

CLONING OF THE GENE ENCODING SAG1 OF LOCAL ISOLATE *TOXOPLASMA GONDII*  
IN *ESCHERICHIA COLI DH5 $\alpha$*

KLONING GEN ANTIGEN PERMUKAAN (SAG1) *Toxoplasma gondii* ISOLAT LOKAL  
PADA *Escherichia coli DH5 $\alpha$*

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ABSTRACT

The aim of this research was to improve diagnostic method of toxoplasmosis using recombinant surface antigen SAG1 from a local isolate. Total ribonucleic acid (RNA) was isolated from tachyzoites that were grown up in mice using *RNA gent total RNA isolation system*. Isolation of mRNA and cDNA synthesis were carried out using *mRNA purification kit* and *Time Saver cDNA synthesis kit* respectively. The DNA encoding SAG1 was amplified by PCR with the specific primers of mature SAG1 then inserted in pCR @ 2.1-TOPO vector and transfected into *E. coli DH5 $\alpha$* . The resulting plasmids were analyzed by *BamHI-EcoRI* to establish the positive clones. The amplification of the DNA encoding mature SAG1 gave rise to a DNA fragment of the expected size i.e. 0.8 kbp. That has been successfully cloned in pCR @ 2.1-TOPO into *E. coli DH5 $\alpha$* .

**Key words :** *T. gondii*, tachyzoite, cDNA, SAG1

ABSTRAK

Kloning gen antigen permukaan (SAG1) *Toxoplasma gondii* isolat lokal dilakukan dengan tujuan untuk mendapatkan antigen rekombinan spesifik untuk pengembangan diagnosa. *Toxoplasma gondii* stadium takizoit diperbanyak secara *in vivo* pada mencit. RNA total takizoit diisolasi menggunakan *RNA gent total RNA Isolation system*, mRNA diisolasi dengan *mRNA purification kit* dan cDNA disintesis menggunakan *Time saver cDNA synthesis kit*. Gen SAG1 dari cDNA diamplifikasi dengan tehnik PCR menggunakan primer spesifik gen SAG1 *mature*. DNA hasil amplifikasi diinsersikan pada plasmid pCR @ 2.1-TOPO dan ditransfeksi ke dalam *Escherichia coli DH5 $\alpha$* . Plasmid rekombinan dianalisis dengan enzim *BamHI-EcoRI* untuk menentukan klon positif. Hasil amplifikasi DNA dengan PCR diperoleh fragmen DNA sebesar yang diperkirakan yaitu 0.8 kbp. Fragmen gen SAG1 ini berhasil diklon pada pCR @ 2.1-TOPO pada *E. coli DH5 $\alpha$* .

**Kata kunci :** *T. gondii*, takizoit, cDNA, SAG1

## INTRODUCTION

*Toxoplasma gondii* is an Apicomplexan parasite that is related to other members of the phylum *Apicomplexa*, such as *Plasmodium* (the cause of malaria) and *Eimeria* (the cause of coccidiosis). It is an obligate intracellular parasite, agent of toxoplasmosis, that infects humans and a broad spectrum of vertebrate hosts. Whereas infection with *T. Gondii* is usually innocuous or asymptomatic in most individuals, it causes serious morbidity and mortality in fetuses of primarily infected pregnant women (Sharma, 1990) and immunocompromized individuals (Israelski and Remington, 1993). It is estimated that toxoplasmosis exists in a chronic, asymptomatic form in 500 million to 1 billion of the world's human population (Savva, 1992). The transmission of *T. gondii* occurs by ingestion of oocysts shed from feline feces, by ingestion of *T. gondii* cysts from chronically infected tissues, or by vertical transmission (Wong and Remington, 1994).

