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Full Length Research Paper

Mutation of A918C embB gene in ethambutol resistant *Mycobacterium tuberculosis* in Bandung, Indonesia

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***Mycobacterium tuberculosis* is the agent that causes infectious and contagious tuberculosis disease. The resistance of *M. tuberculosis* to ethambutol is caused by emb gene mutations. Our research group had isolated and characterized 42 clinical isolates of Multi-Drug-Resistant Tuberculosis using multiplex PCR. Some of these isolates are also resistant to ethambutol, but have not been characterized yet. The purpose of this work is to determine the genotypic basis of the MDR-TB clinical isolate which is resistant to ethambutol (L20). The steps in this work consist of sample selection, amplification of the embB gene, nucleotide sequencing, and *in silico* analysis. The result showed a substitution mutation, adenine to cytosine at position 918 of the embB gene, which is a first base of codon 306, corresponding to mutation from methionine to leucine.**

Key words: *Mycobacterium tuberculosis*, ethambutol resistance, embB, A918C substitution.

INTRODUCTION

Tuberculosis (TB) commonly affects the lungs, but also can affect other body parts such as brain, skin, bones, and lymph nodes (WHO, 2008). Source of transmission of TB is patient sputum containing *Mycobacterium tuberculosis*, which floated in the air and inhaled by others (Singh *et al*., 2006). Tuberculosis can be cured with proper, complete, and orderly treatment. WHO recommended treatment consists of a combination of isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and streptomycin (STR) or ethambutol (EMB) during the first two months, followed by a combination of INH and RIF for four months (Ducati *et al*., 2006). Treatment of irregular and incomplete drug combination in the past led to Multi-Drug-Resistant TB (MDR-TB) if *M. tuberculosis* resistant to INH and RIF. The Extensively-Drug-Resistant TB happen if MDR-TB is also resistant to fluoroquinolones and at least one second-line drugs, namely amikacin, kanamycin, and capreomycin (Rattan *et al*., 1998; WHO, 2008; Tuberculosis, 2008).

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Previous studies estimated the resistance of *M. tuberculosis* of EMB caused by mutations in the embB gene codon 306 that converts methionine to valine, leucine, or isoleucine (Alcaide *et al*., 1997; Lee *et al*., 2002; Sreevatsan *et al*., 1997; Mokrousov *et al*., 2002; Ramaswamy *et al*., 2000; Garg et al., 2006). EMB is arabinose analog which inhibit the arabinosiltransferase, so the synthesis of arabinan to the formation of cell wall is disrupted and led to the accumulation of mycolate acid that causes *M. tuberculosis* death (Wolucka *et al*., 1994; Maddry *et al*., 1996; Belanger *et al*., 1996; Rattan *et al*., 1998; Mokrousov *et al*., 2002a; Mokrousov *et al*., 2002b). EMB target is EmbB protein that encoded by the embB gene of the embCAB operon in *M. tuberculosis*. Mutations primarily on amino acid position 306 embB associated specifically with EMB-resistant, so embB expressed as ethambutol resistance determining region (ERDR) (Deng *et al*., 1995; Alcaide *et al*., 1997; Sreevatsan *et al*., 1997; Telenti *et al*., 1997; Mokrouov *et al*., 2002a; Sharma *et al*., 2006). Telenti *et al.* (1997) estimated that amino acids 306 of EmbB located in the cytoplasm grooves that form ERDR, and Alcaide *et al*. (1997) showed that amino acids in this region are

