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Improvements of PCR Amplification of Guanine plus Cytosine-Rich Constructs of *Mycobacterium tuberculosis* Gene using DMSO

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Abstract

Vaccine research entered a new era when several useful molecular research tools were established. Instead of attenuated virulent microorganisms or killed virulent microorganisms, effective subunit vaccines were developed using recombinant DNA technology. By using the technology, selected genes of the virulent microorganisms can be amplified, cloned, expressed, and evaluated as vaccine components in challenge studies. However, a major bottleneck with the amplification of functional genes from *Mycobacterium tubeculosis* containing guanine plus cytosin-rich templates is often hampered by the formation of secondary structures like hairpins and higher melting temperatures. To solve this problem in this research, the amplification reaction was modified by addition of dimethyl sulfoxide (DMSO) into amplification reaction mixtures. It was found that 10% (v/v) of DMSO in the reaction mixture improved the amplification of GC-rich template of *M. tuberculosis* gene. This result indicating that amplification of unbalanced content of G and C deoxyribonucleotides genome could be improved using low-cost organic molecule, DMSO. Therefore, the DMSO should be widely useful as an enhancer to improve the amplification of GC rich construct from other genome.

Keywords: *Mycobacterium tubeculosis,vaccine, dimethyl sulfoxide, Guanine-Cytosine*

Introduction

Tuberculosis (TB) is still one of the deadliest human infectious diseases, included together with AIDS and malaria (known collectively as The Big Three) in the poverty-related diseases that correlate with the tremendous imbalance between rich and poor countries. World Health Organization (WHO) reported that in 2006 more than 1.5 million people died of TB, and new cases were estimated at 9.1 million [1]. Total number of TB cases worldwide is about 14 million, with most of the TB cases occur in Southeast Asia's most populous countries, namely India which has the highest number of TB cases (1.9 million new cases and 3,4 million total number), followed by China and Indonesia[1].

In the last few years, multi-drug resistant of *M. tuberculosis* (MDR-TB) has also emerged and reported. These *M. tuberculosis* strains are resistant to two antibiotics used most frequently, isoniazid and rifampicin. Therefore, these *M. tuberculosis* strains are associated with a very high mortality rate as witnessed in an outbreak that ravaged New York City almost two decades ago. More recently, *M. tuberculosis* strains that show an extended spectrum of antibiotic resistance (Extensively Drug Resistant TB strains, XDR-TB) have been isolated. These are MDRTB strains that are also resistant to a fluoroquinolone and at least one second-line injectable drug such as kanamycin, capreomycin or amikacin . The emergence of XDR-TB is posing a major threat in many regions of the world[2,3].

Bacillus Calmette-Guerin (BCG), the only available vaccine for tuberculosis discovered nearly 100 years ago and generated from attenuated strain of *Mycobacterium bovis* by serial passages in potato slices imbibed with glycerol, has demonstrated varying levels of efficacy in different clinical trials and geographically distinct population. Moreover, the vaccine not only can cause disseminated disease in immunocompromised individuals

but also does not consistently prevent the development of pulmonary tuberculosis in adults. Therefore, development of new and more effective vaccine for tuberculosis is urgently needed[4,5].

Vaccine research entered a new era when several molecular research tools were established. Instead of attenuated virulent microorganisms or killed virulent microorganisms, effective subunit vaccines were developed using recombinant DNA technology. Vaccine production using the DNA recombinant technology not only replaced the traditional technologies using the attenuated or killed virulent microorganisms, but also permit simple and rapid approaches to generate a novel vaccine against the new outbreak pathogens. Therefore, whole protein vaccine produced by traditional technique which associated with problems and withdrawn from the market can be improved.

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 of 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 intrastrand folding due to increased hydrogen bonding with neighboring guanines. This complication also occurred when genomes of *Mycobacterium tubeculosis* which contain guanine plus cytosin-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%[6]. In amplification result, this phenomenon is marked by the appearance of shorted bands following gel electrophoresis. 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[4].

In this research, the amplification reaction was modified by using dimethyl sulfoxide (DMSO). DMSO was chosen because of previously reported success in PCR amplification of GC-rich construct in *de novo* synthesis[7]. It was found that 10% (v/v) of DMSO in the reaction mixture greatly improved the amplification. Since DMSO is very inexpensive and easily obtainable, make the reagent is very useful for any gene synthesis assay[7].

Materials And Methods

Genomic DNA isolation and PCR confirmation

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). PCR confirmation of the genome was conducted using primers TB-F (5'-TACTACGACCACATCAACCG-3') and TB-R (5'-GGGCTGTGGCCGGATCAGCG-3'). 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 1 ng/ul of genome as template. DMSO (99.9%) was added 0% (control) and 10% into reaction mixture. The primers used in this research are listed in Table 1.

