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Cloning of cDNA Encoding GRA1 Protein of Tachyzoite *Toxoplasma Gondii* Local Isolate

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Abstract

Gene encoding GRA1 protein is potent DNA-vaccine candidate against toxoplasmosis. The aim of the research was to clone the gene encoding GRA1 protein of tachyzoite *Toxoplasma gondii* local isolate by DNA recombinant technology. Tachyzoite was grown in Balb/c mice *in vivo*. Messenger RNA was isolated from total RNA and it was used to synthesis cDNA. Complementary DNA encoding GRA1 protein of tachyzoite *Toxoplasma gondii* local isolate was amplified and cloned in a prokaryote cloning vector. The recombinant GRA1-encoding gene was then digesting using *Eco*RI restriction endonuclease and sequencing. The result showed that the recombinant GRA1-encoding gene consisted of DNA sequences encoding all signal peptide and mature peptide of GRA1 protein. Alignment of recombinant GRA1 sequence to gene encoding GRA1 protein of *Toxoplasma gondii* RH isolate showed 100% homologous.

Keywords: GRA1 protein, *Toxoplasma gondii*, tachyzoite, cloning, cDNA

Introduction

Toxoplasma gondii is an obligate intracellular parasite that infects most of warm-blooded animals and important opportunistic pathogen in humans (Sibley *et al*., 1995; Carruthers, 1999). Although infection is usually asymptomatic in immunocompetent individuals, it can cause severe disease manifestations and even death in immunocompromised patients. If infection acquired during pregnancy it can cause abortion and various congenital anomalies in the child (Singh, 2003). The prevalence of positive *Toxoplasma gondii* antibody in human in Indonesia is 2-63 % (Gandahusada, 1998).

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Vaccination is the best way to prevent serious toxoplasmosis. DNA vaccine against *Toxoplasma gondii* would be valuable because it can elicit both humoral and cellular immune responses (Vercammen *et al*., 2000). So far, the only developed vaccine is the live vaccine, attenuated tachyzoite S48. However, this vaccine is not widely accepted because of its side effect, short half life and high cost. Live vaccines also carry a risk of accidental infection of humans and unexpected harmful reverse mutations (Ismael *et al*., 2003).

Several trial DNA vaccine against toxoplasmosis have been conducted, mainly with mice and various *Toxoplasma gondii* antigens, such as SAG1 (Angus *et al*., 2000), GRA1 (Vercammen *et al*., 2000; Scorza *et al*., 2003), GRA7 (Vercammen *et al*., 2000), GRA4 (Desolme *et al*., 2000), ROP2 (Vercammen *et*

al., 2000; Leyva *et al*., 2001), ROP1 (Chen *et al*., 2001) dan MIC3 (Ismael *et al*., 2003). GRA1 can elicit potentially protective immune responses. The GRA1 antigen is secreted by tachyzoites and bradyzoites, it induces humoral and cellular immune responses in mice and humans in the chronic phase of the infection. DNA vaccination using GRA1 gene elicits a typical type 1 immune response, characterized by an IgG2a-biased antibody response and production of IFN-γ. Epitopes from GRA1 are processed and presented in MHC class I molecules during infection and that the GRA1 DNA vaccine induces specific CTLs (Vercammen *et al*., 2000; Scorza *et al*., 2003).

Therefore, the objective of this research was to clone the gene encoding GRA1 protein of tachyzoite *Toxoplasma gondii* local isolate by DNA recombinant technology and to compare the gene encoding GRA1 protein of tachyzoite *Toxoplasma gondii* local isolate with RH isolate.

Materials and Methods

In vivo tachyzoites cultivation

Tachyzoites *Toxoplasma gondii* local isolate were obtained from Medan's sheep by Dr. Wayan T. Artama. BALB/c mice were obtained from laboratory of Biotechnology GMU. Tachyzoites were harvested from peritoneal fluids of BALB/c mice that had been intraperitoneally infected with 1 x 10^7 tachyzoites 3 to 4 days earlier. In order to get enough tachyzoites (1x10^9) many passage were done.

