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Original Article

Optimization of DNA amplification using PCR method in mce1a gene *Mycobacterium tuberculosis* strain H37RV

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ABSTRACT

Tuberculosis is a disease that is classified as a global emergency and is caused by infection of *Mycobacterium tuberculosis*. *M. tuberculosis* has a mammalian cell entry protein or Mce which is important for the virulence of these bacteria. The Mce gene has an important role in the entry and survival of *M. tuberculosis* in macrophages. Mce protein is expressed and is immunogenic in natural infection by *M. tuberculosis*. Polymerase chain reaction (PCR) is a method used to identify an organism. Molecular identification using PCR-based methods needs to be done on the Mce1A gene from *M. tuberculosis* because this gene has four different operons (Mce 1–4) and this gene is also present in *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, *Mycobacterium leprae*, *Mycobacterium bovis*, and *Mycobacterium smegmatis* which are saprophytic species. There are not many DNA amplification methods for all *M. tuberculosis* complex members. This study aims to determine the optimal conditions for DNA amplification using the PCR method on the Mce1A *M. tuberculosis* strain H37Rv gene. Amplification optimization was carried out on two parameters, namely, the annealing temperature gradient and the polymerase enzyme. The results showed that the optimum conditions for amplification were related to the annealing temperature gradient; whereas the replacement of polymerase enzyme reagent did not provide a significant difference.

Keywords: H37RV, mammalian cell entry protein 1A, *Mycobacterium tuberculosis*, tuberculosis

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INTRODUCTION

Tuberculosis (TB) has long been one of the leading causes of human death due to infectious diseases. TB bacteria attack many organs in the human body, but the majority are the lungs. In general, primary bacterial infections in cases of pulmonary TB are not marked by symptoms of the disease or are asymptomatic until the patient is sufficiently ill to require treatment. The majority of TB patients live in countries with high population, for example, in Asia such as Bangladesh, China, India, Indonesia, and Pakistan where 48% of TB cases occur each year. Approximately 2 million deaths/year are caused by this disease. About one-third of the 2 billion human population in the world is infected by the causative agent of TB, namely, *Mycobacterium tuberculosis*. About 90% of the infected population is latent, that is, they store this pathogen in a dormant form and the remaining 10% suffer from an active disease process.[1-3]

In Indonesia, it is estimated that the number of new cases and recurrent infections (relapses) ranges from 340,000 to 520,000 cases, while the number of deaths is estimated at 62,246 cases/year.[4] The most common cause of TB is *M. tuberculo*sis, which is a member of *M. tuberculosis* complex (MTBC) with other members: *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium pinnipedii*, *Mycobacterium caprae,* and *Mycobacterium canettii*. All members of MTBC are obligate pathogens and can cause TB by giving different phenotypic features and host ranges. Genetically, members of the MTBC are so close that the genome of *M. tuberculosis* shows >99.9% similarity to that of *M. bovis*, a species that primarily infects cattle but can also infect other mammals including humans.[5] It was found that a DNA segment, 450 base pairs of *M. tuberculosis*, can give the non-pathogenic non-invasive *Escherichia coli* strain the ability to enter mammalian cells and survive in macrophage cells. The expression of specific proteins in transformed *E. coli* is

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related to their ability to invade and survive in HeLa cells and human macrophages. The encoded protein is then termed the mammalian cell entry protein or Mce. Expression of these Mce genes is important for the virulence factor of mycobacteria. In each operon, the genes that precede Mce, better known as YrbE, code for two integral membrane proteins while mceA and the next five genes are thought to encode proteins that have the signaling sequence or hydrophobic amino acid region (hydrophobic stretch) on the N-terminus. This protein sequence is then secreted or exposed to the cell surface (mycobacterial cell surface). This supports the theory of the role of Mce in the invasion of host cells, implying the involvement of this protein at an early stage after infection by *M. tuberculosis* and the induction of an immune response. Multiple gene copies of Mce clearly demonstrate the important role played by these proteins in the entry and survival of *M. tuberculosis* in human macrophages. Mce protein is expressed and is immunogenic in natural infection with *M. tuberculosis*. Besides being found in *M. tuberculosis*, these Mce operons were also found in *M. bovis*, BCG, *M. leprae*, and *Mycobacterium avium* complex. *In vitro*, *M. tuberculosis* does not need any components from the host cell to be able to express Mce protein.[6-9]