Table 1. List of primers used in this research

Primers	Sequence (5' 3') ⟶
Mtb32Cf	caattacatatgcatcaccatcaccatcacacggccgcgtccgataacttc
Mtb32Cr	ctaatcgaatccggccgggggtccctcggccaa
Mtb39F	ctaatcgaattcatggtggatttcggggcgtta
Mtb39R	Ctaatcgatatcgccggctgccggagaatgcgg
Mtb32Nf	Ctaatcgatatcgccccgccggccttgtcgcaggac
Mtb32nR	Ctaatcgatatcctaggacgcggccgtgttcatac

Twenty five cycles of PCR were performed as follows: 10 s of denaturation at 94°C, 10 s of annealing at 50°C, and 40 s of elongation at 72°C, and were 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 products purification was conducted using MinElute Purification Kit (Qiagen, USA). These PCR products were then ligated into pGEMT-Easy vector (Promega, USA) and sequenced using standard procedures[8].

Results And 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. In addition, 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[9].

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 secondary structure like hairpins and higher melting temperature because of *M. tuberculosis* genome GC-rich gene.

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. Musso *et al*., 2006 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%. Furthermore, DMSO and betaine were used successfully to improve *de novo* synthesis of 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)[7,10,11].

In this research, DMSO only was tried to obtain three GC-rich fragments from *M. tuberculosis* genome. Results of this research shows no observable target band of the Mtb gene fragments when DMSO was not added into reaction mixtures (Figure 1A). On the other hand, primer dimer or mis-annealing amplification fragments were appeared in large amount in electrophoresis results when no DMSO in the reaction mixtures (bold arrow in Fig 1A). 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 depend 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[10].

Therefore, several steps of PCR optimization, especially melting time and temperature, were also applied to change intramolecular stable stem loops in 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 be need higher annealing temperature than low GC primers. The time of melting also considered because it is based mainly on the 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[12]. However, these steps have no effect to produce unique specific target bands.

Conversely, when 10% of DMSO was included in the reaction mixtures, a unique specific PCR product with right bands size corresponding to Mtb32C, Mtb39, and Mtb32N without no nonspecific product were obtained. The size of Mtb32C (Fig $1B_1$),

Mtb39 (Fig 1B$_2$), and Mtb32N (Fig 1B$_3$) are 396, 1173, 585 base pairs respectively.

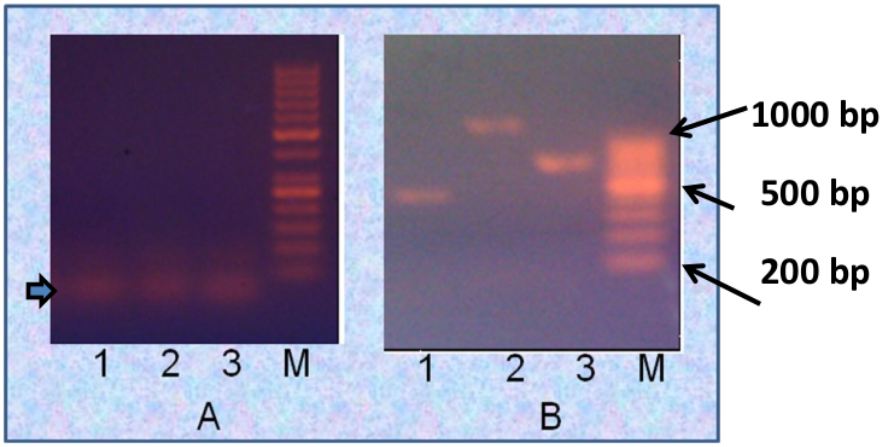

Fig 1. Agarose gel images showing the effects of DMSO during amplification (B) and control (no DMSO, B). 1 = Mtb32C, 2 = Mtb39, 3 = Mtb32N, M = Marker

This result indicated that DMSO greatly improved amplification of three GC-rich gene fragments of *Mycobacterium tuberculosis* (Mtb32C, Mtb32N, and Mtb39) without having to modify nucleotide sequence composition. It is because DMSO has ability to 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[11].

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

A

atgacggccgcgtccgataacttccagctacggccgcg
tccgataacttccagctgtcccagggtgggcagggattcgcc
attccgatcgggcaggcgatggcgatcgcgggccagatcc
gatcgggtgggggtcacccaccgttcatatcgggcctacc
gccttcctcggcttgggtgttgtcgacaacaacggcaacggc
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cggcgctccgatcaactcggccaccgcgatggcggacgc
gcttaacgggcatcatcccggtgacgtcatctcggtgacctg
gcaaaccaagtcgggcggcacgcgtacagggaacgtga
cattggccgagggacccccggcc