Complementary DNA synthesis

The first step of cDNA synthesis was total RNA isolation from tachyzoites. Total RNA was isolated using RNAgents Total RNA Isolation System (Promega). Messenger RNA was isolated from total RNA using PolyATract mRNA Isolation System (Promega), and cDNA was synthesized from mRNA using Universal Riboclone cDNA Synthesis System (Promega). All procedures were essentially as described by manufacture instructions.

Complementary DNA amplification

Complementary DNA were amplified using Pure Taq RTG-PCR and specific primer of GRA1 protein : GF1 (5'-CGGTTTGCTTGTGTTGTTTG-3') and GR1 (5'-CATGGGGTACGATCACAACA-3') (Cybergene AB). The amplified DNA fragment consisted of all coding sequence (signal sequences and sequences of mature peptide). Reaction was performed under the following condition : initial denaturation at 94°C for 5 min and continued with 32 cycle of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min. The last extension phase was prolonged for 5 min at 72°C. Following amplification, the PCR fragment were purified using phenol extraction.

Cloning of PCR product

The extracted PCR fragment were cloned into the cloning vector (pGEM-T Easy). The recombinant plasmids were then used to transform *E. coli* XL1-Blue using the heat-shock method. All cloning procedures were essentially as described by Sambrook *et al.* (1989).

Isolation and analysis of recombinant plasmid

Transformant carrying recombinant plasmids were isolated using alkali lysis method as described by Sambrook *et al*. (1989). Recombinant plasmids were analysed by digesting restriction endonuclease *Eco*RI and sequencing using *ABI PRISM 3130 Genetic Analyser.*

Results and Discussion

In vivo tachyzoites cultivation

The result of *in vivo* tachyzoites cultivation was 1.9 x 10^9 /ml. According to

the previous study, this result was enough for preparing total RNA isolation.

Complementary DNA

Concentration of total RNA was 4.392 µg/µl and ratio of the purity was 1.8. Consentration of mRNA was 0.310 µg/µl and the total mRNA was 6.2 mg. Messenger RNA could be separated from total RNA because in most mature eukaryotic mRNA had 3′-poly(A) tail that can hybridize with oligo(dT) probe, but the other RNA component did not. The mRNA was only 0.56 % of total RNA (typically, 1 – 5 % of total RNA). Preparation of mRNA was hard enough because the characteristic of RNA that degraded easily by extremely stable RNases in the laboratory environment.

Consentration of cDNA was 1.877 µg/µl and ratio of the purity was 1.76. The quality and quantity of cDNA synthesized by any method is critically depend on the integrity of the mRNA used as a template. Most cDNA molecule produced lack a few nucleotides corresponding to the 5′ end of the mRNA because second-strand replacement only proceeds from 3′-OH RNA primers. The most 5′ nick in the mRNA generally occur several nucleotides from the end, and the remaining RNA oligonucleotide may be too short to remain hybridized. The 3′ to 5′ exonuclease activity of DNA polymerase I remove the last few nucleotides of the cDNA fisrt strand. Because all eukaryotic mRNA have 5′ noncoding leader sequences, which commonly range from 40 – 60 nucleotides, it is likely that the majority of double-stranded cDNA contain all coding sequences present in the initial cellular mRNA.

Amplification of GRA1 cDNA

Figure 1 showed the amplification fragment. Electrophoresis analysis of PCR product showed that a single band was obtained. The size of the amplified DNA fragment is 692 bp (line 2). Spesific primer of GRA1 gene amplified cDNA of local isolate up to 692 bp (nucleutides position 527 to 1218) (according to the genbank, 692 bp). This fragment consisted of signal sequences (nucleutides position 613 - 684) and sequences of mature peptide (nucleutides position 685 – 1182).

