

AMPLIFICATION OF 0.7KB FRAGMENT KATG GENE FROM CLINICAL MULTI DRUG RESISTANT TUBERCULOSIS ISOLATE IN BALI

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ABSTRACT

During last decade has seen a particular increase in the occurrence of drug-resistant of tuberculosis (DR-TB) and multi-DR strains, such as Isoniazid (INH) resistant strains of *M. tuberculosis*. INH resistance is more frequently associated with mutations in the *katG* gene. Detection of *katG* gene mutations can be performed by PCR technique, followed by sequences. The aim of this study is to amplify *katG* gene region (0,7 Kb) from clinical isolate of MDR-TB in Bali. DNA isolation for PCR was done by Boom method and *katG* gene amplification was performed under the following conditions: predenaturation at 95^oC for 15 min; fourty cycles of denaturation at 94^oC for 1 min, annealing at 56^oC for 1 min, extension at 72^oC for 2 min; final extension at 72^oC for 10 min. The amplicons were detected by 1.5% agarose gel electrophoresis and showed a specific band size at 0.7 kb. This suggests that the fragment of *katG* gene has been successfully amplified in these areas.

Keywords: amplification, *katG* gene, MDR-TB, 0.7 Kb

INTRODUCTION

Tuberculosis, caused by *M. tuberculosis* is one of the most important re-emerging infectious diseases.¹ Prevalence of tuberculosis in Indonesia (1990-2010) reached 289 cases per 100,000 population.² Particularly, in Bali, prevalence of TB cases occurred by 64 of 100,000 population based on a survey in 2004³. The last decade has seen a particular increase in the occurrence of drug-resistant (DR-TB) and multi-DR strains, such as Isoniazid (INH) resistant strains of *M. tuberculosis*.⁴ Isoniazid (INH) is a prodrug which is converted into a biological active form by *M. tuberculosis* catalase-peroxydase, enzyme *katG*.⁵ INH resistance in *M. tuberculosis* is caused by gene mutation that involves several genes, there are *katG*, *inhA*, *oxyR-ahpC* and *kasA*.⁶ INH resistance is more frequently associated with mutations in the *katG* gene.⁷ Mutation at codon 315 of *katG* gene is most prevalent in isoniazid resistant *M. tuberculosis*.⁸

Detection of *katG* gene mutations can be performed by PCR technique, followed by sequencing. In some studies, there are several options oligonucleotide primers *katG* gene region, a case of regional 209 bp, 351 bp and 580 bp.^{4,5,9} In this study,

we designed new primers with a longer area of amplification, that is 724 bp.

The frequency and type of mutations in the *katG* gene specific because of differences in geography^{10,11}. In addition, no studies have revealed about *katG* gene mutation from clinical isolate of MDR-TB in Bali. Therefore, research on the matter is needed. To achieve these goals, this study will be performed *katG* gene amplification with primers that have been designed to amplify 724 bp (2437-3160 bp) *katG* gene region from clinical isolate of MDR-TB in Bali.

MATERIALS AND METHODS

DNA Isolation for PCR

DNA isolation was carried out by Boom method for one clinical isolate of MDR-TB in Bali. Bacterial was grown on Lowenstein Jensen medium that had been dissolved in PBS (*Phosphate Buffer Saline*). The lysis cell was carried out using lysis buffer L6 and washing buffer L2. DNA resulted in isolation method was used as template DNA in the PCR process.

katG Gene Amplification

Amplification was done with *Taq DNA polymerase* as the main enzyme. Primers was designed to amplify a fragment of *katG* gene which size was 724 bp. Oligonucleotide primers that used for this PCR, consisted of forward primer (KG24F): 5' GAA GTA CGG CAA GAA GCT CTC 3' and reverse primer

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(KG60R): 5' CGT GAT CCG CTC ATA GAT CG 3'. The reaction of PCR was performed under the following conditions: pre denaturation at 95°C for 15 min; forty cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 2 min; final extension at 72°C for 10 min.

Agarose Gel Electrophoresis

The amplicons were detected by 1,5% agarose gel electrophoresis with TBE 1X and visualized by staining with 2,8 µL ethidium bromide. Running of electrophoresis was done at 65 V for 45 min. Visualization was carried out by UV transilluminator (Gel Doc®).

RESULTS

The study begins with DNA isolation and continued with quality test of DNA to ensure DNA isolation outcome. Quality test of DNA done by PCR amplification using standard primer for *M. tuberculosis*. Result showed the band formed (Figure 1) and means that positive results containing DNA.

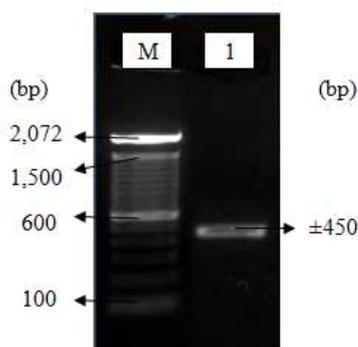


Figure 1 PCR products using standard primers *M. tuberculosis*: M. marker (Invitrogen®) 100 bp DNA ladder, 1: fragment DNA from amplification of *katG* gene of clinical isolate *M. tuberculosis*

Next step is a PCR optimization to amplify our targeted 724 bp fragment. The following thermocycler parameters were applied with following condition: pre denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 1 min, variation of annealing temperature at 53°C, 56°C, 59°C, 62°C, 65°C and 72°C for 1 min, extension at 72°C for 1 min; final extension at 72°C for 10 min. No band resulted from optimization above these conditions. Therefore, it is needed to re-optimize PCR condition to produce a band at 0.7 kb region. The improved conditions were: pre denaturation at 95°C for 15 min; forty cycles of denaturation at 94°C for 1 min, annealing at 48°C, 50°C, 52°C, 54°C, 56°C, 58°C for 1 min, extension at 72°C for 2 min; final extension at 72°C for 10 min. Based on these conditions produced band at 0.7 kb (Figure 2).

