

Cloning and sequencing of *Toxoplasma gondii* major surface antigen (SAG¹) gene

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ABSTRACT

Genetic typing methods of *T. gondii* strains have been extensively perfected in recent years. From a technical point of view, many tools usable for genetic studied on single-copy loci have been used: RFLP, PCR-RFLP, sequencing, RAPD-PCR and isoenzyme analysis. We described the cloning and sequence analysis of the gene which encodes the major surface antigen (SAG¹ or P^{π}) of *T. gondii*. SAG¹ is the immunodominant antigen of *Toxoplasma gondii* tachyzoites being considered as the most promising molecule for a recombinant vaccine or such as DNA vaccine against toxoplasmosis. In the present work, first, genomic DNA of *Toxoplasma gondii* was extracted and used for amplifying of SAG¹ gene as a template. Then PCR product was cloned into pTZ^{Δ}^VR/T vector and plasmid containing SAG¹ gene (pT-SAG¹) was extracted from transformed bacteria and SAG¹ gene cloned into pTZ^{Δ}^VR/T was sequenced. Results showed that the P^{π}· gene contains no introns and can extract it from genomic DNA of tachyzoite stage. Results showed also that SAG¹ gene is cloned in pTZ^{Δ}^VR/T plasmid, forming pT-SAG¹ recombinant plasmid and *E. coli* TG¹ strain is the best host for pT-SAG¹ transformation. Sequence analysis of SAG¹ gene cloned into pTZ^{Δ}^VR/T vector showed that SAG¹ gene sequence from a high virulent strain of *T. gondii* (Known as RH strain) has ¹··³</sup> sequence identity with P-Br strain, P strain and C strain and high homology of ⁹A¹/. with RH strain and ZS¹ strain.

Keywords: Cloning, Sequencing, Toxoplasma gondii, SAG¹, P^r.

INTRODUCTION

Toxoplasmosis, caused by an intracellular protozoan parasite, *Toxoplasma gondii*, is widespread throughout the world (Bhopale (...)). The disease is

of major medical and veterinary importance, being a cause of congenital disease and abortion in humans and in domestic animals (Bhopale (., ., ., .)). There are marked biological differences among *Toxoplasma gondii* stocks concerning their pathogenicity to mice: most of the stocks are avirulant in mice producing asymptomatic chronic infections, while few which are highly virulent in mice stocks produce acute

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toxoplasmosis killing all mice with less than \. tachyzoites. Isoenzyme analysis using six different enzyme systems allowed the identification of 17 zymodemes (Z¹-Z¹) among a population of $\lambda \hat{\gamma}$ stocks (Ajzenberg *et al* $\gamma \cdot \cdot \gamma a$). For biologic and epidemiologic studies, three main genotypes are generally recognized in the T. gondii population: type I, II, and III (Ajzenberg et al ۲۰۰۲b). Lekutis et al $(\gamma \cdot \cdot \gamma)$ believed that in addition to developmentally regulated differences in SAG expression, there is measurable allelic variation between the three prototypic strains of T. gondii. Interestingly, just to alleles were identify at the SAG¹ and SAG⁷A luci when Type I, II and III strains were compared. In fact, most SAGs are dimorphic. SAG' or P", protein has an apparent M.W. $\forall \cdot kDa$ (Kasper *et al* $19\Lambda\Delta$) and is stage specific, being detected only in the tachyzoite stage, but absent in the sporozoite and bradyzoite stages (Burg et al 19AA, Hunter et al 1999, Kimbita et al (\dots)). This Antigen is abundant and homogeneously distributed on the surface of both extracellular and intracellular tachyzoites (Burg et al 19AA). SAG1 has two glycoforms (Zienker *et al* (\cdots)) and is a highly conformational antigen (Chen *et al* (\cdot, \cdot)). The gene encoding SAG¹ occurs as a single copy, without introns (Kimbita et al Y...), Biemans et al 199A) and is highly conserved in T.gondii strains (Letscher-Bru et al 1991, Burg et al 1911). We are interested in the role of P^{τ} . in the parasite's life cycle. Because of this and its importance in the immune response to T. gondii infection (and therefore its potential as a diagnostic tool and/or subunit or DNA vaccine), we have studied further molecular characterization of this protein through cloning and sequencing of the P^{ψ} . gene.

MATERIALS AND METHODS

Production of *T. gondii* tachyzoites. A high virulent strain of *T. gondii* (presented in experimental laboratory of Parasitology Department

of Medical sciences Faculty of Tarbiat Modarres University, Known as RH strain), maintained in BALB/c mice by serial intraperitoneal inoculation of about $1 \times 1.^{\circ}$ tachyzoites, was used for production of tachyzoites.