These Mce proteins differ in terms of regulation of expression or the degree of expression depending on the pathogenicity of the pathogen. Its function can also be affected by hosts infected with specific pathogens, for example, *M. tuberculosis* generally infects humans, whereas *M. bovis* is the main pathogen for cattle. *M. bovis* has three Mce operons, the Mce3 operon located within the RD7 deletion region, which is present in *M. tuberculosis* genome but not in *M. bovis* genome. This could mean that the protein encoded by the Mce3 operon is specifically involved in the entry and survival of *M. tuberculosis* in human macrophage cells.[10-12] Mce1A and Mce2A genes are expressed by pathogens that are actively growing and also in dormant conditions; non-replicating dormant bacilli, while the Mce3A and Mce4A genes are only expressed by pathogens that are actively growing.[12,13]

The phylogenetics of *M. tuberculosis* is closely related to phylogeographics, where based on the largest population, it is often grouped into the Beijing and non-Beijing families with the majority of *M. tuberculosis* strains appearing to differ from one area to another. In Indonesia, the Beijing strain of *M. tuberculosis* is believed to have the most widespread spread compared to other strains. The results of Van Crevel's *et al*. (2001) study using the spoligotyping technique showed that 34% of *M. tuberculosis* isolates tested from TB patients in Indonesia were Beijing strains. Meanwhile, in several countries, such as China, Mongolia, South Korea, Thailand, and Vietnam, Beijing has a strain percentage of 85%, 50%, 43%, 37%, and 34%, respectively. TB is more virulent and based on its characteristics tends to cause resistance to several OAT at once.[13]

MATERIALS AND METHODS

M. tuberculosis

This study used *M. tuberculosis* H37Rv strain grown on Lowenstein–Jensen (LJ) agar slant medium. The H37Rv strain is the standard strain in the laboratory.

Isolasi DNA Genom

Genomic DNA was obtained by extracting it using a DNA isolation kit according to the applicable work procedure.

Primers

The primary pair used was the sense and antisense primer pair specific to the open reading frame Mce1A *M. tuberculosis* strain H37Rv. This primary design was carried out with the GENETYX-WIN version 5.1.1 2001 software. The primary sequence used was:

S. No.	Name	Sequence
1	Mce1A F	5'GAATTCATGACGACGCCGGGG3'
2	Mce1A R	5'CTCGAGTCATGGGTTGATCGTG3'

Amplification of the Mce1A Gene

Amplification of the Mce1A gene was carried out using the polymerase chain reaction (PCR) technique using the primary pairs mentioned above. The reaction mixture used was 10× FastStart Buffer, 10 mM dNTP mixture, primer mixture, DNA template, FastStart DNA Polymerase (Roche Applied Science) enzyme, and 20 mM $MgCl_2$. The temperature cycle used is initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min, and final extension at 72°C for 7 min, with 35 cycles repetition. The amplification process was carried out using the Applied Biosystems GeneAmp PCR System 9700 (Bio-Rad) device.

Amplicon Visualization

The results of synthetic Mce1A DNA amplification or amplicon were then analyzed by 1.5% agarose gel electrophoresis followed by visualization using ethidium bromide.

RESULTS

M. tuberculosis isolates are routinely cultured on LJ agar slant media. LJ medium was prepared by weighing 18.65 g of LJ base medium and dissolved in 300 ml distilled water. Six milliliters of glycerol and 10 ml of 2% green malachite solution were added. The solution was sterilized in autoclaved at 121°C for 30 min and cooled. Subsequently, 500 ml of homogenized eggs were added and mixed. The medium is distributed into a tube covered with a groove as much as 6–8 ml. This culture tube was heated at 85°C for 50 min. To check sterility status, the culture medium was prepared by incubating at 37°C for 48 h and stored in the refrigerator if no contaminants were

detected. All tubes are tightly closed to prevent evaporation during storage. Bacteria were grown in an incubator at 37°C for 6–8 weeks.

Bacterial isolates (10–20 colonies) were taken using sterile ose and stored in 400 µl of RNAse-free water. The total bacterial genome was isolated by the minipreparation DNA isolation technique. A total of 1.5 ml of cell culture were centrifuged at 5000 g for 2 min. The pellets obtained were then resuspended in 540 µl TE buffer (0.1 M Tris-HCL, 0.1 M EDTA pH 8), then added 30 µl 10% SDS and incubated at 37°C for 60 min, added 100 µl 5 M NaCl and 80 µl CTAB/NaCl, then homogenized and incubated at 65°C for 10 min. Next, 750 µl of chloroform isoamyl alcohol (24:1) was added and centrifuged at 12,000 g for 5 min. The top layer formed was transferred to a new 1.5 ml Eppendorf tube then added 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged 12,000 g for 5 min. The top layer was transferred to a new 1.5 ml Eppendorf tube. Then, 0.6 × volume of isopropanol was added and centrifuged again at 12,000 g for 5 min. The pellets formed were washed with 70% ethanol and dried, then resuspended in 20 µl of TE buffer.

