An Efficient Polymerase Chain Reaction (PCR) Enhancer for Highly Guanine-Cytosine (GC)-Rich DNA Sequences

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An Efficient Polymerase Chain Reaction (PCR) Enhancer for Highly Guanine-Cytosine (GC)-Rich DNA Sequences

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ABSTRACT

Background: Polymerase chain reaction (PCR) has become a fundamental technique in molecular biology. Nonetheless, PCR amplifications are frequently impaired by high GC content of the target sequence, leading to low yield and specificity of products, with no product at all in the worst cases. Locally high-temperature melting regions within the template can act as permanent termination sites.

Method: Here we designed and tested an effective and low-cost PCR enhancer, a combination of dimethyl sulfoxide (DMSO) 10% (v/v) and magnesium chloride (MgCl2) 1,5 mM that broadly enhanced the qualitative output of PCRs. We used *Mycobacterium tuberculosis* strain H37vR as a PCR template.

Result: It was found that PCR enhancer containing 10% (v/v) of DMSO and 1,5 mM of MgCl2 improved the amplification of GC-rich template of *M. tuberculosis* gene other than without the PCR enhancer.

Conclusion: Therefore, this PCR enhancer could be widely useful to improve the amplification of GC rich construct from another genome.

Keywords: *DMSO, GC-rich template, MgCl2, PCR, PCR Enhancer*

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INTRODUCTION

Polymerase chain reaction (PCR) is believed as the most an indispensable and widely used tool to accomplish amplification of DNA sequences that can be used for many purposes, such as sequencing for molecular diagnosis or cloning into vectors and for vaccine production. Combination of the techniques and others molecular methods available now in molecular research are enhancing the ability to engineer, assemble, and derive proteins or antigens of interest to the development of new vaccine design.

Despite these achievements has revolutionized the field of biotechnology, amplification of GC-rich fragments remains a major obstacle because of secondary structure like hairpins and higher melting temperature. Sequence populated with G repeats produce complex inter and intra strand folding due to increased hydrogen bonding with neighboring guanines. This complication also occurred when genomes of *Mycobacterium tuberculosis* which contain guanine plus cytosine-rich templates was used as a template to amplify several genes-encoding proteins for vaccine generation. According to Dale *et al.* (1990), mycobacterial genes have a GC content of 65 to 70%. In amplification result, this phenomenon is marked by the appearance of shorted bands following gel electrophoresis.[1] These truncated versions of the target amplicon are primarily the consequence of arrest sites (hairpins) introduced into the template causing premature termination to polymerase extension.[1]

To overcome the problems associated with the amplification of GC-rich gene, several approaches have been developed. Organic molecules such as DMSO, glycerol, polyethylene glycol, formamide, betaine have been included in the reaction mixture and have shown to improve the amplification of GC-rich DNA sequences.[2,3] In this research, the amplification reaction was modified by using PCR enhancer containing DMSO and MgCl2. DMSO was chosen because of previously reported success in PCR amplification of GC-rich construct in *de novo* synthesis.[4]

MATERIALS AND METHODS

Genomic DNA isolation

In this research, *Mycobacterium tuberculosis* strain H37Rvwas used to isolate genome of the bacteria as a template. Before use, the bacteria were grown on solid Luria-Bertani media. Genome isolation was conducted using Plasmid Isolation Kit (Machenery, Germany). The isolated genome then diluted with Tris-EDTA (TE) buffer and stored at -20°C for further used.

PCR Amplification reactions

The amplification reactions were carried out in a total volume of 10 ul with 0.1 units of Pfu TurboTM DNA polymerase (Stratagene, L Jolla, CA, USA) and reaction buffers, 0.2 mM each of dNTPs, 0.5 uM primers, and one ng/ul of genome as a template. PCR enhancer containing DMSO 10 % and 1,5 mM MgCl2was added into reaction mixture. The primers used in this research are listed in Table 1.

Twenty-five cycles of PCR were performed as follows: 10 s of denaturation at 94°C, 10 s of annealing at 60°C, and 40 s of elongation at 72°C, and then continued with electrophoresis on 1.0% agarose gel.

Gel Purification, cloning, and Sequencing of PCR products

To remove miss-amplification products (primer-dimer) and PCR reaction components which not used after amplification processes (dNTPs mix etc.), PCR product purification was conducted using MinElute Purification Kit (Qiagen, USA). These PCR products were then ligated into a pGEMT-Easy vector (Promega, USA) and sequenced using standard procedures.[5]

Table 1 List of primers used in this research

Primers	Sequence (5' 3')
Mtb32cF	caattacatatgcatcaccatcaccatcacacggccgcgtccgataacttc
Mtb32cR	ctaatcgaatccggccgggggtccctcggccaa
Mtb39F	ctaatcgaattcatggtggatttcggggcgtta
Mtb39R	ctaatcgatatcgccggctgccggagaatgcgg