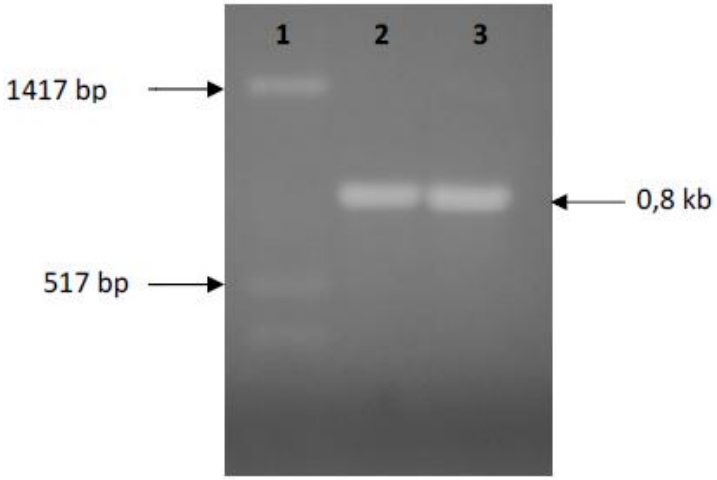

Figure 1. The results of agarose gel electrophoresis *M. tuberculosis* H37Rv and L20 isolate. Lane 1 is pUC19/HinfI DNA marker, lane 2 is L20 isolate, and lane 3 is *M. tuberculosis* H37Rv wild type.

sustainable, which is found in EmbB protein of *M. tuberculosis*, *M. leprae, M. smegmatis,* and many other species of mycobacteria (Alcaide *et al*., 1997; Telenti *et al*., 1997; Tuberculosis, 2008).

Our research group that focus in MDR-TB in Bandung, Indonesia had been succeeded to isolation and characterization of 42 clinical isolates of MDR-TB using the multiplex PCR. Several clinical isolates of MDR-TB is also resistant to EMB but the cause of the resistance at the genotype level has not been characterized (Noviana, 2007). The purpose of this work is to identify the presence or absence of mutations at the embB gene in L20 isolate which MDR-TB that resistant to ethambutol.

MATERIALS AND METHODS

Reagents: L20 MDR-TB isolates which has been characterized in previous studies and *M. tuberculosis* H37Rv (ATCC 25618), master mix (MDBio. Inc.), embF 5'-GGTGCGCGCCATGCCACC-3' and embR 5'-GGTCTGGCAGGCGCATCC-3' (Eurogentec Ait, Singapore) based on Ramaswamy *et al*. (2000) research, agarose (Merck), ethidium bromide (Merck), Tris acetate (Merck), EDTA (Merck), sucrose (Merck), bromophenol blue (Pharmacia), and plasmid pUC19/HinfI (MDBio. Inc.).

Sample selection: The sample was L20 isolate which isolated from patient sputums of BPLK Laboratory. Phenotype of L20 isolate was resistant to RIF, INH, EMB, PZA, kanamycin, and mutated in rpoB and katG genes, but the genotype of the embB gene have not been characterized yet.

PCR and product determination: DNA templates were prepared by suspending a loop of the sputum patient in 100 mL TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and then subjecting in to 20 min of deactivation at 80 °C and 30 min of boiling (Telenti and Persing, 1996). For PCR, 0.2 μL of DNA template was added to 25 μL PCR mixture (MDBio. Inc.) containing reverse primer embR 5'-GGTCTGGCAGGCGCATCC-3' and forward primer embF 5'-GGTGCGCGCCATGCCACC-3', targeting 800 bp conserved embB sequence region. Reaction mixtures were then heated in thermal cycler (GeneAmp PCR system 2700) as follows: 95 °C for 5 min followed by 30 cycles at 95 °C for 1 min, annealing at 72 °C for 1 min, and an extension at 72 °C for 1 min. Final extension was done at 72 °C for 4 min. Efficient amplification was confirmed by electrophoresis on 1.5% agarose gel.

Sequencing and *in silico* analysis: Sequencing of amplified PCR products was carried out using the ABI PRISM 377 Genetic Analyzer (Perkin Elmer). Sequences generated using the program were compared to their respective wild type (H37Rv, Accession : AP012340.1 GI: 379026087) sequences using MegAlign program (DNASTAR, USA).