Genetic analysis of strains indicates that the propagation of *T. gondii* is primarily by clonal, asexual, or uniparental sexual reproduction, while sexual recombination between different strains of the parasite is exceptional in natural populations (Sibley and Boothroyd, 1992). Population genetic studies have established that the number of *T. gondii* genotypes is remarkably limited in nature, the majority of which fall into one of only three distinct lineages, i.e. type I, type II and type III (Howe and Sibley, 1995). Type I is highly virulent in mice (Sibley and Boothroyd, 1992). Type II strains seem to predominate in infections of immunocompromised patients while type I or type I-like strains seem to be relatively over represented in congenital infections (Fuentes *et al.*, 2001; Grigg and Boothroyd, 2001; Howe *et al.*, 1997). SAG-1 (P30) is the most predominant antigen of *Toxoplasma* tachyzoites (Couvreur *et al.*, 1988; Tomavo, 1996). It is thought to play an important role in adherence and infection though the exact function remains far from understood (Mineo and Kasper, 1994). The nucleotide sequence of SAG-1 gene has been established (Burg *et al.*, 1988). The remarkable feature of SAG-1 gene is that it does not contain introns. SAG-1 gene is expressed as a precursor which is then processed to mature SAG-1 protein. We describe in this paper the isolation of mature and precursor SAG-1 gene by PCR reaction and its cloning by a topoisomerase-based cloning methodology.

## MATERIALS AND METHODS

### Preparation of *Toxoplasma* tachyzoites

*Toxoplasma* tachyzoites were cultured in mice. Tachyzoites of *T.gondii* were harvested on the 4<sup>th</sup> days post infection from 60 mouse infected intraperitoneally with  $1 \times 10^7$  tachyzoites of local Indonesian isolate per mice. The tachyzoites, then, was washed three times in PBS pH 7.4 ( *phosphat buffer saline* ) solution contained 0,1% DEPC ( *diethylpirocarbonat* ). The washing was done by centrifugation 3500 rpm, 4°C for 5 minutes, respectively.

### Total RNA dan mRNA isolation

A number of  $1,3 \times 10^9$  tachyzoites of the *Toxoplasma* was used to Total RNA and m-RNA extractions. The former was carried out by using RNeasy® Total RNA Isolation System ( *Promega, Cat : Z5110* ) and the latter was carried out by using m-RNA Purification Kit® ( *AmarshamPharmacia Biotech. Cat: 27-9258-02* ).

### cDNA syntesis

A number of 5 µg mRNA of *T. gondii* was used to cDNA syntesis. The procedure of the syntesis was carried out by using Time Saver cDNA Syntesis Kit® ( *AmarshamPharmaciaBiotech. Cat : 27-92620-01* ).

### PCR reaction

Primers used in PCR reactions will be described in Results and Discussion. PCR reactions were carried out using Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech) in 25 µL buffer, 0.4 µM of each primer, variable amounts of matrix (genomic DNA or cDNA), with the following conditions: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 95°C for 40 sec, annealing at 60°C for 40 sec, and elongation at 72°C for 1 min 30 sec; finally an additional elongation step at 72°C for 10 min then 4°C. One fifth of the reaction products were analyzed by electrophoresis on a 0.8% agarose gel.

### Cloning in pCR2.1-TOPO

Cloning in pCR2.1-TOPO plasmid was carried out using TOPO TA Cloning system (Invitrogen), a methodology based on topoisomerase reaction, according to the instruction manual. This system allows direct cloning of PCR reaction

products. Topoisomerase reaction was done in 6  $\mu\text{L}$ , containing 1 to 2  $\mu\text{L}$  of the PCR reaction products and 1  $\mu\text{L}$  TOPO vector, for 5 min at 22°C. Reaction products were kept on ice or at -20°C until use.

#### Transfection in *E. coli* DH 5 $\alpha$

Transformation was done using TSS method in *E. coli* DH 5 $\alpha$ . TSS-competent *E. coli* DH 5 $\alpha$  bacteria were obtained by 10 times-concentrating fresh exponential phase bacterial culture (OD<sub>600nm</sub> around 0.6) in LB containing 10% PEG 6,000 (w/v), 5% DMSO (v/v) and 35 mM MgCl<sub>2</sub>. Different amounts of the transformation were spread on LB agar plate containing 50  $\mu\text{g}/\text{mL}$  ampicillin, 40  $\mu\text{L}$  of 40 mg/mL X-Gal and 40  $\mu\text{L}$  of 100 mM IPTG and incubated at 37°C, for one night (OVN). White bacterial colonies were cultured in 5 ml LB-ampicilline for OVN. Bacteria were harvested by centrifugation (Sorvall, 4,000 rpm, 10-15 min, 4°C). The bacterial pellet was used for plasmid preparation.

