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MUTATIONS IN 1700 BP FRAGMENT OF RPOB GENE OF MULTI-DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATE

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ABSTRACT

This research aimed to amplify a 1700 bp fragment of rpoB gene of multidrug resistance M. tuberculosis (MDR-TB) isolate and determine types of mutation beyond the core region (hot-spot region). DNA sequencing studies indicate that more than 95% of rifampin-resistant M. tuberculosis strains have mutations within the 81-bp hot-spot region (codons 507 to 533) of the RNA polymerase β-subunit (rpoB). Since almost 90 % of rifampicin resistant isolate are also resistant to isoniazid, mutation in rpoB gene become important as a surrogate marker for MDR-TB. MDR- TB isolates used for this research, namely isolate 885, was collected by Regional Health Laboratory of Surabaya. PCR was used to amplify the gene, on described steps : a cycle of preheating at 95°C for 15 minutes, amplifying in 45 cycles (1 minute at 94°C, 1 minute at 58°C, 1 minute 72°C) and post extension for 5 minutes at 72°C. The mutations were detected by sequencing and alignment using MEGA4. The result of this research showed that there were new mutations downstream of the core region of rpoB. Sequence analysis showed some mutations such as S594A, S626V, T629A. In conclusion, it is reported for the first time, new mutations at downstream region of the core region of rpoB.

Keywords : M.tuberculosis; rifampicin resistance; MDR-TB.

INTRODUCTION

Tuberculosis is a major cause of death among people all around the world. Indonesia ranks third on the list of 22 high-burden tuberculosis (TB) countries in the world. According to the World Health Organization's (WHO's) Global Tuberculosis Control Report 2008, there was an estimated 535,000 new TB cases and an estimated incidence rate of 105 new sputum smear-positive (SS+) cases per 100,000 people in 2006. Based on WHO disability-adjusted life-year calculations, TB is responsible for 6.3 percent of the total disease burden in Indonesia, compared with 3.2 percent in the Southeast Asian region.[1]

Eventhough, regiment of four drug combination has been implemented, single-drug resistant and multi-drug resistant of Mycobacterium tuberculosis (MDR-TB) are continuously increasing. A major challenge to TB control in Indonesia is the containment of multidrug- resistant (MDR) TB. Though the MDR rate is still relatively low, the total number of MDR cases is considerable due to the large numbers of TB patients. It is estimated that yearly there are at least between 5,000 and 10,000 new SS+ MDR-TB patients. MDR-TB is generated mainly due to the large numbers of TB patients who are inadequately treated. The private sector also provides TB treatment but the treatment outcomes are not captured by the National TB Control Program (NTCP) surveillance system. Based on the WHO 2008 report, MDR-TB rates among previously treated cases is 19 percent. The high defaulter rate and the misuse of second-line drugs in hospitals are contributing to the increase of drug-resistant TB. The first representative drug resistance survey in Java is currently being completed, with support from USAID. Ninety-eight percent of 1,226 samples have been tested for drug resistance. Preliminary results of this survey indicate that the level of MDR-TB is around 1.5 percent among new patients and 14 percent among re-treatment cases.[1]

According to the World Health Organization (WHO), MDR-TB is a form of TB that does not respond to the standard six month treatment using first line-drugs (i.e. at least resistant to isoniazid and rifampicin). Rifampin resistance is of particular epidemiologic importance, since it represents a valuable surrogate marker for multidrug-resistant (MDR) tuberculosis strains, and the prevalence of MDR strains is a significant obstacle to tuberculosis therapy. DNA sequencing studies indicate that more than 95% of rifampin-resistant M. tuberculosis strains

have mutations within the 81-bp hot-spot region (codons 507 to 533) of the RNA polymerase β-subunit (rpoB) gene.[2]Over the last 15 years, Kapur et al. and Telenti et al. have identified the molecular basis of rifampin resistance in M. tuberculosis. Some researchs reported that almost 90 % of rifampicin resistant isolate are also resistant to isoniazid. Therefore, resistance to rifampicin is used to be a surrogate marker of MDR-TB.[3,4]

Lingala et al,2010 reported, for the first time multiple silent mutations, between the codons 145-184 (outside the hot spot region). Present study aims to study amplify a 1700 bp fragment of rpoB gene of multidrug resistance M. tuberculosis (MDR-TB) isolate and determine types of mutation beyond the core region.[5]

MATERIALS AND METHODS

Materials

Mycobacterium strains of MDR-TB 885 was obtained from culture collection of Regional Health Laboratory, Surabaya, East Java, rifampicin 4 ppm, Lowenstein-Jenssen medium (L-J medium), Miniprep DNA isolation kit (Invitrogen)

Methods

Subculture on L-J medium: A loopful of isolate MDR-TB was inoculated on L-J media with 4 ppm rifampicin and incubated at 370 C for 21 days.