RESULTS AND DISSCUSSION

The target of amplification was the area before and after the embB gene codon 306, which is ERDR. The product of EmbB gene amplification of *M. tuberculosis* H37Rv and L20 isolate performed at 72 °C annealing temperature produces a 0.8 kb-sized band with the same intensity to the intensity of the first band of the marker PUC/HinfI (Figure. 1).

Figure.1 showed the estimated size of 0.8 kb DNA band (Ramaswamy *et al*., 2000). The location of the band is positioned between the first and second band of pUC19/HinfI DNA marker, i.e.1.4 kb and 0.5 kb. The size

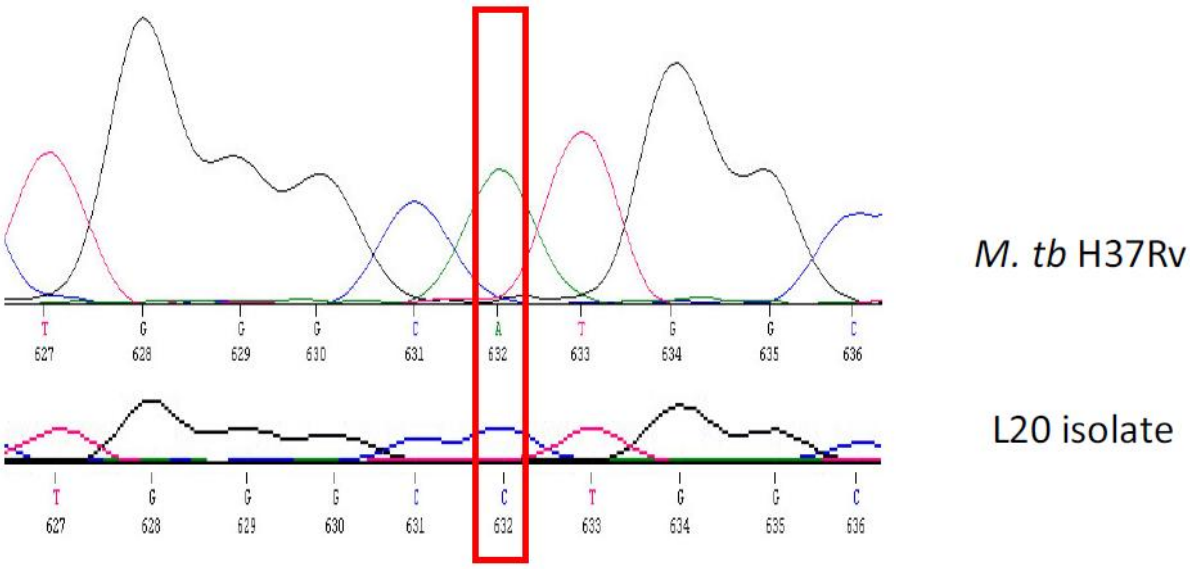

Figure 2. Electropherogram of the embB gene fragment of *M. tuberculosis* H37Rv and L20 isolate

of EmbB gene fragments agreed with the estimation of the length fragment for embF and embR primers on Editseq program, DNASTAR.

The results of alignment between the two samples indicate different bases at position 918, namely adenine (A) in *M. tuberculosis* H37Rv wild type and cytosine (C) to L20 isolate, which indicates a substitution mutation of A to C (Figure. 2). In the present study, the aim was to identify the presence or absence of mutations at the embB gene in L20 isolate that MDR-TB that resistant to ethambutol. Our result exhibited substitution mutation at codon 306 in the embB gene. Other studies have also reported similar trend, e.g Korean study (Lee *et al*., 2002), American study (Ramaswamy *et al*., 2000), German study (Rinder *et al*., 2001), and Russian studies (Mokrousov *et al*., 2002a; Mokrousov *et al*., 2002b).