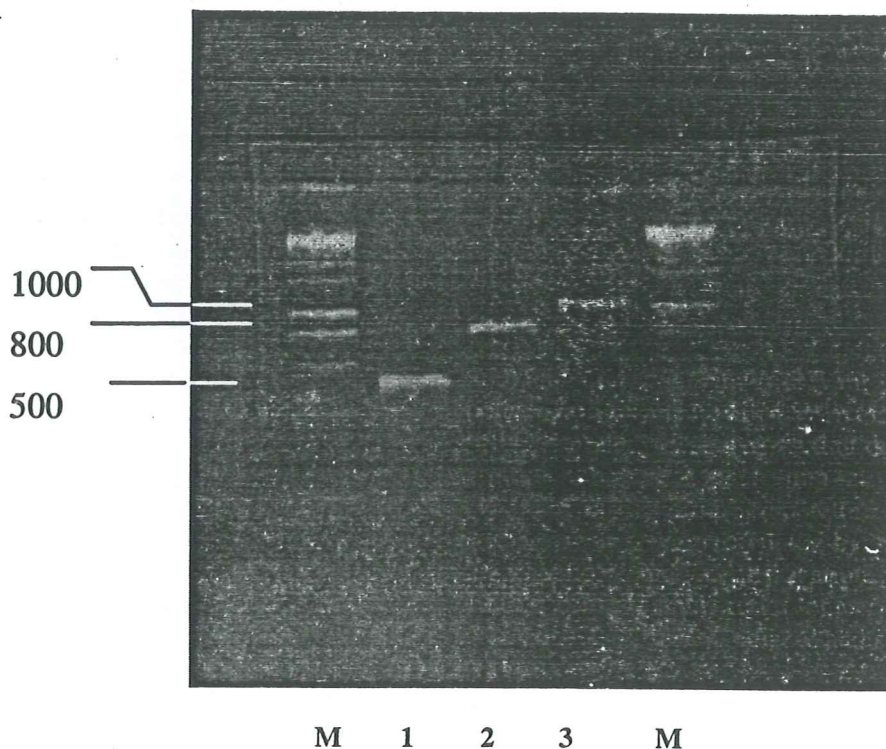
#### Preparation of plasmid

Plasmids were prepared using alkaline lysis method. Briefly, pelleted bacteria were first resuspended in 0.3 ml buffer 1 (50 mM Tris-HCl, pH

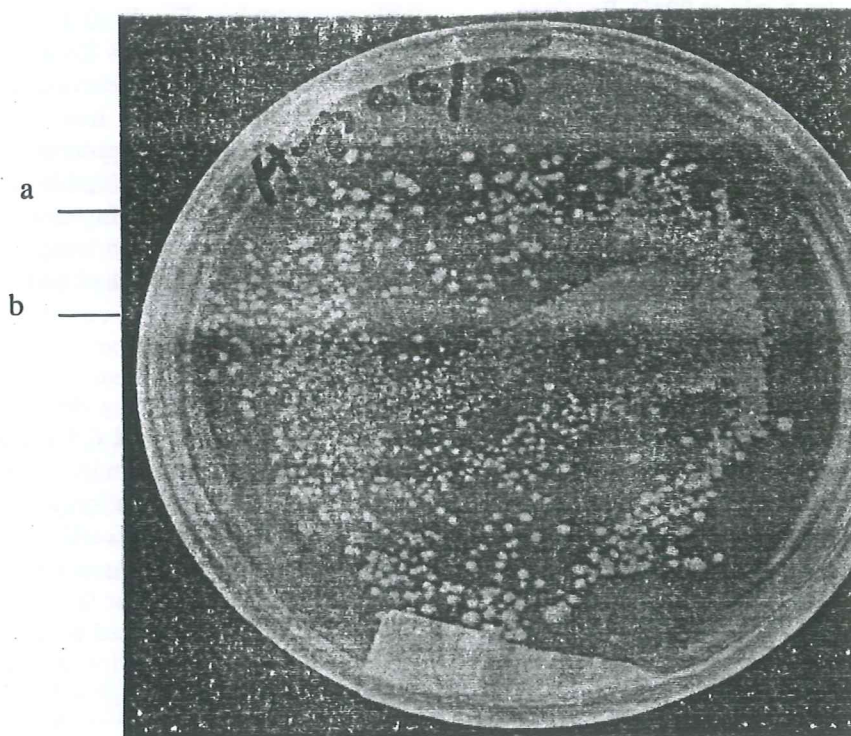
7.5, 10 mM EDTA). 0.3 ml buffer 2 (0.2 M NaOH, 1% SDS) was then added and the solution mixed without vortexing. Finally 0.3 ml buffer 3 (2.55 M potassium acetate, pH 4.8) was added and the solution mixed without vortexing then centrifuged in a minicentrifuge for 15 min at maximum speed (13,000 rpm) at room temperature. The supernatant (0.8 ml) was then precipitated by addition of 0,7 ml isopropanol and centrifugation (minicentrifuge, 13,000 rpm, 15 min, room temperature). The pellet was washed with 70% ethanol and slightly dried.

