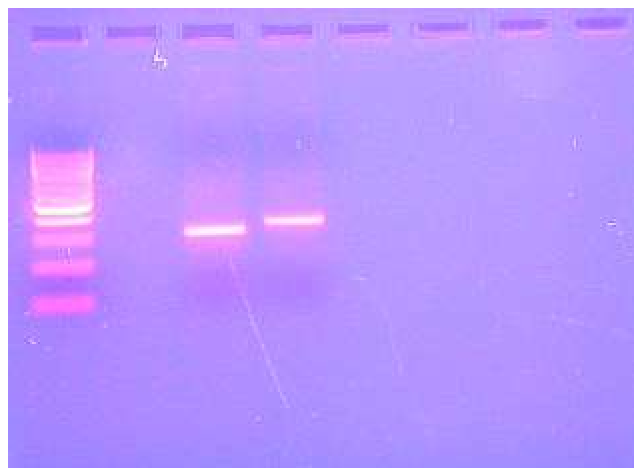

Fig. 2: PCR products of emb *CB* genes, Lane 1: 100bp ladder, Lane 3: *emb C (318bp)* and Lane 4: *emb B (355bp)* PCR product

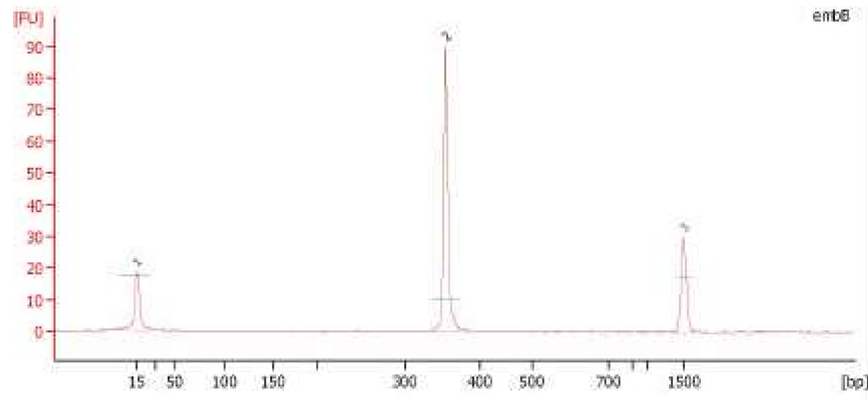

Fig. 3: Electropherogram of PCR amplified *emb B* gene (355 bp) Peak 1: Lower Marker, Peak 2: PCR product of *emb B* gene and Peak 3: Upper marker

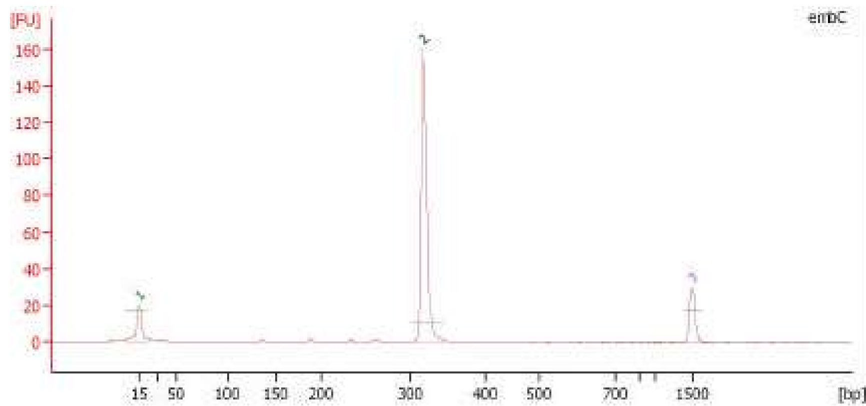

Fig. 4: Electropherogram of PCR amplified *emb C* gene (318 bp) Peak 1: Lower Marker, Peak 2: PCR product of *emb C* gene and Peak 3: Upper marker

