

DIGoxigenin (DIG) Labeled Probe Candidate of *Surface Antigen 1 (SAG1)* for *Toxoplasma gondii* Detection

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Abstract

Toxoplasma gondii is one of the opportunistic pathogen that causes toxoplasmosis. Infection of *Toxoplasma gondii* has been estimated as high both in human and animal. The manifestation of infection were abortion, hydrocephalus, brain calcification, chorioretinal scar, and loss of productivity even to death in patients with acquired immunosuppression. Early diagnostic method which are rapid and accurate is essential for *T. gondii* detection because of its high prevalence. The purpose of this study was to develop a sensitive probes derived from *Surface Antigen 1 (SAG1)* for detection *T. gondii* and to examine the specificity and sensitivity of probe as diagnostic tool for toxoplasmosis. This research used *SAG1* gene of *T. gondii* local isolate IS-1 that was cloned into pGEX-2T and transformed into *Escherichia coli* DH5 α . The sequence of *SAG1* was labeled with DIGoxigenin (non radioactive labeled) using PCR *DIG Labeling Mix* to derive 213 bp (*probe-TS*). BLAST and dot-blot hybridization analyses showed that probes had high specificity with other strains of *T. gondii*. Probe was able to detect *T. gondii* DNA up to 10 ng/ μ l of total sample DNA.

Keywords : probe, *SAG1*, *Toxoplasma gondii*, dot-blot hybridization

Introduction

Toxoplasmosis is a zoonotic disease caused by obligate intracellular parasite *Toxoplasma gondii*, which infects all warm-blooded animals including humans (Carruthers and Boothroyd, 2007; Craeye *et al.*, 2008; Ma *et al.*, 2009; Sibley *et al.*, 2009). It is estimated that Toxoplasmosis infects 30% to 50% of the entire human population in the world (Aspinall *et al.*, 2002; Weiss and Dubey, 2009). In Indonesia, the prevalence of toxoplasmosis in humans ranges from 2% -63% (Gandahusada, 1998).

In a person with a good immune system, infections caused by *T. gondii* does not cause serious problems even no symptoms

(asymptomatic) but can be fatal if the infection is congenital and occur to patients with immunosuppression (Chintana *et al.*, 1998), as well as to patients whose immune systems are interrupted such as encephalitis, AIDS, transplantation, chemotherapy, and ocular lesions (Yamamoto *et al.*, 2000; Sibley *et al.*, 2009). In animals, infection of *T. gondii* could reduce the productivity of livestock and be a source of transmission of infection to humans (Dubey, 2008).

Humans could be infected with *T. gondii* through variety ways, such as (1) through the placenta from mother to fetus if the mother receives primary infection during pregnancy, (2) consuming raw or undercooked meat if the meat contains tissue cysts or tachyzoite, (3) to a person who works in the laboratory and works with experimental animals infected through

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needles and other contaminated laboratory equipment, (4) organ transplantation from donors who suffer from latent toxoplasmosis, and (5) a complete blood transfusion from an infected donor (Gandahusada, 1998; Kasper LH, 2001; Hiswami, 2003; Sibley *et al.*, 2009).

Rapid technique of early diagnosis that is appropriate for toxoplasmosis is needed because of its high prevalence rate. So far, the diagnosis of toxoplasmosis is detected from clinical symptoms, blood or a patient's body tissue tests, and serological examination. Diagnosis of clinical symptoms is sometimes rather difficult because most of patients do not show any symptoms (asymptomatic) (Montoya and Liesenfeld, 2004; Robert-Gangneux *et al.*, 1999; Wu *et al.*, 2009). Diagnosis by discovery of parasites in sufferer's tissue or body fluids directly is rarely performed because of its difficulty in terms of taking the specimen to be studied. Serological tests which are currently most developed technique are important diagnostic methods for detecting *T. gondii*. Toxoplasma on the basis of antigen-toxoplasma will form specific antibodies in the blood serum of patients, but this method has a weakness that could not show the active phase of *T. gondii* infection. In addition, serological tests do not always show quick and accurate diagnosis because IgM is not found in the neonatal phase, or because IgM can be found for months or a year (Gandahusada, 1998).

Diagnosis methods through molecular approaches are possibly used to improve the accuracy, specificity and sensitivity of early detection of *T. gondii*. DNA can be used to detect *T. gondii* by dot-blot hybridization technique. Diagnosis of various types of pathogens such as *T. gondii*, *Plasmodium falciparum*, *Neisseria gonorrhoeae*, *cytomegalovirus*, enterotoxigenic *Escherichia coli* using DNA probes have been widely used (Savva and Holliman, 1990), but not yet much developed in Indonesia.