#### Analysis of clones

Plasmid pellet was dissolved in 50  $\mu\text{L}$  10 mM Tris-HCl pH 7.5 and 0.5 mg/mL RNAses and incubated at 37°C for 30 min. Plasmid analysis was carried out by single digestion with *EcoRI* or double digestion with *BamHI-EcoRI*, in 20  $\mu\text{L}$  buffer containing 2.5-5,0  $\mu\text{L}$  plasmid solution, 5 units of each enzyme, at 37°C for 1 h 30 min. Digestion products were then analyzed by electrophoresis on a 0.8% agarose gel. Revelation was done by ethidium bromide and observation under UV lamp.



**Figure 1.** Isolation and amplification of mature and precursor SAG-1 genes by PCR. The reaction products was analyzed on agarose gel. M: Molecular weight markers, 1:  $\lambda$  DNA control, 2: Mature SAG-1, 3: precursor SAG-1



**Figure 2.** Transfection of *E. coli DH5 $\alpha$*  with pCR-mSAG-1 and pCR-pSAG-1 constructs.

a. White colonies with insert, b. Blue colonies without insert.

## RESULTS AND DISCUSSION

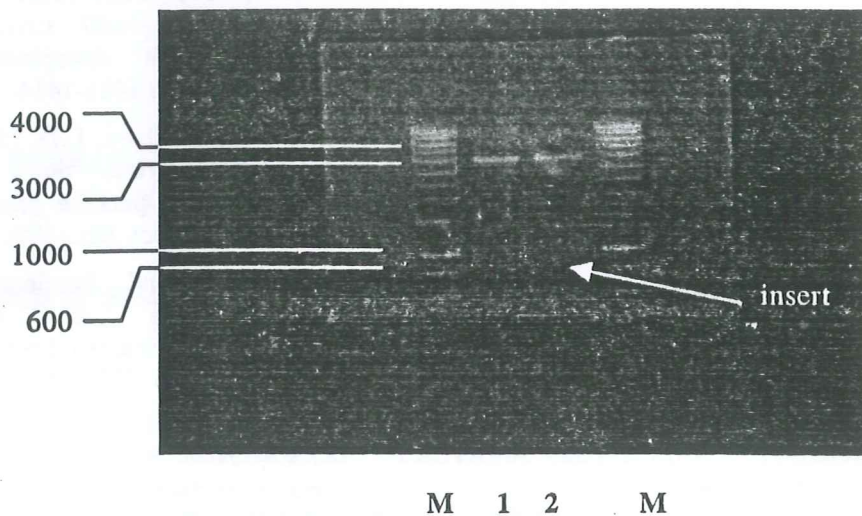
### Conception of primers for mature and precursor SAG-1 gene isolation-amplification

Primers used in PCR reactions were derived from a sequence data (Burg, *et al.*, 1988) and are as follows. a) Mature SAG-1 gene: upstream primer ATTAGGATCCATGTTCACT-CTCAAGTGCCCT (additional initiation codon ATG in bold letter, introduced *Bam*HI site underlined); downstream primer TTGAGAATTCAGCACAACGGTGATCAC TC (additional *Eco*RI site underlined). The entire mature SAG-1 gene will be amplified as a DNA fragment of about 0.8 kb. b) Precursor SAG-1 gene: upstream primer ATTAGAATTCGT-ATGTCGGTT TCGCTGCAC (initiation codon ATG in bold letters, introduced *Eco*RI site underlined); downstream primer as above. The amplified DNA fragment will be around 1.0 kb in length.