Genomic DNA extraction. About $\Delta \times 1 \cdot^{\vee} T$. gondii (**\..**µl) were concentrated tachyzoites bv centrifugation, washed with phosphate buffer saline (PBS), then lysed in \mathfrak{lysis} buffer (\mathfrak{lysis} Tris-HCl pH A, containing 1% sodium dodecyl sulphate, ·, M NaCl and l·mM EDTA) and then treated with $1 \cdot \mu l$ proteinase K ($1 \cdot \cdot \mu g/ml$) at $\Delta \circ C$ for hr(Kimbita *et al* (\cdot, \cdot)). The lysate was then added to an equal volume of phenol/chloroform $(\Upsilon \Delta; \Upsilon \Delta)$ to remove proteins. This mixture was centrifuged at Nr...rpm for Vomin and an equal volume of chloroform was added to the supernatant which was then re-centrifuged. The supernatant was mixed with $1/1 \cdot$ volume of "M sodium acetate and two volumes ١٠٠٪ of ethanol to precipitate DNA by centrifugation at 17...rpm for 1.min. The DNA pellet was washed with \vee . ? ethanol, dissolved in sterile distilled water and stored at -Y · °C until use (Sambrook et al 1949). DNA extraction products were detected in \cdot, λ' . agarose gel and photographed.

PCR amplification and gel electrophoresis. Genomic DNA isolated from tachyzoites was used as a template to amplify the SAG¹ gene by PCR performed in ^{YA} µl of solution containing ^Wµl of template DNA, •, ^a µl dNTP, •, ^a µl Taq DNA polymerase, $7, \Delta \mu l \rightarrow X$ PCR buffer, $\cdot, \nabla \Delta \mu l$ MgCl₁, 12, V2 µl distilled water and 1 µl each of primers [Forward primer, YV nt: introduced Hind III recognition site, underlined: &'-ATT AAG CTT ATG TTT CCG AAG GCA GTG-" (1-1/ nt); Reverse primer, $\forall \hat{\gamma}$ nt; introduced EcoRI recognition site, underlined: &'-ATT GAA TTC TCA CGC GAC ACA AGC $TG^{\gamma}(9^{\gamma}, 9^{\gamma})$ under the following conditions: After an initial ²min denaturation at 99 °C, each cycle consisted of 9 · s at 9° °C, 7° s at 2° °C and 4° s at 7° °C at the end of

the γ cycles of amplification, a final extension was continued for Δ min at $\forall \gamma \circ C$.

The PCR products analyzed by electrophoresis on a 1, agarose gel and photographed. The size markers used to estimate PCR products were the $1 \cdot bp$ and kbp DNA ladders (Fermentas). The DNA sequence of gene encoding the surface antigen $P^{r} \cdot (SAG^{1})$ of *T. gondii* was obtained from the GenBank database (http://www.ncbi.com) with accession No. AY^r 1, 0 of 7. Bor forward and reverse primers were designed according to the nucleotide sequence in GenBank database and GenRuner Software.

Extraction of PCR products (SAG) gene band) from agarose gel. PCR products were purified using a DNA Extraction Kit from agarose gel (Ferments), following the manufacturer's recommendations.

Ligation of SAG¹ gene to $pTZ^{\Delta \vee}R/T$ Cloning vector. The purified PCR products were ligated to $pTZ^{\Delta \vee}R/T$ cloning vector (InsT/AcloneTM PCR product cloning kit, Fermentas), following the manufacturer's protocol.

First ligation reaction was prepared $" \rightarrow \mu l$ of solution, containing $\[Delta \mu l\]$ of pTZ $\[Delta VR/T\]$ plasmid, $\[Delta \mu l\]$ purified PCR products (SAG¹ gene), $\[Delta \mu l\]$ T[¢] DNA ligase, " $\[Delta ll\]$ X buffer, " $\[Delta ll\]$ PEG and " $\[Delta ll\]$ Nuclease free D.W. After vortex and spin, this mixture was incubated at '' °C for overnight, and then ligation reaction product was stored at $\[-"] ' \cdot °C$ until use.

Transformation and Screening. Preparation of competent cells from *Escherichia coli* TG¹ strain was performed by calcium chloride method (Sambrook *et al* 19Å9).

For transformation, $\gamma \cdot \mu l$ of ligation reaction product was added to $\gamma_{\bullet} \cdot \mu l$ competent cells, after vortex and spin the mixture was incubated at $\gamma_{\bullet} \circ C$ for $\gamma \cdot s$, and immediately was placed on ice for $\gamma - \gamma_{min}$. The transformed cells were allowed to recover in $\gamma \cdot \mu l$ LB broth medium free antibiotic by incubated at $\gamma_{\bullet} \circ C$ for $\gamma - \gamma$ hr with shaking. These recovered cells were plated onto LB agar plates containing ampicillin, IPTG (Fermentas) and X-Gal (Fermentas) to screening of blue and white colonies and incubated at ^{wv} °C for ¹/_h hr. Several white and blue colonies were randomly selected from each agar plate and inoculated in a LB medium containing ampicillin and incubated at ^{wv} °C for ¹/_h hr.