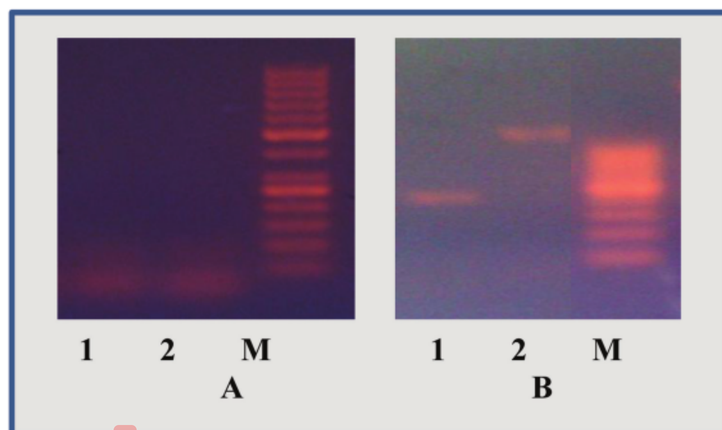

Figure 1 Agarose gel images showing the effects of DMSO and MgCl2 after PCR process. (A) no observable target band of the Mtb gene in reactions without PCR enhancer. (B) unique specific PCR product with right bands size corresponding to Mtb32C and Mtb39. 1 = Mtb32C, 2 = Mtb39, M = Marker

RESULTS

In this research, PCR enhancer containing DMSO and MgCl2 was used to amplify two GC-rich fragments from *M. tuberculosis* genome. Results of this research showed no observable target band of the Mtb gene fragments when PCR enhancer was not added into reaction mixtures (Figure 1A). On the other hand, primer dimer or miss-annealing amplification fragments appeared in large amount in electrophoresis results when no PCR enhancer in the reaction mixtures (bold arrow in Figure 1A). Conversely, when PCR enhancer containing 10% of DMSO and 1,5 mM MgCl2 was included in the reaction mixtures, a unique specific PCR product with right bands size corresponding to Mtb32C and Mtb39. The size of Mtb32C (Figure $1B_1$) and Mtb39 (Figure $1B_2$) are 396 and 1173 base pairs respectively.

The amplification results were then confirmed by DNA sequencing in which BLAST analysis showed these sequences of two genes completely overlapping with *M. tuberculosis* genome. The complete nucleotide sequences of these genes are illustrated in Figure 2 (A and B).

DISCUSSION

Several *M. tuberculosis* antigens were identified by Skeiky *et al.* (2004) in the context of controlled infection in human and mice. Of these, two proteins, Mtb32 and Mtb39 were expressed as a single recombinant polyprotein with a predicted size of 72 kDa (Mtb72F). Immunization of mice with the antigen resulted in the elicitation of immune responses. Also, immunization of guinea pigs with the antigen prolonged the survival of the animals after aerosol challenge with virulent *M. tuberculosis*. Therefore, Mtb72 antigen is very prospective TB vaccine candidate for ease the tuberculosis pandemic.[6]

Since the Mtb72 construct is generated by fusing at either end of Mtb39, the N- and C-terminal portions of Mtb32 (designated Mtb32N and Mtb32C), amplification of each fragment should be performed using *M. tuberculosis* genome as a template. As mentioned previously, however, amplification of these fragments from *M. tuberculosis* genome was hampered by a secondary structure like hairpins and higher melting temperature because of *M. tuberculosis* genome GC-rich gene.

Musso reported that combination of betaine, DMSO, and 7-deaza-dGTP are powerful mixture for amplification of DNA sequences of three disease genes showing GC content ranging from 67 to 79%.[4] Furthermore, DMSO and betaine were used successfully to improve *de novo* synthesis of

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A

atgacggccgcgtccgataacttccagctacggccgcgtccgataacttccagctgtcccagggtgggcag-
ggattcgccattccgatcgggcaggcgatggcgatcgcgggccagatccgatcgggtggggggtcacccac-
cgttcatatcgggcctaccgccttcctcggcttgggtgttgtcgacaacaacggcaacggcgcacgagtccaac-
gcgtggtcgggagcgctccggcggcaagtctcggcatctccaccggcgacgtgatcaccgcggtcgacgg-
cgctccgatcaactcggccaccgcgatggcggacgcgcttaacgggcatcatcccggtgacgtcatctcggt-
gacctggcaaaccaagtcgggcggcacgcgtacagggaacgtgacattggccgagggacccccggcc