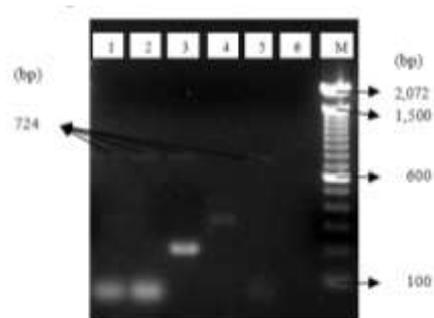


Figure 2

PCR product of segment 0,7 Kb *katG* gene in several annealing temperature: 1. 48°C, 2. 50°C, 3. 52°C, 4. 54°C, 5. 56°C, 6. 58°C, M. marker (Invitrogen®) 100 bp DNA ladder

Results from these optimization conditions formed a thin band with dimers. Above these condition, annealing temperature 56°C was selected and amplification at 56°C (Figure 3).

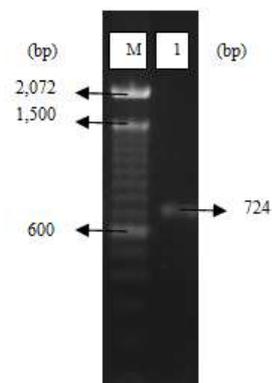


Figure 3

PCR product with annealing temperature at 56°C: 1. is a fragment DNA (724 bp), M. marker (Invitrogen®) 100 bp DNA ladder

DISCUSSION

PCR optimization could be done by varying the conditions used in the PCR. Optimization of conditions related to factors such as the type of DNA polymerase, temperatures, concentration of dNTPs, MgCl₂ and DNA polymerase, PCR buffer and time.¹² In this study, temperature was optimized, especially annealing temperature.

The first optimization is based on several criteria, including cycles of PCR, the temperature of denaturation, annealing and extension. PCR process consists of pre denaturation; 30-50 cycles, comprising the steps of denaturation, annealing, and extension; final extension.¹³

During the denaturation, the double strand DNA will separate to form single-stranded DNA due to hydrogen bonding disconnected. Time required to process usually 1-2 minutes.¹³⁻¹⁵ The next process is the annealing stage which usually occurs 5°C below the T_m primer (45-60°C). T_m primers used in this

study was 62°C. Therefore, the annealing temperature is estimated to range at 57°C. In the annealing step, the primers will move and stick with one single strand of complementary DNA template. Time required on the annealing process usually 1-2 minutes.^{13,15} PCR process ends with the extension phase occurs at 72°C. At this stage DNA was synthesized by a DNA polymerase. Time required at this stage is 1 minute for every 1000 base pairs.^{13,16}

Based on these references, the researchers used the initial optimization conditions as follows: pre denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 1 min, gradient of annealing temperature at 53 °C, 56 °C, 59 °C, 62 °C, 65 °C, 72°C for 1 min, extension at 72 °C for 1 min; final extension at 72 °C for 10 min.

On the initial optimization condition, band is not formed. It can be caused by template concentration is too low, the annealing temperature is too high, extension time is too short and lack of cycle.¹⁷

Template concentrations that are too low cause the primer could not find the target easily in an annealing process.¹⁸ In this study, the concentration of DNA templates used by diluting 25 times in an initial optimization, so that need to increase template concentrations in a further optimization by using template DNA concentration without dilution. Increased of concentration of DNA template need to be improved is a reason why low quantity DNA produced.

Annealing temperature is too high can cause the primer does not bind to the template¹⁹. Therefore, variations in the annealing temperature should be lowered to 48-58°C.

Extension time is too short affects not perfected polymerization process. So, it is needed to increase the extension time with the addition 1 min¹⁷. Therefore an extension time for further optimization increased from 1 min to 2 min.

Lack of cycle amplification, especially on a low quantity of DNA will result in low DNA amplicons. So, it could not be detected by electrophoresis on agarose gel. Under these conditions, the addition of cycle needs to be done 3-5 cycles.¹⁷ So that, in a further optimization, the addition of the cycle was 35 cycles to 40 cycles.

Based on the above, be re-optimized in order to obtain the band at 0.7 kb by increase sample concentration without diluted sample, decrease variation of annealing temperature be 48°C up to 58°C, increase extension time be 2 min and increase cycles of PCR be 40 cycles.

Results from the further optimization conditions, produces a thin band with dimers at 48°C, 50°C and 56°C. Whereas at 52°C occurred misspriming and at 54°C and 58°C did not produce a band at 724 bp fragment. Annealing temperature at 48°C and 50°C

produced specific band (724 bp) with thicker dimers than 56°C. Thus, selected annealing temperature was set at 56°C.

The last stage with these conditions, amplification performed at 56°C to make sure the band formed. Results showed that the thin band formed at 0.7 kb fragment (724 bp).

CONCLUSION

KatG gene region at 0.7 Kb fragment has been successfully amplified under the following conditions: pre denaturation at 95°C for 15 min; forty cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72 °C for 2 min; final extension at 72°C for 10 min.

PCR amplification results showed thin band formed. Therefore, further research needs to be done to get ready for a thick band that was sequenced and help to detect *katG* gene mutations in region 0.7 kb.

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