Table 1: Characteristics of EMB-resistant clinical *M. tuberculosis* isolates

Codons	Amino acid changes *emb B gene*	Strain no
268	Leu →Phe (CTC→TTT)	1
269	Ile →Leu (ATC → CTC)	1
272	Ser→Arg (AGC→AGA)	2
274	Arg→Trp (CGG→TGG)	1
275	Thr→Ser (ACC→AGC)	2
276	Phe→Thr (TTC→ACC)	1
277	Thr→Gly (ACA→GGA)	1
278	Leu→Gly (CTA→GGA)	2
279	Thr→Leu (ACG→CTG)	1
281	Ala→Thr (GCA→ACA)	1
280	Val→Leu (GTA→TTA)	1,2
282	Val→Leu (GTT→CTT)	1,2
284	Gly→Val(GGC→GTC)	1,2
285	Phe→Ala (TTC→GCC)	2
286	Gly→Val (GGA→GTA)	1,2
287	Phe→val (TTT→GTT)	1
288	Leu→Val (CTT→GTT)	2
289	Leu→Trp (GTG→TGG)	2

Table 1: Continued

Codons	Amino acid changes *emb B gene*	Strain no
290	Val→Phe (GTT→TTT)	1,2
291	Tlu→Val (ATA→GTA)	1
292	Val→Phe (GTC→TTC)	1,2
293	Ile→Val (ATT→GTT)	2
295	Ser→Thr (TCC→ACC)	1,2
297	Ser→Thr (TCA→ACA)	1,2
303	Gly→Thr (GGC→ACC)	1,2
305	Gly→Thr (GGA→ACA)	1,2
308	Ala→Ser(GCA→TCA)	1,2
309	Asp→Glu(GAC→Glu)	2
310	Ala→Ser (GCC→TCC)	1,2
311	Asp→Glu (GAT→GAG)	1,2
315	Ser→Ala(TCC→GCC)	1,2
316	Phe→Tyr(TCC→TAC)	1,2
317	Ser→Ala (TCA→GCA)	1,2
320	Phe→Tyr (TTC→TGG)	1,2
321	Ser→Thr(TCA→ACA)	2
324	Asp→Ala(GAC→GCA)	2

We analyzed two strains of EMB resistant clinical *M. tuberculosis* isolates for potential mutations in the *emb CB* gene to further define the molecular basis of ethambutol resistance and determine the frequency of *emb CB* mutations among EMB-resistant *M. tuberculosis* strains. The results of the sequence analysis of the *emb CB* gene from various ethambutol-resistant strains are presented in Table 1. These two strains with 1% (the critical proportion) of bacilli resistant to ethambutol drug (2µg/ml) are classified as resistant. The resistant pattern of the clinical isolates due to mutation through the PCR mediated DNA sequencing results were compared with in vitro sensitivity tests on ethambutol drug (2µg/ml) slants. This suggests that mutations in the *emb CB* gene are indicative of emb resistance, an important feature for the detection of ethambutol resistance on the basis of identifying mutations in the *emb CB* gene.

DISCUSSION

M. tuberculosis is often acquired early in life with acute infection and with developing immunity, granuloma formation and calcification. This is followed by a long latent period, which continues until reactivation occurs in a proportion of the individuals. This means that individual strains of *M. tuberculosis* have little opportunity to interact and exchange genetic information with other strains compared with, for example, organisms that colonize the nasopharynx or the gastrointestinal tract. In these locations, other bacteria may transmit antibiotic resistance determinants through transmissible genetic elements, transposons, integrons and plasmids, by transduction or transformation. This option is not available for *M. tuberculosis*, so resistance can only occur through chromosomal mutation although rarely movement of mobile genetic elements, such as the insertion sequence IS*6110*, has been associated with new resistance emerging through the inactivation of critical genes [26, 27].

The mechanism of resistance to antibiotics may involve chromosomal mutations or expression of a latent chromosomal gene as a result of exposure to the drug. Substantial progress has been made in our understanding of the molecular basis of drug - resistance in *M. tuberculosis* in the last decade [2]. EMB targets the mycobacterial cell wall through interaction with arabinosyl transferases involved in arabinogalacton (AG) and lipoarabinomannan (LAM) biosynthesis. It specifically inhibits polymerization of cell-wall arabinan, thereby leading to accumulation of b-D-arabinofuranosyl-1-monophosphoryldecaprenol (DPA). 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 [28]. 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) [29-31].