### Isolation and amplification of mature SAG-1 gene by PCR

We used as matrix for PCR reactions genomic DNA or cDNA prepared from mRNA. PCR reactions were performed as described in Materials and Methods. One fifth of the reaction products was analyzed by electrophoresis on a 0.8% agarose gel to control the PCR reactions. Figure 1 shows gel analysis of PCR products. The expected size of mature SAG-1 is *circa* 0.8 kb whereas that of precursor SAG-1 gene is about 1.0 kb in length. As seen, DNA fragments migrating at the expected positions were clearly obtained, i.e. about 0.8 kb and about 1.0 kb. These two fragments were obtained either when genomic DNA was used as matrix or when cDNA served as matrix. It appeared nevertheless that a denser band was obtained when cDNA was used as matrix.

This may arise from differences in SAG-1 gene number in the sample. For use in nucleic acid amplification, SAG-1 gene presents the advantage of being intronless. Therefore genomic DNA as well as



**Figure 3.** Clone analysis by restriction digestion (doubly digested with *Bam*HI-*Eco*RI)

M: molecular with markers, (1,2): pCR-mSAG-1

cDNA can be used as matrix. The use of DNA is particularly interesting as less steps are required for matrix preparation. By fine-tuning DNA extraction, more efficient gene amplification can be easily attained.

**Cloning in pCR2.1-TOPO: construction of pCR-matSAG-1 and pCR-precSAG-1**

Cloning in pCR2.1-TOPO was done by direct use of PCR products. The system used is based on topoisomerase reaction for exogeneous DNA insertion in the plasmid, instead of ligation with DNA ligase. Topoisomerase reaction is more rapid and efficient and easy to perform. For insertion, the exogeneous DNA fragments have to possess a single 3' adenine overhang that interact with the single 5' thymidine overhang of the plasmid. The single 3' adenine overhang results from amplification by Taq DNA polymerase.

Topoisomerase reaction products were transfected in *E. coli* DH 5 $\alpha$  and spread on agar-LB-ampicilline containing IPTG and X-Gal. Positive recombinant bacterial colonies are white or light blue. Figure 2 shows bacterial colonies obtained. More than 600 bacterial colonies were obtained for each construction.

Cloning using pCR2.1-TOPO methodology allows easy determination of positive bacterial transformants i.e. bacteria harvesting a plasmid containing an insert. Gene insertion occurs in the  $\beta$ -galactosidase gene so that it will result in the production of non-active enzyme. Therefore negative bacterial transformants are blue. Positive bacterial

recombinants were easily characterized. However, white or light blue coloration may also result from insertion or deletion of short sequences. Therefore, analysis of plasmids was necessary.

**Clone analysis of the pCR-SAG-1 constructs by restriction digestion**

Mature SAG-1 gene, isolated and amplified by PCR, contains additional sequences at the upstream and downstream ends. A new site was introduced for *Bam*HI upstream and for *Eco*RI downstream the SAG-1 gene. The gene can thus be excized by a double-digestion with *Bam*HI and *Eco*RI. The isolated precursor SAG-1 gene contains an additional *Eco*RI site upstream and downstream the gene. Therefore positive clones could be easily characterized by *Eco*RI digestion. White bacterial colonies were cultured and plasmids prepared as described in Materials and Methods. The results of plasmid digestions are given in Figure 3.

It clearly shows that the double-digestion of mature SAG-1 construct (called pCR-mSAG-1) resulted in two DNA fragments of 3.9 kb (vector) and of about 0.8 kb (mature SAG-1 gene). *Eco*RI digestion of precursor SAG-1 construct (called pCR-pSAG-1) gave rise to two fragments of 3.9 kb (vector) and of about 1.0 kb (precursor gene). This analysis definitely establishes that the positive clones actually contain pCR2.1-TOPO plasmid harvesting mature SAG-1 or precursor SAG-1 gene.

Cloning in pCR2.1-TOPO using a methodology based on topoisomerase reaction is easy and rapid to perform and efficient. Positive clones were rapidly

determined, solely by the bacterial colony coloration. Analysis with restriction enzymes was needed to definitely establish clone characteristics. Isolation-amplification by PCR and cloning in pCR2.1-TOPO constitute the first step for the production of recombinant proteins in *E. coli*. The gene we isolated will be excized and introduced in a prokaryotic expression vector. The primer conception was also intended for correct insertion and without shifting the reading frame.

#### ACKNOWLEDGEMENTS

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