Detection of *Toxoplasma gondii* based on sequence r529 and sag1 gene probe

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ABSTRACT: *Toxoplasma gondii* is an obligate intracellular protozoan parasite that infects all warm-blooded animals, including humans. It is the pathogenic agent of toxoplasmosis which is one of the main causes of infectious reproductive failure in small ruminants in the world. It causes fetal resorption, abortion, stillbirth and neonatal mortalities resulting in great economic losses. Diagnostic tool based on nucleic acid is an attractive alternative for parasite detection. Several target genes of *T. gondii* have been cloned and expressed. Two kinds of potential target sequence for detection are sequence repetitive 529 bp (R529) and *Surface antigen*1 (SAG1) gene. SAG1 is the most immunodominant and stage-specific antigen of tachyzoite and is highly conserved in most isolated *T. gondii* strains examined. SAG1 has been proven to be a good candidate for diagnosis and vaccine development. R529 is a high copy number fragment and conserved within genom of *T. gondii* during evolution. The nucleotide sequence of R529 and SAG1 gene has been established. The purpose of this study is to developed probe from R529 and SAG1 gene that have high specificity and sensitivity. R529 and SAG1 was isolated from genome of *T. gondii* and Amplified by PCR using *sense* and *antisense* to derived 237 bp (probe-TR) and 213 bp (probe-TS), respectively. The sequence was labeled with anti-digoxigenin (non radioactive labeled) using PCR *Dig Labeling Mix*. The dilution of probe-TR can be detect until the quantities of 2,9 pg whereas probe TS until 56.7 pg/µl.

Key words: *Toxoplasma gondii*, SAG1, R529, *Probe*

INTRODUCTION

Toxoplasmosis is a disease caused by the protozoan parasite *Toxoplasma gondii* (Montoya and Liesenfeld, 2004), an ubiquitous protozoan parasite that is estimated to infect one-third of the world's human population. It can infect many species of warm-blooded animals and is a significant zoonotic and veterinary pathogen (Weiss and Dubey, 2009).

There are four groups of individuals in whom the diagnosis of toxoplasmosis is most critical: pregnant women who acquire their infection during gestation, fetuses and newborns who are congenitally infected, immunocompromised patients, and those with chorioretinitis (Remington et al., 2004). Maternal toxoplasmosis if acquired during the first trimester of pregnancy can cause significant morbidity and mortality in developing fetuses (Thiebaut et al., 2007).

Toxoplasmosis in human has high economic impact due to severity of infection, associated complications, treatment and social costs (Kijlstra and Jongert, 2008). In verterinary, toxoplasmosis deserves special attention because of its economic lost (Dubey, 2008), it is one of the main causes of infectious reproductive failure in small ruminants in the world (Ahmed et al., 2008).

The diagnosis of *T. gondii* infection is most commonly made by detecting IgG and IgM antibodies in the blood; however, these tests can not indicate infection at some indeterminate time and not necessarily acute or current infection (Iqbal and Khalid, 2007).

The SAG-1 (P-30) single-copy sequence has shown to be a satisfactory target for the detection of *T. gondii* (Contini et al., 2006). In the last years, many scientists have been using repetitive DNA sequences to increase the sensitivity and spesificity of detection. Repetitive 529-bp DNA fragment (R529) is repeated more than 300-fold in the genome of *T. gondii*. Since individual intragenic copies of the target are conserved on sequence level, the high copy number leads to an ultimate level of analytical sensitivity in routine practice (Reischl et al., 2003).

This paper describes PCR digoxigenin (DIG) labeling method for for generating probes base on R529 and *SAG1* gene. The probes have potentially used for detecting nucleic acid of *T. gondii* from various sample by hybridization techniques.

MATERIALS AND METHODS

Tachizoites Production and DNA Extraction

Indonesian *T. gondii* isolate (IS-1) (Iskandar, 1988) was manitained by i.p. passage in Balb/C mice. IS-1 tachizoites 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). Tachizoite's DNAs were extracted using PureLink Genomic DNA Kit (Invitrogen) as described mamalian cells lysate protocol accoding to the manufacturer recommandation.

R529 and SAG1 Gene of T. Gondii Amplifikation Using PCR

R529s and *SAG1* genes from genomic DNA *T. gondii* were amplified using Pure Taq Ready-To-Go PCR Beads (Amersham Bioscience). Upstream primer T1 5'-CTGCAGGGAGGAAGACGAAAG and downstream primer R1: 5'-CTGCAGACACAGTGCACTCGG were selected to amplified R529s. PCR was performed on a Thermal cycler, Eppendorf by 2 min incubation at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 74°C, and a final 5 min incubation at 72°C. *SAG1* genes were amplified using uperstream primer S1: 5'-ATTAGGATCC -**ATG**TTCACTCTCAAGTGCCCT dan downstream primer G1: 5'-TTGAGAATTCAGCACAACGGTGATCACTC through initian 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 a final 7 min incubation at 72°C.