In our study, the mutations were dispersed along the *emb B* genes, but some degrees of clustering of mutations were found at the following regions: 280,282Val →Leu: 284,286 Gly→Val: 290,292Val→Phe: 295,297Ser→Thr: 303, 305Gly→Thr: 308,310 Ala→Ser: 309,311 Asp→Glu: 315,317 Ser→Ala: 316,320Phe→Tyr. No amino acid changes were observed in *emb C* gene. Molecular methods to detect the most frequent mutations in the gene encoding functions that are targets for ethambutol drugs have provided encouraging results for early diagnosis of drug resistance.

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REFERENCES

1. Berg, S., D. Kaur, M. Jackson and P.J. Brennan, 2007. The glycosyltransferases of *Mycobacterium tuberculosis*-roles in the synthesis of arabinogalactan, lipoarabinomannan and other Glycoconjugates. Glycobiol., 17: 35R-56R.
2. Ramaswamy, S. and J.M. Musser, 1998. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*. Tubercle Lung Dis., 79: 3-29.
3. Frieden, T.R., T. Sterling, A. Pablos-Mendez, J.O. Kilburn, G.M. Cauthen and S.W. Dooleyl, 1993. The emergence of drug resistance tuberculosis in NY City. N Eng. J. Med., 328: 521-526.

4. Mahmoudi, A. and M.D. Iseman, 1993. Pitfalls in the care of patients with tuberculosis: common errors and their association with the acquisition of drug resistance. J. Am. Med. Assoc., 270: 65-68.
5. Telenti, A., W.J. Philipp, S. Sreevatsan, C. Bernasconi, K.E. Stockbauer, B. Wieles, J.M. Musser and W.R. Jacobs, 1997. The emb operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. Nature Med., 3: 567-570.
6. Besra, G.S., K.H. Khoo, M.R. McNeil, A. Dell, H.R. Morris and P.J. Brennan, 1995. A new interpretation of the structure of the mycolyl-arabinogalactan complex of *Mycobacterium tuberculosis* as revealed through characterization of oligoglycosylalditol fragments by fast-atom bombardment mass spectrometry and 1H nuclear magnetic resonance spectroscopy. Biochemistry, 34: 4257-4266.
7. Takayama, K. and J.O. Kilburn, 1989. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. Antimicrob Agents Chemother, 33: 1493-1499.
8. Telenti, A., W.J. Phillip and S. Sreevatsan, 1997. The *emb* operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. Nature Med., 3: 567-570.
9. Sreevatsan, S., K.E. Stockbauer and Z. Pan, 1997. Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of *embB* mutations. Antimicrob Agents Chemother, 41: 1677-1681.
10. Cohen, J., 2006. Extensively drug-resistant TB gets foothold in South Africa. Sci., 313: 1554.
11. Espinal, M.A., S.J. Kim, P.G. Suarez, K.M. Kam, A.G. Khomenko, G.B. Migliori, J. Baez, A. Kochi, C. Dye and M.C. Raviglione, 2000. Standard short-course chemotherapy for drug-resistant tuberculosis: treatment outcomes in 6 countries. JAMA, 283: 2537-2545.
12. DeRiemer, K., L. Garcia-Garcia, M. Bobadilla-del-Valle, M. Palacios- Martinez, A. Martinez-Gamboa, P.M. Small, J. Sifuentes-Osornio and A. Ponce-de-Leon, 2005. Does DOTS work in populations with drug-resistant tuberculosis. Lancet, 365: 1239-1245.
13. Espinal, M.A., A. Laszlo, L. Simonsen, F. Boulahbal, S.J. Kim, A. Reniero, S. Hoffner, H.L. Rieder, N. Binkin, C. Dye, R. Williams and M.C. Raviglione, 2001. Global trends in resistance to antituberculosis drugs. N. Engl. J. Med., 344: 1294-1303.
14. Petroff, S.A., 1915. A new and rapid method for isolation and cultivation of tubercle bacilli directly from sputum and faeces. J. Exp. Med. (USA), 21: 38.
15. Laboratory services in tuberculosis control: Part II: Microscopy. WHO/TB/98.258 (1998).
16. Allen B.W. and F.J. Baker, 1968. Mycobacteria Isolation, Identification and Sensitivity testing. Butterworths, pp: 10.
17. Venkataraman, P. and R. Prabhakar, 1977. Niacin production test in mycobacteria; Replacement of benzidine-cyanogen bromide reagent by o-tolidine-cyanogen bromide. Ind. J. Tub., 24: 153.
18. Kubica, G.P. and G.I. Pool, 1960. studies on the catalase activity of acid fast bacilli-1: An attempt to subgroup these organisms on the basis of catalase activities at different temperatures and pH. Am. Rev. Resp. Dis., 81: 287-91.
19. Tsukamura, M. and S. Tsukamura, 1964. Differentiation of Mycobacterium tuberculosis and Mycobacterium bovis by p-nitro benzoic acid susceptibility. Tubercle, pp: 45-64.
20. PAHO/WHO, 1998. Advisory committee on Tuberculosis Bacteriology. Manual of technical standards and procedures for tuberculosis bacteriology. PartIII Sentisivity of *M. tuberculosis* to anti-TB drugs. PAHO. Marlinez. Argentina.
21. Mani, C., N. Selvakumar, V. Kumar, S. Narayanan and P.R. Narayanan, 2003. Comparison of DNA sequencing, PCR-SSCP and PhaB assays with indirect sensitivity testing for detection of rifampcin resistance in *Mycobacterium tuberculosis,* Int. J. Tuber Lung Dis., 7: 625-659.
22. Helen, Fletcher, A., D. Helen Donoghue, G. Michael Taylor, G.M. Adri Van der Zanden and Mark Spigelman, 2003. Molecular analysis of *Mycobacterium tuberculosis* DNA from a family of 18th century Hungarians. Microbiol., 149: 143-151.
23. Sreevatsan, S., 1997. Ethambutol resistance in *M. tuberculosis*: critical role of *embB* mutation. Antimicrob Agents Chemother, 4: 1677-1681.
24. Lucy, Hathaway, J., Silvio Brugger, Alina Martynova, Suzanne Aebi and Kathrin Muhlemann, 2007. Use of the Agilent 2100 Bioanalyzer for Rapid and Reproducible Molecular Typing of *Streptococcus pneumoniae.* J. Clinical Microbiol., pp: 803-809.
25. Altschul, Stephen, F., L. Thomas, A. Madden, Alejandro, Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller and J. David Lipman, 1997. Gapped Blast and PSI-BLAST: A new generation of protein database search programs, J. Nucleic acids Res., 25: 3389-3402.