B

gtggatttcggggcgttaccaccggagatcaactccgcgaggatgtacgccggcccgggttcggcctc-
gctggtggccgcggctcagatgtgggacagcgtggcgagtgacctgttttcggccgcgtcggcgtttcagtcg-
gtggtctggggtctgacggtggggtcgtggataggttcgtcggcgggtctgatggtggcggcggcctcgccg-
tatgtggcgtggatgagcgtcaccgcggggcaggccgagctgaccgccgcccaggtccgggttgctgcggc-
ggcctacgagacggcgtatgggctgacggtgcccccgccggtgatcgccgagaaccgtgctgaactgatgat-
tctgatagcgaccaacctcttggggcaaaacaccccggcgatcgcggtcaacgaggccgaatacggcgagat-
gtgggcccaagacgccgccgcgatgtttggctacgccgcggcgacggcgacggcgacggcgacgttgctg-
ccgttcgaggaggcgccggagatgaccagcgcgggtgggctcctcgagcaggccgccgcggtcgaggag-
gcctccgacaccgccgcggcgaaccagttgatgaacaatgtgccccaggcgctgcaacagctggcccagc-
ccacgcagggcaccacgccttcttccaagctgggtggcctgtggaagacggtctcgccgcatcggtcgccgat-
cagcaacatggtgtcgatggccaacaaccacatgtcgatgaccaactcgggtgtgtcgatgaccaacacctt-
gagctcgatgttgaagggctttgctccggcggcggccgcccaggccgtgcaaaccgcggcgcaaaacggg-
gtccgggcgatgagctcgctgggcagctcgctgggttcttcgggtctgggcggtggggtggccgccaacttgg-
gtcgggcggcctcggtcggttcgttgtcggtgccgcaggcctgggccgcggccaaccaggcagtcaccccgg-
cggcgcgggcgctgccgctgaccagcctgaccagcgccgcggaaagagggcccgggcagatgctgggcgg-
gctgccggtggggcagatgggcgccagggccggtggtgggctcagtggtgtgctgcgtgttccgccgcgac-
cctatgtgatgccgcattctccggcggccggc

Figure 2 Nucleotide sequences of Mtb32C (A) and Mtb39 (B)

two GC-rich gene fragments implicated in tumorigenesis, the Insulin-like Growth Factor 2 Receptor (IGF2R) and V-raf murine sarcoma viral oncogene homolog B1 (BRAF).[4]

In this research, PCR enhancer containing DMSO and MgCl2 was used to amplify two GC-rich fragments from *M. tuberculosis* genome. Results of without PCR enhancer showed large amount of primer dimer or miss-annealing amplification fragments in electrophoresis. According to Mammedov *et al.* (2008), three contributing events are considered occurs in this reaction. Firstly, primers may anneal at incorrect sequence of template, the probability of such an occurrence depends on the difference between the melting rates of primers at correct versus incorrect sites. Secondly, DNA polymerases molecules may bind to annealed primers, including ones in incorrect sites to further stabilize the formed complexes. Lastly, the complexes begin to elongate, albeit at reduced rates (at the annealing temperature), and further stabilize the double stands DNA.[7]

Therefore, several steps of PCR optimization, especially melting time and temperature, were also applied to change intramolecular stable stem-loops in the GC-rich template due to the strong G-C pairing in the *M. tuberculosis* genome. This effort was conducted since it is well known that GC content influences both optimal annealing temperatures and primer specificity. GC-rich primers may need higher annealing temperature than low GC primers. The time of melting also considered because it is based mainly on time it takes to reach the proper temperature for annealing. Optimum annealing times for GC-rich genes lie in the range 3 to 6 second and depend on annealing temperature.[7] However, these steps do not affect to produce unique specific target bands.

A unique specific PCR product with right bands size corresponding to Mtb32C and Mtb39 result indicated that this PCR enhancer greatly improved amplification of two GC-rich gene fragments of *Mycobacterium tuberculosis* (Mtb32Cand Mtb39) without having to modify nucleotide sequence composition. It is because that DMSO can prevent intramolecular stable stem-loops in GC-rich template due to the strong G-C pairing. Musso *et al.* (1998) reported that DMSO disrupts base pairing of DNA duplex which then can facilitate the amplification.[3]

Based on PCR products and their sequencing confirmation, addition of PCR enhancer in the PCR reaction mixture was successfully used for amplification of DNA sequences of three GC-rich genes of *M. tuberculosis*. Therefore, inclusion of the low-cost organic molecule in the amplification reaction mixture will substantially enhance gene target-specific amplification from moderately high GC-rich content of genome. This result will provide a low cost, general and reliable means to improve the molecular analysis of DNA sequences that are otherwise refractory to amplification.

CONCLUSION

We demonstrated that the use of PCR enhancer containing 10% (v/v) of DMSO and 1,5 mM MgCl2 in the reaction mixture was essential to achieve amplification of DNA sequences of two GC-rich genes of *M. tuberculosis*. Therefore, inclusion of the this low-cost PCR enhancer in the amplification reaction mixture will substantially enhance gene target-specific amplification from moderately to highly GC-rich content of genome. Since PCR enhancer in this research is inexpensive and easy to obtain, make the reagent is very suitable for any gene synthesis assay.

CONFLICT OF INTEREST

The authors declare that they don't have any conflict of interest regarding manuscript

ETHICAL APPROVAL

This study has been approved by the ethics committee of Faculty of Medicine, Mataram University prior to study was conducted.

FUNDING

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AUTHOR'S CONTRIBUTION

Ima Arum Lestarini and colleges were contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

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