In Toxoplasmosis, a variety of target genes can be used for the detection of *T. gondii* and the choice of targets is very important because *T. gondii* found in three infective forms, which are: oocyst, tachyzoite and

cyst. (Homan *et al.*, 2000). One of genes which is easy to be isolated from tachyzoite of *T. gondii* is *SAG1* (Surface Antigen1) gene (Wu *et al.*, 2009, Kazemi *et al.*, 2007; Wang *et al.*, 2009). *SAG1* antigen is distributed homogeneously in the tachyzoite surface that makes up approximately 3-5% of total tachyzoite protein and is a major protein (Wu *et al.*, 2009). *SAG1* gene is highly conserved gene (Wu *et al.*, 2009), then a labeled DNA probe that can detect the existence of complementary nucleic acids in field samples (clinical sample) through dot-blot hybridization technique can be made.

In this research, probe was labeled with DIGoxigenin (DIG) used PCR method to avoid radiation risk of a radioactive element. This method allows tens to hundreds of samples to be analyzed at the same time by using a membrane. Therefore, time efficiency can be achieved and it can be applied for various purposes, including clinical use in the laboratory, as an effective method of molecular diagnosis and for epidemiological studies to determine prevalence of cases of Toxoplasmosis.

The purpose of this research was to create probes derived from the *SAG1* gene sequences and to test the specificity and sensitivity of probe sequences of *SAG1* gene to detect *T. gondii*.

Materials and Methods

Tachyzoites Production and DNA Extraction

Indonesian *T. gondii* isolate (IS-1) was maintained by intraperitoneal passage in Balb/C mice. IS-1 tachyzoites were harvested from peritoneal exudate that had been infected 6 days earlier and purified from host cellular material as described by Garberi *et al* (1990). Tachyzoite's DNA were extracted using PureLink Genomic DNA Kit (Invitrogen).

SAG1 Gene of T. gondii amplification using PCR

SAG1 gene from genomic DNA of *T. gondii* were amplified using Pure Taq

Ready-To-Go PCR Beads (Amersham Bioscience). Upstream primer F1: 5'-ATTA GGATCCATGTTCACTCTCAAGTGCCT-3' and downstream primer B1: 5'-TTGAGAATTCAGCACAACGGTGATCACTC-3' through initial incubation 2 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and final 7 min incubation at 72 °C.

Cloning SAG1 gene in a plasmid vector

PCR product of *SAG1* genes were cloned in pGEX-2T plasmid (Amersham Bioscience) according to manual instruction. PCR product of *SAG1* gene and pGEX-2T plasmid were both cutted using *Bam*HI and *Eco*R1 restriction enzyme in separated tubes prior to ligate. Transformation into DH5α *E. coli* strain were done using TSS method described by Chung *et al* (1989). TSS competent cells of DH5α were obtained by 10 times-concentrating fresh exponential phase bacterial culture (OD600nm around 0.6) in LB containing 20 % PEG 6000 (w/v), 10 % DMSO (v/v) and 70 mM MgCl₂. Different amount of transformation were spreaded on LB agar plate containing 100 µg/mL ampicilin, 40 µL of 40 mg/mL X-Gal and 40 µL of 100 mM IPTG and incubated overnight at 37 °C. Possitive transforman that contained plasmid with an insert will appear as white or light blue colonies. The succesful construction of transformant were named pGEX-SAG (pGEX-2T plasmid containing *SAG1* gene). A mixture of pGEX-SAG positive clones were then cultured overnight and recombinant plasmids were isolated using High Pure Plasmid Isolation Kit (Roche).

Designing and syntesizing of DIG labeled probe

Basic Local Alignment Search Tool (BLAST), an online software from NCBI: <http://www.ncbi.nlm.nih.gov/BLAST/>, was utilized to generate probe TS (213 bp probe derived from *SAG1* gene). PCR DIG Labeling mix was used to label probe TS. Upstream primer (TS-

F): 5'-AAGCATTTCAGCCGAGTC-3' and downstream primer (TS-B): 5'-GCACAACGGTAATCACTCA-3' through initial incubation 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 30 sec at 55°C, 25 sec at 72°C, and final 5 min incubation at 72°C.

Probe and dot-blot analysis

Probe TS were diluted into 10⁻¹ to 10⁻⁵. 1 µl of diluted probe were blotted into membrane of DIG Quantification Test Strip (Roche). Quantification was performed according to the manufacturer as briefly described: DIG Control Test Strip and DIG Quantification Test Strip, which arranged back to back, was blocked using blocking solution for 2 min, dipped into antibody binding for 5 min, blocked again with blocking solution for 1 min. The process was continued to wash the strips with washing buffer for 1 min, then dipped into detection buffer for 1 min. The strips were subsequently incubated in colour substrate solution for 5-30 min. The blot signal of DIG-DNA probes were revealed using enzyme-catalized colour reaction solution (one NBT/BCIP tablet (Roche), in 10 ml dH₂O) for producing blue-purple precipitate.