B

gtggatttcggggcgttaccaccggagatcaactccgc
gaggatgtacgccggcccgggttcggcctcgctggtggcc
gcggctcagatgtgggacagcgtggcgagtgacctgttttcg
gccgcgtcggcgtttcagtcggtggtctggggtctgacggtg
gggtcgtggataggttcgtcggcgggtctgatggtggcggc
ggcctcgccgtatgtggcgtggatgagcgtcaccgcgggg
caggccgagctgaccgccgcccaggtccgggttgctgcgg
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aggaggcctccgacaccgccgcggcgaaccagttgatga
acaatgtgccccaggcgctgcaacagctggcccagccca
cgcagggcaccacgccttcttccaagctgggtggcctgtgg

aagacggtctcgccgcatcggtcgccgatcagcaacatgg
tgtcgatggccaacaaccacatgtcgatgaccaactcgggt
gtgtcgatgaccaacaccttgagctcgatgttgaagggctttg
ctccggcggcggccgcccaggccgtgcaaaccgcggcg
caaaacggggtccgggcgatgagctcgctgggcagctcg
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gagggcccgggcagatgctgggcgggctgccggtggggc
agatgggcgccagggccggtggtgggctcagtggtgtgct
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ggccggc

C

gccccgccggccttgtcgcaggaccggttcgccgactt
ccccgcgctgcccctcgacccgtccgcgatggtcgcccaa
gtggggccacaggtggtcaacatcaacaccaaactgggct
acaacaacgccgtgggcgccgggaccggcatcgtcatcg
atcccaacggtgtcgtgctgaccaacaaccacgtgatcgcg
ggcgccaccgacatcaatgcgttcagcgtcggctccggcc
aaacctacggcgtcgatgtggtcgggtatgaccgcaccca
ggatgtcgcggtgctgcagctgcgcggtgccggtggcctgc
cgtcggcggcgatcggtggcggcgtcgcggttggtgagcc
cgtcgtcgcgatgggcaacagcggtgggcagggcggaac
gccccgtgcggtgcctggcagggtggtcgcgctcggccaa
accgtgcaggcgtcggattcgctgaccggtgccgaagaga
cattgaacgggttgatccagttcgatgccgcgatccagccc
ggtgattcgggcgggcccgtcgtcaacggcctaggacagg
tggtcggtatgaacacggccgcgtcctaa

Fig. 2. Nucleotide sequences of Mtb32C (A), Mtb39 (B), and Mtb32N (C)

Based on PCR products and their sequencing confirmation, addition of DMSO 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 10% (v/v) of DMSO in the reaction mixture was essential to achieve 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. Since DMSO is very inexpensive and easily obtainable, make the reagent is very useful for any gene synthesis assay.

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References

1. World Health Organization. Global Tuberculosis Control. Surveillance, Planning, Financing. 2007.
2. Moss,A.R., Alland,D., Telzak,E., Hewlett,D., Jr., Sharp,V.,Chiliade,P., LaBombardi,V., Kabus,D., Hanna,B., Palumbo,L., Brudney,K., Weltman,A., Stoeckle,K., Chirgwin,K., Simberkoff,M.,

Moghazeh,S., Eisner,W., Lutfey,M., and Kreiswirth,B. (1997) A city-wide outbreak of a multiple-drugresistant strain of *M. tuberculosis* in New York. Int.J.Tuberc.Lung Dis. 1, 115-121

3. Singh,J.A., Upshur,R., and Padayatchi,N. 2007. XDR-TB in South Africa: no time for denial or complacency. PLoS.Med. 4, e50
4. Colditz, GA, Brewer, TF., Nerkey CS., Wilson ME., Burdick E., Fineberg HV., and Mosteller F. 1994. Efficacy of BCG vaccine in the prevention of tuberculosis: meta-analysis of the published literature. JAMA 271; 689.
5. Ginsberg AM., 1998. The tuberculosis epidemic: scientific challenges and oppurtunities. Public Health Rep. 113; 128.
6. Dale JW, and Patki. 1990. Mycobacterial gene expression and regulation. In: McFadden J (Ed) Molecular Biology of the Mycobacteria. Surrey University Press, London, United Kingdom.
7. Jensen MA., Fukushima M., and Davis RW. 2010. DMSO and betaine greatly improve amplification of GC-rich constructs in de novo synthesis. PloS One, 5; e11024.
8. Sambrook J., Fritsch EF., and Maniatis T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
9. KangJ., Lee MS, Gorenstein DG. 2005. The enhancement of PCR amplification of a random sequence DNA library by DMSO and betaine: application to in vitro combinatorial selection of aptamers. J. Biochem Biophys Methods, 64; 147-151.
10. Musso, M., Bocciardi R., Parodi S., Ravazzolo R., and Ceccherini I. 2006. Betaine, dimethyl sulfoxide, and 7-deaza-dGTP, a powerful mixture for amplification of GC-rich DNA sequences. J Mol Diagn; 8; 544-550.
11. TG. Mammedov1, E. Pienaar1, SE. Whitney1, JR. TerMaat1, G. Carvill2, R. Goliath2, A. Subramanian1, and HJ. Viljoen. 2008. A Fundamental Study of the PCR Amplification of GC-Rich DNA Templates. Comput Biol Chem. 32: 452–457.
12. Skeiky YAW., Alderson MR., Ovendale PJ., Guderian JA., Brandt L., Dillon DC., Campos-Neto A., Orme IM., and Reed SG. 2004. Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as nacked DNA or recombinant protein. J. Immun., 172; 7618-7628.

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