DNA extraction: A loopful of MDR-TB growth on L-J medium, was added to NaCl 0.9%. and lysed by TE(Tris-EDTA) 1X, lysis cell solution (guanidin thiocyanate, Tris-HCl, EDTA and Triton-X) and 20 µl ddH2O. The mixture solutions was shaked for 10 minutes and centrifuged at 100 rpm for 2 minutes. Genomic DNA was extracted by modified Boom methods using buffer solution, 70% ethanol, acetone, and TE as described previously. DNA concentration yields was detected by nanogram.[6]

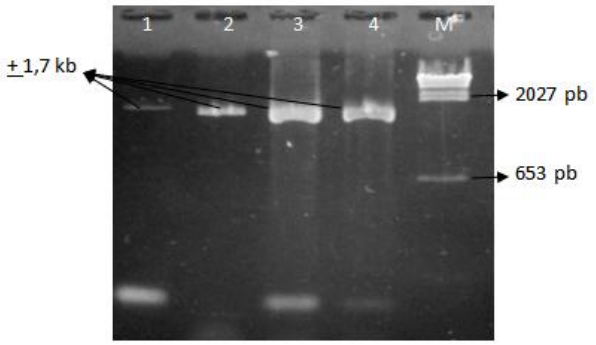
Amplification and Sequencing 1700 bp fragment: Amplification of fragment rpoB gene was carried out using primers FNdeR1 5'-GCC ATA TGA TGT CTC CGA TCG ACC ACT TC 3' and RBamR1 5' GTG GAT CCT GTC GTG CAT CAC AGT GAT GTA G 3'. Total volume of PCR were 20 µL Amplification conditions was set on described steps : a cycle of preheating at 95°C for 15 minutes, amplifying in 45 cycles (1 minute at 94°C, 1 minute at 58°C, 1 minute 72°C) and post extension for 5 minutes at 72°C. Amplification product of 1700 bp was visualized on 1,5 % agarose. Sequencing was done at First Base Laboratory, Singapore using specific primers as described previously.

DNA sequence analysis: DNA sequences were analyzed after DNA sequencing by BLAST using multiple sequence alignments (http://www.ncbi.nlm.nih.gov/BLAST) and MEGA4. Mutations were detected in the respective gene by comparison with M. tuberculosis H37Rv (wild-type reference laboratory strain).

RESULTS

During PCR condition was optimized, based on advice given from the program Clone Manager 6 and adapted to the tool. The purpose of PCR was to amplify the rpoB gene. The result of PCR consisted of PCR product of + 1.7 kb that were detectable trough electrophoresis as presented in Figure 1.

Figure1. Electroforegram of PCR Product on 1,5% agarose



1. H37Rv isolate; 2. Biomolecular lab.MDR-TB isolate; 3. 836 isolate; 4. 885 isolate, 5. Ladder gene marker HindIII / λ; The target rpoB gene fragment of 1707 bp.

Nucleotide sequencing results were analyzed using the program MEGA 4. Sequence alignment of the sample PCR product and a few sequences of MDR-TB in some countries around the world that are available in data base (www.ncbi.nlm.nih.gov) were performed at the nucleotide codons 585-732. The results showed some differences with the alignment of nucleotide sequences of wild type M. tuberculosis H37Rv as listed in Table 1.