The results of nucleotide sequence alignment found an A918C mutation that changes the amino acid residues Met306Leu, this result supports the previous research which reported that the most frequent mutations in the embB gene mutations was adenine substitution at position 918 to cytosine, thymine or guanine residues that would alter amino acid residues of methionine to leucine or valine at codon 306. This mutation known as "hot spots" cause of resistance of *M. tuberculosis* to EMB and the most frequently found in publications (Lee *et al*., 2002; Sreevatsan *et al*., 1997; Mokrousov *et al*., 2002a; Mokrousov *et al*., 2002b; Ramaswamy *et al*., 2000).Nucleotide mutation in 918 position is located on the first base 306, ATG to CTG, which altered the amino acid residue from methionine to leucine. The Met306 mutations cause the area around ERDR, i.e. amino acid residue at 302-310 position, had more hydrophobic characteristic, so EMB was difficult to achieve the binding site of EmbB protein (Lety *et al*., 1997; Sonnhammer *et al*., 1998; Grag *et al*., 2006; Das *et al*., 2006). The alteration of EMB-EmbB interaction is estimated to cause the resistance of *M. tuberculosis* to EMB.

Conclusion

Substitution mutation (A918C) in the embB gene L20 isolate made base alteration, from methionine to leucine. This mutation known as "hot spots" cause of resistance of *M. tuberculosis* to EMB and the most frequently found in publications.

REFERENCES

Alcaide F, Pfyffer GE, Telenti A (1997). Role of emb*B* in Natural and Acquired Resistance to Ethambutol in Mycobacteria. Antimicrob. Agents Chemother., 41: 2270–2273.

Belanger AE, Besra GS, Ford ME, Mikusova K, Belisle JT, Brennan PJ, Inamine JI (1996). The emb*AB* Genes of *Mycobacterium avium* Encode an Arabinosyl transferase Involved in Cell Wall Arabinan Biosynthesis that is the Target for the Antimycobacterial Drug Ethambutol. Proc. Natl. Acad. Sci., 93: 11919–11924.

Das R, Katoch K, Jadauan GPS, Gupta P, Sharma VD, Malhotra B, Garg A, Ayyagari A, Chauhan DS, Singh HB, Yadav P, Katoch VM (2006). Studies On Mutations In Emb*b* Locus In Indian Clinical Isolates Of *Mycobacterium Tuberculosis* Having High Degree Of Ethambutol Resistance. Current Science, 91(7): 293-295.

Deng L, Mikusova K, Robuck KG, Scherman M, Brennan PJ, McNeil MR (1995). Recognition of Multiple Effects of Ethambutol on Metabolism of Mycobacterial Cell Envelope. Antimicrob. Agents Chemother, 39: 694–701.

Ducati RG, Netto AR, Basso AA, Santos DS (2006). The Resumption of Consumption. A Review on Tuberculosis. Mem Inst Oswaldo Cruz, 101: 697-714.

Garg A, Srivastava S, Ayyagari A, Katoch VM, Dhole TN (2006). embB Gene Mutations Associated with Ethambutol Resistance in Indian Strain of *Mycobacterium tuberculosis*. Current Science, 91**(7)** : 1512-1517.

Lee HY, Myoung HJ, Bang HE, Bai GH, Kim SJ,Kim JD, Cho SN (2002). Mutations in the embB locus among Korean clinical isolates of *Mycobacterium tuberculosis* resistant to ethambutol. Yonsei Med. J., 43: 59–64.

Lety MA, Nair S, Berche P, Escuyer V (1997). A Single Point Mutation in the embB Gene is Responsible for Resistance to Ethambutol in *Mycobacterium smegmatis*. Antimicrob. Agents Chemother., 41: 2629–2633.

Maddry JA, Suling WJ, Reynolds RC (1996). Glycosyltransferases as Targets for Inhibition of Cell Wall Synthesis in *M. tuberculosis* and *M. avium*. Res. Microbiol., 147: 106–112.

Mokrousov I, Narvskaya O, Limeschenko E, Otten T, dan Vyshnevskiy B (2002a). Detection of Ethambutol-Resistant *Mycobacterium tuberculosis* Strains by Multiplex Allele-Specific PCR Assay Targeting embB306 Mutations. J. Clin. Microbiol., 40: 1617-1720.