Confirmation of Cloning of SAG¹ gene into $pTZ^{\Delta V}R/T$ vector. The plasmid was purified from white and blue colonies of bacteria by Accuprep plasmid Extraction Kit (BioNEER), following the manufacturer's protocol. After plasmid extraction, following experiments were performed for improving cloning of SAG¹ gene into $pTZ^{\Delta V}R/T$ vector:

¹) Comparison of extracted plasmids on \cdot, h ? agarose gel. "µl of each plasmid extracted from white (pT-SAG¹) and blue (pTZ^Δ^VR/T) colonies bacteria were loaded on a \cdot, h ? agarose gel and were electrophoresis and photographed. Then, plasmid bands on agarose gel were compared.

*) PCR amplification of SAG' gene using by pT-SAG' plasmids. Plasmid DNA extracted form white colonies bacteria (pT-SAG') was used as a template to amplify the SAG' gene by PCR performed in Υ^{0} µl of solution under condition previously description in part Υ . The PCR product were analyzed by electrophoresis on a Υ' agarose gel and photographed. The size marker used to estimate PCR products were the $\Upsilon \mathcal{O}$ part here $\Upsilon \mathcal{O}$ has a solution of the size marker used to estimate PCR products were the $\Upsilon \mathcal{O}$ product were analyzed by DNA ladders (Fermentas).

^(v) Enzyme digestion of pT-SAG¹ plasmids. With regard to designed HindIII and EcoRI restriction enzymes sites respectively on forward and reverse primers and present them in recombinant plasmids extracted from white colonies bacteria (pT-SAG¹), these plasmids were digested by HindIII and EcoRI enzymes. For this propose each enzyme digestion reaction was performed in ^(v) µl of solution containing ^(v) µl plasmid, ^(v) µl restriction enzyme, ^(v) µl ^(v) × buffer and ^(v) µl D.W, after spin and vortex, this mixture was incubated in ^(v) °C for overnight. Because of being different of restriction enzyme buffers, each enzyme digestion was performed

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separately. Total of enzyme digestion products by EcoRI was loaded on a 1? agarose gel and the band resulting from digestion by EcoRI enzyme containing SAG1 fragment was extracted from agarose gel by DNA extraction Kit from agarose gel (Fermentas) and second enzyme digestion by HindIII was performed on it. Products from digestion by HindIII were loaded on a 1? agarose gel and the band resulting from digestions by two enzymes was analyzed by electrophoresis.

^{*}) Nucleotide sequencing of the SAG¹ cloned in pTZ^Δ^VR/T vector. The plasmids extracted from white colonies bacteria (pT-SAG¹) were sequenced by Faza Biotech Company.

RESULTS

DNA extraction. Fig.^Y shows that genomic DNA has been extracted by using lysis buffer and proteinase K followed by phenol /chloroform method.

PCR amplification. Fig.^r shows that DNA fragment PCR amplified was about ^{$9,\hat{r}$}·bp and similar to the *Toxoplasma gondii* SAG¹ gene size and no any genes was amplified exception with SAG¹ gene. Thus, these designed primers are specific for amplifying of SAG¹ gene. Results from electrophoresis of PCR products with extracted plasmids pT-SAG¹ using specific primers and remembered conditions showed that a ^{$9,\hat{r}$}·bp fragment of SAG¹ gene was amplified and this gene has been cloned into PTZ^{2,V}R/T plasmid (Figure \hat{r}).

Ligation of SAG¹ gene into pTZ^{Δ}^VR/T Cloning vector. According to the figure ¹, there were two patterns for ligation of SAG¹ gene into pTZ^{Δ}^VR/T cloning vector. In pattern ¹, introduced HindIII recognition site of SAG¹gene is near to EcoRI restriction site of pTZ^{Δ}^VR/T cloning vector but in pattern ⁴, introduced HindIII recognition site of SAG¹gene is far from EcoRI restriction site of pTZ^{Δ}^VR/T cloning vector.



Figure '. Ligation patterns for construction of recombinant pT-SAG' plasmid with $pTZ^{\Delta V}R/T$ (cloning vector) and SAG gene.



Figure '. Genomic DNA extraction from *Toxoplasma gondii* tachyzoites was performed by lysis buffer and phenol : chloroform method and electrophoresed in •, h? agarose gel.