The process of breaking a double strand of DNA into single strand is called denaturation of template DNA, usually occurs at a temperature of 95°C for 1–2 min so that DNA in the form of double strands (double stranded) will separate into single strands, then proceed with the process of attaching (annealing) the primer to the DNA prints at a temperature of 56°C for 1 min. The primer will form a hydrogen bond with the mold in the complementary sequence region. The next step is DNA synthesis, where new DNA will be formed based on the information on the printed DNA with the help of the DNA polymerase enzyme. This synthesis stage usually occurs at a temperature of 72°C for 1–2 min. These stages are repeated 25–35 cycles. At the end of the PCR process, the results are stored at 4°C. Modifications to the annealing temperature and the reagents used, namely, the replacement of the reagent from DreamTaq Polymerase (Thermo Scientific) to FastStart Polymerase (Roche) was carried out in this study.

The amplified DNA was visualized by electrophoresis (Bio-Rad) on 1.5% agarose gel in TE buffer with ethidium bromide dye (0.5 µg/ml). Electrophoresis was carried out at 100 V for 30 min and DNA was observed with a UV transilluminator (Gel Doc/Bio-Rad). The size of the DNA from the PCR was compared with the marker (ladder) to determine the length of the DNA sample. The ladder used is a high mass ladder with a length ranging from 250 to 1 kbp [Figure 1].

Optimization is done using the temperature gradient method; thus, the annealing temperature shows a positive amplification result. The amplification results will be seen as DNA bands. These results are then used as a new annealing benchmark for

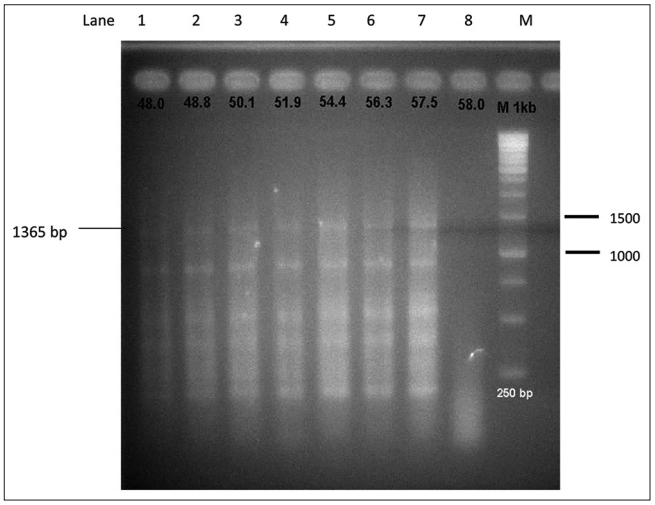

Figure 1: The results of annealing temperature optimization for the Mce1A gene strain H37Rv using the DreamTaq Polymerase enzyme (Thermo Scientific). M: Marka 1 kb; Lane 1: PCR results with annealing temperature of 48.0 ° C; Lane 2: PCR results with annealing temperature of 48.8°C; Lane 3: PCR results with annealing temperature of 50.1 ° C; Lane 4: PCR results with annealing temperature of 51.9 ° C; Lane 5: PCR results with annealing temperature of 54.4 ° C; Lane 6: PCR results with annealing temperature of 56.3 ° C; Lane 7: PCR results with annealing temperature of 57.5 ° C; Lane 8: PCR results with annealing temperature of 58.0 ° C.

amplification. From the overall temperature gradient carried out, temperatures that show positive results are at temperatures of 48.0°C, 48.°C, 50.1°C, 51.9°C, 54.4°C, and 56.3°C. While for temperature of 57.5 C showed different results in both polymerase enzyme reagents. At 58.0°C, the two polymerase enzyme reagents did not show positive results. Optimization using two different polymerase enzyme reagents showed different results where the DNA bands produced by the FastStart polymerase enzyme (Roche) were involved thicker than those produced by the DreamTaq (Thermo Scientific) enzyme. From the optimization of the annealing temperature, it was found that the optimal annealing temperature was 55°C using the FastStart polymerase enzyme (Roche).

In the electrophoretic gel, it was seen that there were DNA bands that migrated between the 1000 bp and 1500 bp DNA markers [Figures 1-3]. The expected size of the synthetic Mce1A DNA fragment is 1365 bp.