Cloning R529 and SAG1 Gene in a Vector Plasmid

PCR product of R529s and *SAG1* genes were cloned in pCR®2.1-TOPO® plasmid (Invitrogen) and pGEX-2T plasmid (Amersham Bioscience) respectively, accoding to those each manual instruction. Transformations into DH5α *E.coli* strain were done using TSS method described by Chung et al. (1989). Positive transforman, containing plasmid with an insert are white or light blue colonies. The succesful constructs were named pCR-TR (pCR®2.1-TOPO® plasmid containing R529) and pGEX-SAG (pGEX plasmid containing *SAG1* gene). A mixture of respective pCR-TR and pGEX-SAG positive clones were then cultured ON and recombinant plasmids were isolated using High Pure Plasmid Isolation Kit (Roche) accoding to manual instruction.

Designing and Synthesis DIG Berlabel Probe

Basic Local Alignment Search Tool (BLAST), an online sofware from NCBI: http://www.ncbi.nlm.nih.gov/BLAST/, was utilized to generate both probe-TR (237 bp probe derived from R529) and probe-TS (210 bp probe derived from *SAG1* gene). PCR Dig labeling mix (Roche) was used to label either probe-TR or probe-TS.

Probe and Dot Blot Analysis

Probe-TR and probe-TS were diluted into 10^{-1} to $10^{-}5$ each, 1 µl of diluted probe were blotted onto membran of Dig Quantification Test Strip (Roche). Quantification of two probes 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 bocking 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 color substrate solution for 5-30 min. The blot signal

of Dig-DNA probes were revealed using enzim-catalized color reaction solution [one NBT/BCIP tablet (Roche®) in 10 ml dH_2O] for producing blue-purple precipitate.

RESULTS AND DISCUSSION

Tachizoites Production and DNA Extraction

Light-microscope examination of extracellular parasites obtained from peritoneal exudates of infected mice showed that *T. gondii* was in tachizoite stage which can be as free organism or within host cells such as leukocytes. DNAs extracted from tachizoites, blood and various organs of infected mice were all positively contained genomic DNA of *T. gondii* as confirmed by PCR using primer couple T1/R1. On the contrary PCR using primer S1 and G1 failed to amplify *SAG1* gene from extracted DNAs from blood and various organs of infected mice, the positive result was only obtained from tachizoites' DNA. Both R529 and *SAG1* gene sequences of IS-1 isolate have been listed in GenBank with *Acession Number* EF195646.1 and AY651825 respectively.

Cloning R529 and SAG1 Gene into Vector Plasmid

R529s obtained by PCR using DNA tachyzoite as template and primer couple T1/R1 were cloned in pCR2.1-TOPO vector plasmids. *Taq* polymerase adds single deoxyadenosine (A) to 3'-ends of PCR products, whereas the liniarized vector has single overhanging 3'deoxythyminidine (T) residues. This allows PCR inserts to ligate efficienltly with the vector which facilitated by Topoisomease I from *Vaccinia* virus (Shuman, 1994).

Both cutted *SAG1* gene sequence and pGEX-2T plasmid using *Bam*HI and *Eco*RI were mach each others. An *Bam*HI sticky end of *SAG1* gene which was a peptide signal and coding sequence could anneal to *Bam*HI cuted site of pGEX-2T. The same way, an *Eco*RI sticky end of *SAG1* gene anealled to the same *Eco*RI sticky end of the plasmid. These cloning method served a correct orientationally ligation of target gene into plasmid vector.

Probes Designing from R529 and SAG1 gene of Toxoplasma gondii

BLASTN analysis of R529 from IS-1 isolate and all published sequences of R529 in GenBank indicated a highly conserved nucleotide among various strains and isolates of *T. gondii*. The same analysis to the *SAG1* gene showed the similar properties to those the R529. Primers for generating probe were designed using BLAST program base on all of the published sequence of R529 and *SAG1* gene in GenBank.

By applying the two probe sequent candidates (a 237 bp selected sequence from R529 and a 210 bp selected sequence from *SAG1* gene) to BLAST program, we found no significant matches through all possible host DNA and only poor homology to curently published DNA from other parasites (*Echinococcus granulosis*, *Giardia duodenalis*, *Plasmodium falciparum*, *Sarcocystis* spp., *Trichinella spiralis*, *Tricomonas vaginalis*, *Neospora caninum*, *Isospora suis*, *Besnoitia besnoiti* and *Cryptosporidium parvum*). The BLAST analysis results indicated that both of two selected sequences were unique and have specificity as probe candidates.

Synthesis and Quantification of the Probe

Probe labeling with DIG may be added by random primed labeling, nick translation, 3'-end labeling/tailing, direct chemical labeling, and PCR labeling. In this study we used PCR labeling because of some advantages of the mothod i.e: requires only small amount of template, requires less optimation than other methods, high yield of labeled probe, suit for very short probe(<100 bp), and produces very sensitive probe.

Digoxigenins which linked to C-5 position of uridine nucleotides via spacer arm containing elevent carbon atom are incoperated into nucleic acid probes by DNA polymeases during PCR. Since

the DIG system is very sensitive, it is important to check the efficiency of each labeling reaction and to define amounts of probe in oder to get optimal detection result.

Blots from probe-TR with dilution of 10^{-1} to 10^{-4} gave a strong signal, otherwise that from 10^{-5} gave weak signal, the same results were also shown from probe-TS. Ass a whole probe-TR produced stronger signal than did probe-TS. DIG concentration of the probe using equation obtained from Dig control test strips indicated that concentration of probe-TR and probe-TS was 0,296 μg/μl and 56,7 pg/μl respectively. As little as 2,9 pg of probe-TR could still give visible signal.

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