Dot-blot hybridization of pGEX-SAG and hybridization detection

pGEX-SAG was diluted into 10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl, then denatured by boiling for 10 min at 100°C and placed into ice tube. 1 µl of diluted were blotted into Hybond possitive membrane (Roche) and dried for 2 h at 80°C in oven. Prehybridization for 3 to 4 h at 42°C in prehybridization medium (*DIG easy hyb*, Roche) then hibridyztion at 48°C overnight by adding 2 µl/ml probe that were labeled with DIG which had been denatured for 10 min at 100°C in prehybridization medium. Membrane was washed two times with 2X SSC, 0.1% SDS, for 15 min and with 0.5X SSC, 0.1% SDS, for 15 min at 68°C (Roche, 2006). Hybridization detection with alkaline phosphatase-conjugated and

anti-DIGoxigenin antibody according to the manufacturer as briefly described: Wash the hybridization membrane with *washing buffer I* (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.3% Tween 20) for 1 - 3 min, block with *blocking reagent* (100 mM maleic acid, 150 mM NaCl, pH 7.5, 1% blocking reagent) for 1 hour. The process was continued to wash the membrane two times with *washing buffer I* for 15 min and *washing buffer II* (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 15 min. Add BCIP/NBT substrate at room temperature until the best signal to noise ratio. Stop with excess ddH₂O.

Results and Discussion

Tachyzoites production and DNA extraction

Light microscope examination of extracellular parasites that were obtained from peritoneal exudate of infected mice showed that *T. gondii* was in tachyzoite stage, which can be formed as free organism or within host cells like leukocytes (Figure 1). DNA extract from tachyzoites were positively contained genomic DNA of *T. gondii* (Figure 2) and *SAG1* gene was successfully confirmed by PCR using primer pairs of F1/B1 and produced amplified sequences with 800 bp long (Figure 3).

Cloning SAG1 gene into plasmid vector

Both cutted *SAG1* gene sequence and pGEX-2T plasmid using *Bam*HI and *Eco*RI were match to each others. An *Bam*HI sticky end of *SAG1* gene which was a peptide signal and coding sequence could anneal to *Bam*HI cutted site of pGEX-2T. The same way, an *Eco*RI sticky end of *SAG1* gene was annealed to the same *Eco*RI sticky end of the plasmid. These cloning method served a correct ligation orientation of target gene into plasmid vector. *SAG1* gene was successfully conditioned in the vector pGEX-2T and transfected into *E. coli* for propagation of recombinant plasmids (pGEX-SAG). Confirmation was done by PCR and it was proved that the recombinant plasmid pGEX-SAG was inserted with *SAG1* gene (800 bp).

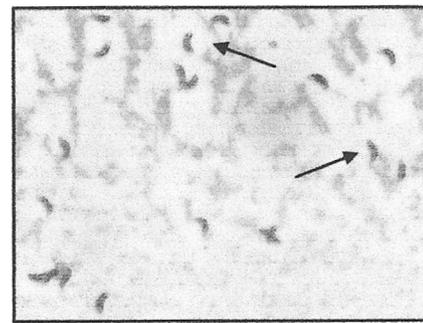


Figure 1. Tachyzoites *Toxoplasma gondii* isolate IS-1 (1000x)

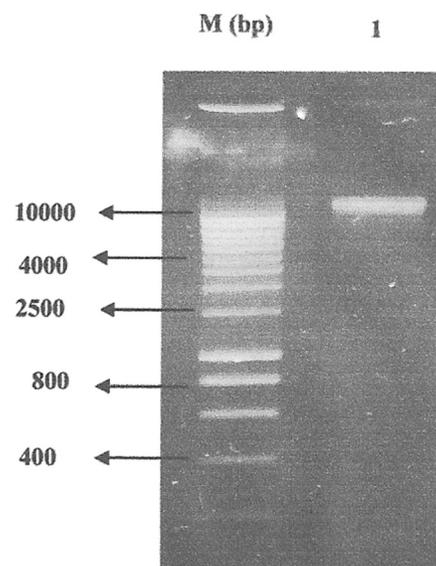


Figure 2. M: Marker DNA, 1. Tachyzoites

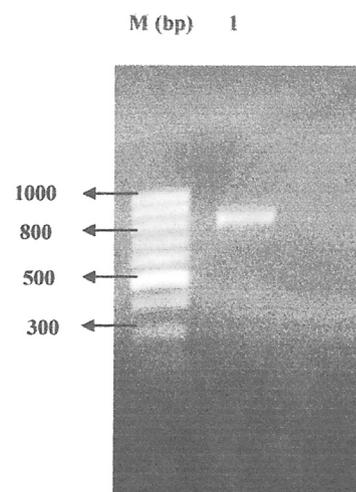


Figure 3. M: Marker DNA, 1: *SAG1* gene amplified using primer F1/B1 (800 bp)