DISCUSSION

The volume amplification of the Mce1A gene used for the preparative scale was 100 ul, which was divided evenly into two tubes so that each tube contained 50 ul of the reaction mixture. This is done so that the heat change process of the reaction mixture solution is more efficient, approaching

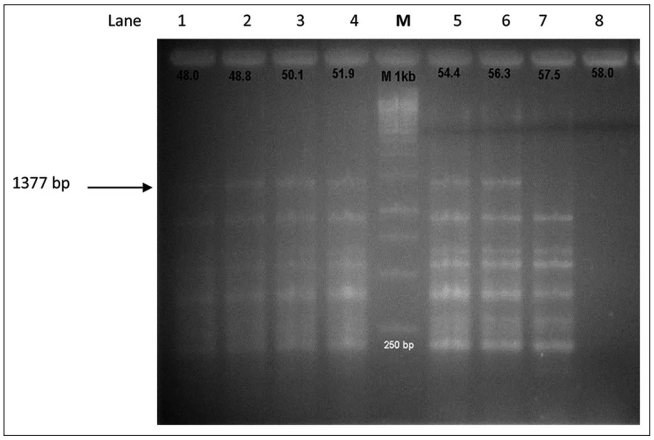

Figure 2: The results of annealing temperature optimization for the Mce1A gene H37Rv strain using the FastStart Polymerase (Roche) enzyme. M: Mark 1 kb; Lane 1: PCR results with annealing temperature of 48.0°C; Lane 2: PCR results with annealing temperature of 48.8°C; Lane 3: PCR results with annealing temperature of 50.1°C; Lane 4: PCR results with annealing temperature of 51.9°C; Lane 5: PCR results with annealing temperature of 54.4°C; Lane 6: PCR results with annealing temperature of 56.3°C; Lane 7: PCR results with annealing temperature of 57.5°C; Lane 8: PCR results with annealing temperature of 58.0°C.

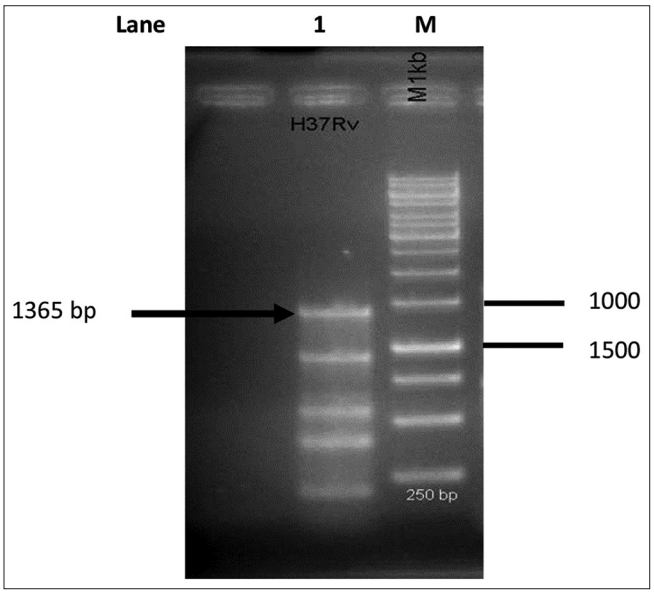

Figure 3: Results of Mce1A DNA amplification strain H37Rv with annealing temperature of 55 ° C and FastStart polymerase enzyme (Roche) on agarose gel electrophoresis.

the conditions set in the Thermocycler so that the resulting amplicons are more specific and present in high concentrations.

The results of visualization of PCR products after electrophoresis showed that the DNA bands were successfully amplified even though they did not show the same thickness of the DNA bands. This could be due to differences in the concentration of extracted DNA in the templates used for PCR and differences in DNA concentrations that were successfully amplified.[14]

Optimization of annealing temperature is one of the methods commonly used to increase the success of amplification. This temperature affects the process of attaching the primer to the DNA template. If the annealing temperature is too high, the primer cannot adhere well to the template, whereas if the annealing temperature is low, the primer will stick to an unspecified attachment site which will then amplify the unwanted gene fragments. Gradient PCR is a method that can be used to determine the optimal annealing temperature in DNA.[15]

Optimization of the polymerase enzyme changes did not give too different results, namely, because it was seen from the presence or absence of amplification results in the replacement of the reagent used from DreamTaq Polymerase to FastStart Polymerase.

CONCLUSION

Amplification optimization can be done by changing the annealing temperature, while the replacement of polymerase enzyme reagent does not have a significant effect. Annealing temperatures that can be used for amplification include 48.0°C, 48.8°C, 50.1°C, 51.9°C, 54.4°C, and 56.3°C.

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CONFLICTS OF INTEREST

There are no conflicts of interests during this study.

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