Table 1. The difference in rpoB gene sequences

Title of sequence	Nucleotide mutation	Predictively amino acid mutations
367031_885_R_rpoB	AGC→ GCC, TCG → TCC, CAC→ ACC,	S594A, S626V, T629A
M._tuberculosis_MDR-TB4_rpoB	-	-
M._tuberculosis_DR-TB2_rpoB	-	-
M._tuberculosis_pan-DR-TB1_rpoB	-	-
M._tuberculosis_MDR-TB1_rpoB	-	-
M._tuberculosis_CJ10_rpoB	ACA → GCA	T652A
M._tuberculosis_JX041_rpoB	TCG → CCG	S627T
M._tuberculosis_H37Rv_Y_rpoB	-	-
M._tuberculosis_MDR-TB3_rpoB	-	-
M._tuberculosis_MDR-Iran-98/1384_rpoB	-	-
M._tuberculosis_MDR-Belarus-369_Minsk	-	-
M._tuberculosis_MDR-Belarus-388_rpoB	-	-
M._tuberculosis_108_Tehran_rpoB	ACA → GCA, CGC → GGG, TGG → GGG, GGC → CAC	T652A R659G W660G G661D

The result of sequence analysis showed that there were mutations such as S594A, S626V, T629A. All detected mutations were beyond the core region of rpoB gene, i.e. in downstream of the core region.

Comparison with some of the sequences of several MDR-TB countries formed a phylogeny tree as can be seen in Figure 2.

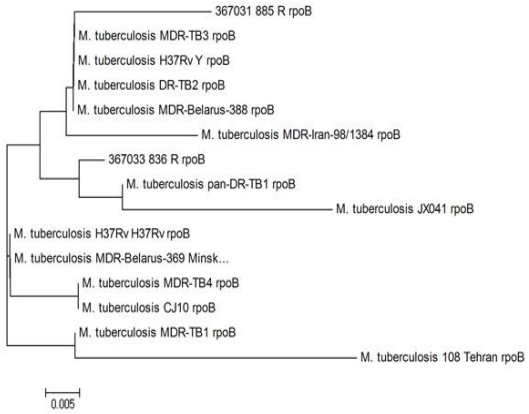


Figure 2
The 885 isolate

DISCUSSION

Culture remains the most sensitive and reliable methods for detection of M. tuberculosis drug resistance. However, phenotypic testing is time-consuming and requires specialized laboratory facilities. Therefore, there is a need for rapid molecular methods for detection of drug resistance. This study illustrates that molecular methods for antimicrobial susceptibility testing of MDR M. tuberculosis isolates test not only primary mutational hot-spot regions of the rpoB.

The analysis in this study revealed several amino acid differences. In the 885 isolates there is a change from the wild type as follows S594A, S626V, T629A. Amino acid changes did not occur in the hot spot region (81 bp, codons 507-533). During 90-98% of rifampin-resistant M. tuberculosis were reported to have mutations in the core area. However, there is also a low level of resistance was reported that showed mutations outside the region, namely the L176F.[7] Multiple silent mutations in codon regions 145 to 148 rpoB gene of MDR-TB were also found in studies in India.[4]

In the 885 isolate, the first mutation is S594A, from serine to alanine which is polar to non polar, with changes in the first two nucleotides of the codon. The next change is the S626V, serine to valine which is non polar, because changes in the third nucleotide of the codons. Other changes are T629A, which are polar threonine to alanine which is polar, due to changes in the first two nucleotides of the codon. Serine and threonine are polar amino acids with side chains are reactive. Serine is containing oxygen atoms which act as potent nucleophiles in several enzymes. Threonine is containing hydroxyl groups which contribute to the formation of hydrogen bonds.[8]

Generally stated that the resistance to rifampicin is due to mutations in the rpoB core region. Mutations in these regions produce a high level resistance (MIC> 32µg/ml). In this study, the concentration of rifampicin were added to the culture medium for optimal growth is 4 ppm and found mutations outside the core area. It is similar to the studies that have been done, that mutations outside the region usually associated with low-level resistance (MIC, 4 µg / ml).[9] Thus it can be said that isolates used in this study were MDR-TB isolates with low resistance levels.

The tree of phylogeny indicated that isolates 885 closer to the MDR isolates from Belarus and Iran with the time difference of less than 5 years (Mega 4). This isolate included in the Beijing family group which is spread widely around the world.[10] It is due to M. tuberculosis is a travel disease as HIV.

CONCLUSION

Some mutations were showed in this study, i.e T629A,S626V,D623P, and Q624K. This region of mutation is reported for the first time, new mutations at downstream region of the core region of rpoB.

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