Probe designing from SAG1 gene of *T. gondii*

A gene sequence that will be used as a template for synthesis probes must have high stability in an organism (Reue, 1998). BLAST analysis was carried out by NCBI website: <http://www.ncbi.nlm.nih.gov/BLAST/> Result of BLAST analysis showed that SAG1 gene sequence of *T. gondii* isolates IS-1 in pGEX-SAG against various strains of other *T. gondii* indicated high level of similarity and high stability that reached 90 to 100%. E-value that reached 0.0 indicated SAG1 gene sequence of *T. gondii* isolates IS-1 could be used as the basis for the design of probe candidate. The result of homology tree analysis through the Geneious program showed that the SAG1 gene of *T. gondii* isolates IS-1 included in the RH strain. Hartati (2007) had also conducted sequencing of SAG1 gene sequence and registered it in GenBank with Acc.no. AY651825.

Probe for detection of a disease must have high genetic similarity with the gene of target organisms and low genetic similarity with the host genome. BLASTN analysis of SAG1 gene probe candidate (probe TS) on various nucleic acids host showed a very low level of similarity, same as the results of BLASTN analysis of TS probe candidate to other various parasites such as *Echinococcus granulosus*, *duodenal Giardia*, *Plasmodium*

falciparum, *Sarcocystis* spp., *trichinella spiralis*, *Tricomonas vaginalis*, *Neospora caninum*, *Isospora suis*, *Cryptosporidium parvum*, and *Besnoitia besnoiti*. The results also showed that the candidate bore no resemblance to TS probe (0%). Therefore, the TS probe candidate that have been designed based on *T. gondii* has the specificity of the parasite *T. gondii* (not cross-reacted with other host and parasite species). Probe candidate of SAG1 gene sequence of *T. gondii* isolate IS-1, which has molecular weight of 213 bp, was proved to be conserved based on BLASTN analysis.

Synthesizing and quantification of the probe

Labeling of probes used recombinant plasmid pGEX-SAG as a template by DIG labeling PCR method. DIG labeled PCR product has a molecular weight of 213 bp. Qualitative analysis of the labeled probe results indicated that DIG labeled probe has a larger molecular weight than those which were not labeled, and DIG labeled PCR product showed lower band intensity than PCR product of non DIG labeled probe (Figure 4). Quantitative analysis result of DIG labeled DNA probes with TS probe yielded concentration of 56.7 pg / μ l.

Dot blot hybridization of pGEX-SAG and hybridization detection

The result of DNA *dot-blot* hybridization with DIG labeled probes on the pGEX-SAG plasmid showed that the TS probe was capable to detect up to 100 pg/ μ l of *T. gondii* with TS probe concentration which was

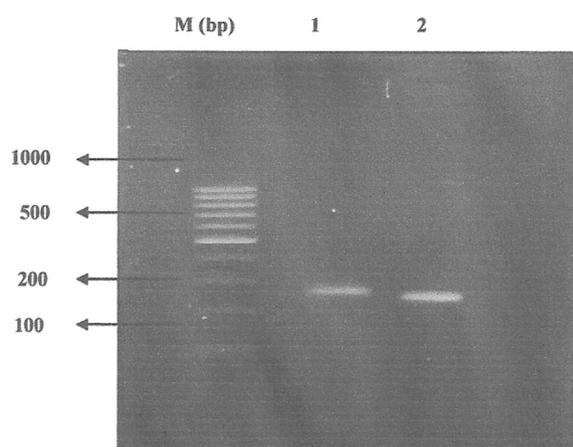


Figure 4. Qualitative analysis DIG labeled probe (213 bp). M: Marker DNA, 1: DIG labeled probe, 2: without DIG label.

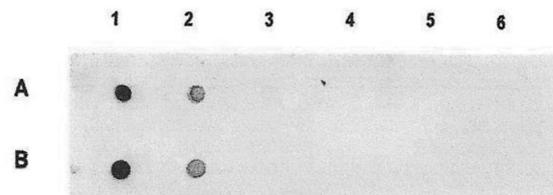


Figure 5. *Dot blot* hybridization of pGEX-SAG with probe TS 2 μ l / ml, followed pGEX-SAG concentration 1-6: 10ng/ μ l, 1 ng/ μ l, 100 pg/ μ l, 10 pg/ μ l, 1 pg/ μ l dan 0 pg/ μ l (negative control)

added to the hybridization solution was 2 μ l/ml (Figure 5).

In summary, *SAG1* gene of *T. gondii* isolate IS-1 has a high homology through the strains. Probe that was derived from *SAG1* gene could be synthesized with high spesificity and can be used to detect *T. gondii* from PGEX-SAG (recombinant plasmid) until concentration 100 pg.

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