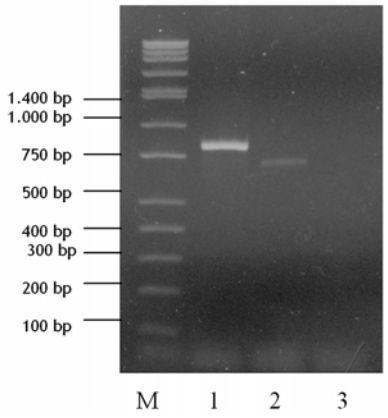

Figure 1. Amplicon of cDNA using spesific primer GF1 dan GR1 on 1% agarose gel. M: DNA marker, 1. positive control (DNA of RH isolate), 2. cDNA of local isolate, 3. negative control (no template).

Cloning of PCR product

The amplified fragment was then cloned into pGEM-T Easy and resulted in a recombinant plasmid designated as pWTA-cG1. The recombinant plasmid was subsequently used for transformation into *E. coli* XL1-Blue using the heat-shock method. The result of transformation plasmid into *E. coli* XL1-Blue demonstrated two kinds of colony, blue and white colony. Blue colony was the result of hydrolysis of chromogenic substrate (X-gal) by active β-galactosidase. Activation of the enzyme because of β-complementation between amino-terminal of β-galactosidase from Lac Z gene of vector and carboxy-terminal of β-galactosidase from the host cell. White colony represented that there was DNA fragment inserted in the cloning site, which can cause the failure of Lac Z gene

expression, therefore there was no α-complementation. Therefore, the white colony was suggested as recombinant colony.

Analysis of recombinant plasmid

Figure 2 showed the result of restriction analysis of the recombinant plasmid using *Eco*RI. It was clearly demonstrated that recombinant plasmid was digested into 2 fragment, 3015 bp and 692 bp (line 4). The vector (pGEM-T Easy) from the blue colony which had no foreign DNA insert was also digested into single fragment, 3015 bp (line 2).

Sequensing of recombinant plasmid was performed by using T7 and SP6 from the construct of pGEM-T Easy. Sequensing using T7 resulted 1101 bp and SP6 resulted 1080 bp. So, the sequencing using both T7 and SP6 resulted the whole 692 bp of sequence insert from both two directions.

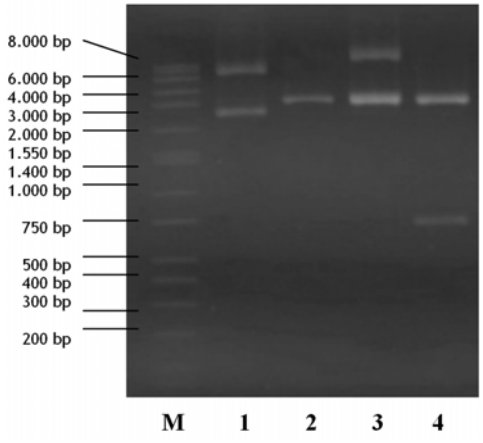

Figure 2. Restriction analysis of recombinant plasmid carrying DNA insert. M. DNA marker, 1. plasmid of blue colony, 2. (1) digested with *Eco*RI, 3. pWTA-cG1, 4. pWTA-cG1 digested with *Eco*RI.

Analysis using BLAST showed that the significant alignment was gene encoding P24 major protein (GRA1 protein) of *Toxoplasma gondii* RH isolate. Alignment recombinant plasmid sequences showed 100 % homologous with 526 to 1218 gene encoding GRA1 protein of *Toxoplasma gondii* RH isolate. These sequences consisted of the whole coding sequence of GRA1 protein, it confirmed that GRA1 protein of *Toxoplasma gondii* local isolate is not different with RH isolate.

In conclusion, we have successfully established a clone that carry 692 bp of the gene encoding GRA1 protein of *Toxoplasma gondii* local isolate by cDNA cloning. Sequences of local isolate showed 100% homologous with the gene encoding GRA1 protein of *Toxoplasma gondii* RH isolate.

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