26. Telenti, A., 1997. The *emb* operon, a gene cluster of *M. tuberculosis* involved in resistance to ethambutol. Nature Med., 3: 567-570.
27. Telenti, A., 1997. The *emb* operon, a gene cluster of *M. tuberculosis* involved in resistance to ethambutol. Nature Med., 3: 567-570.
28. Kilburn, J.O. and J. Greenberg, 1977. Effect of ethambutol on viable cell count in *M. smegmatis*. Antimicrob Agents Chemother, 11: 534-540.
29. Sreevatsan, S., K.E. Stockbauer, X. Pan, B.N. Kreiswirth, S.L. Moghazeh and W.R. Jacobs, 1997. Ethambutol resistance in Mycobacterium tuberculosis: critical role of mutations. Antimicrob Agents Chemother, 41: 1677-81.
30. Telenti, A., W.J. Phillip, S. Srevaatsan, C. Bernasconi, K.E. Stockbauer and B. Wiles, 1997. The emb operon, a gene cluster of M. tuberculosis involved in resistance to ethambutol. Nature Med., 3: 567-70.
31. Ramaswamy, S.V., A.G. Amin, S. Goksel, S.J. Stager, S.J. Dou and E.L. Sahley, 2000. Molecular genetic analysis of nucleotide polymorphism associated with ethambutol resistance in human isolates of Mycobacterium tuberculosis. Antimicrob Agents Chemother, 44: 326-36.