Mokrousov I, Otten T, Vyshnevskiy B, Narvskaya O (2002b). Detection of emb*B306* mutation in susceptible isolates of *Mycobacteriumtuberculosis* from North Russia: Implications for genotyping resistance testing. *Antimicrob. Agents Chemother*. 40: 3810–3813.

Noviana H (2007). Hubungan *Multidrug-Resistant Mycobacterium tuberculosis* dengan Gen-gen yang Terkait dan Pengaruh Mutasi terhadap Tingkat Resistensi Rifampin dan Isoniazid. Institut Teknologi Bandung. pp. 30-45.

Ramaswamy SV, Amin A, Göksel S, Stager CE, Dou S, El Sahly H, Moghazeh SL, Kreiswirth BN, Musser JM (2000). Molecular Genetic Analysis of Nucleotide Polymorphisms Associated with Ethambutol Resistance in Human Isolates of *Mycobacterium tuberculosis*. Antimicr. Agents and Chemother., 326-336.

Rattan A, Kalia A, Ahmad N (1998). Multidrug-resistant *Mycobacterium tuberculosis*: Molecular Perspectives. Emerging Infectious Disease, 4: 195-209.

Rinder H, Mieskes KT, Tortoli E, Richter E, Casal M, Vaquero M, Cambau E, Feldmann K, Loscher T (2001). Detection of emb*B* codon 306 mutations in ethambutol resistant *Mycobacterium tuberculosis* directly from sputum samples: a low cost, rapid approach. Mol. Cell. Probes, 15: 37–42.

Sharma K, Gupta M, Pathak M, Gupta N, Koul A, Sarangi S, Baweja R, dan Singh Y (2006). Transkripsional Control of the Mycobacterial embCAB Operon by PknH through a Regulatory Protein, EmbR, *In vivo*. J. Bacteriol., 188: 2936-2944.

Singh Y, Vohra R, Gupta M, Chaturvedi R (2006). Attack on the Scourge of Tuberculosis: Patented Drug Targets, Benfham Science Publishers Ltd. Recent Patents on Anti-Infective Drug Discovery, 1: 95-106.

Sonnhammer ELl, Heijne GV, Krogh A (1998). A Hidden Markov Model for Predicting Transmembrane Helices in Protein Sequences, In J. Glasgow, T. littlejohn, F. Mayor, R. Lathrop, D. Sankoff, dan C. Sensen (ed.), Proceeding of Sixth International Conference on Intelligent Systems for Molecular Biology, AAAI Press, Menlo Park, Calif, 175-182.

Sreevatsan S, Stockbauer KE, Pan X, Kreiswirth BN, Moghazeh SL, Jacobs (Jr.) WR, Telenti A, Musser JM (1997). Ethambutol Resistance in *Mycobacterium tuberculosis*, Critical Role of embB Mutations. Antimicrob. Agents Chemother., 41: 1677–1681.

Telenti A, Persing DH (1996). Novel strategies for the detection of drug resistance in *Mycobacterium tuberculosis*. Res. Microbiol., 147:73–79.

Telenti A, Philipp WJ, Sreevatsan S, Bernasconi C, Stockbauer KE, Wieles B, Musser JM, Jacobs WR (Jr.) (1997). The emb Operon, A Gene Cluster of *Mycobacterium tuberculosis* Involved in Resistance to Ethambutol. Nature Med, 3: 567–570.

Tuberculosis (2008). 88(2): 102-105. Accessed on 28 July 2008.

WHO Global Tuberculosis Control(2008). Surveillance Planning Financing, WHO Report, Geneva, Switzerland, WHO/HTM/TB/2008, 393.

Wolucka BA, McNeil MR, de Hoffmann E, Chojnacki T, Brennan PJ (1994). Recognition of Lipid Intermediate for Arabinogalactan/arabinomannan Biosyntesis and Its Relation to Mode of Action of Ethambutol on *Mycobacterium*. J. Biol. Chem., 269: 23328-23335.