Figure ". PCR amplification and gel electrophoresis of PCR product. *Lane), ightharpoondown, PCR*product (approximately <math>
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Comparison of extracted plasmids on \cdot, Λ ? agarose gel. Electrophoresis of extracted plasmids showed that both of plasmids (pTZ $^{\Delta V}$ R/T and pT-SAG¹) had three bands (linear, open circular and super-coil plasmids respectively from up to down) in which pT-SAG¹ bands placed above of pTZ $^{\Delta V}$ R/T bands on agarose gel (Figure ⁴).



Figure [•]. Comparison of extracted plasmids on ·, λ ?, agarose gel showed that both of plasmids (pTZ $^{\Delta}$ VR/T and pT-SAG¹) had three bands (open circular, linear and super-coil plasmids respectively from up to down) in which pT-SAG¹ bands placed above of pTZ $^{\Delta}$ VR/T bands on agarose gel : pTZ $^{\Delta}$ VR/T (*Lane 1*) and pT-SAG¹ (*Lane 7*).



Figure ⁴**A.** Agarose gel electrophoresis of Digestion of extracted pT-SAG¹ (according to ligation pattern ¹) after transformation; *Lane 1*: ¹Kbp DNA ladder; *Lane 7*: pT-SAG¹ has three bands (linear, open circular and super-coil plasmids respectively from up to down); *Lane 7*: pT-SAG¹ digested by EcoRI has two bands in that, one is less than ¹···bp (down) and the other is less than ¹···bp (up); *Lane 7*: Second digestion by HindIII on less than ¹···bp band (containing SAG¹); *Lane C*: ¹··bp DNA ladder.

Figure 4B. Agarose gel electrophoresis of Digestion of extracted pT-SAG¹ (according to Ligation pattern ^Y) after transformation; *Lane 1*: ¹Kbp DNA ladder; *Lane 7*: pT-SAG¹ has three bands (linear, open circular and super-coil plasmids respectively from up to down); *Lane 7*: pT-SAG¹ digested by EcoRI has one band (approximately less than ^Y · · · bp); *Lane 7*: Second digestion by HindIII on pT-SAG¹ digested by EcoRI, has two bands in that, one is ⁴⁷pb (down) and the other is less than ^Y · · · · bp (up); *Lane Q*: ¹ · · · bp DNA ladder.

Comparison of bands of extracted plasmids from white and blue colonies bacteria shows that bands of plasmids extracted from white colonies are heavier than plasmids extracted from blue colonies and thus, SAG¹ gene has been cloned into $pTZ^{\Delta V}R/T$ (Figure ^{*}).

Enzyme digestion. According to the ligation patterns (figure 1), when plasmids extracted from white colonies are digested by EcoRI restriction enzyme, digestion products may have two different electrophoresis patterns. Figure 2,1 showed that electrophoresis of digestion products obtained from the first digestion by EcoRI had two bands in that, one was less than *\...* bp and the other was less than $\forall \cdots$ bp. Figure Δ , \forall showed that when plasmids extracted from white colonies were digested by EcoRI restriction enzyme, one band (approximately less than $^{\flat}\cdots$ bp) was observed. In both above sets of enzyme digestion, electrophoresis of digestion products obtained from the second digestion by HindIII (that performed on digestion products obtained from the first digestion by EcoRI) showed a $97 \cdot$ bp band (Figure 2,1 and 2,7). Figure 2,7 showed that second digestion by HindIII on pT-SAG digested by EcoRI, had two bands in that, one was 97 bp (down) and the other was less than 7... bp (up). Results from enzyme digestion revealed that if the plasmid extracted from white colonies bacteria (pT-SAG¹) were digested with EcoRI and HindIII, a $97 \cdot$ bp band was cut and separated that this is SAG γ gene, and thus the SAG' gene has been cloned into $pTZ^{AV}R/T.$



Figure ⁷. Agarose gel electrophoresis of PCR amplification of SAG¹ gene products with pT-SAG¹ and Genomic DNA; *Lane 1*: ¹Kbp DNA ladder; *Lane 7*: PCR amplification of SAG¹ gene with pT-SAG¹; *Lane 7*: PCR amplification of SAG¹ gene with Genomic DNA; *Lane 7*: ¹. ¹.¹bp DNA ladder. **Nucleotide Sequencing.** Nucleotide sequence analysis of the SAG¹ cloned in pTZ^{Δ}^VR/T vector revealed ¹···[?]</sup> sequence identity with P-Br strain (GenBank Accession No. AY¹A^V^YV^A,¹), P strain (GenBank Accession No. S^{Δ}¹V^{Υ},¹), and C-strain (GenBank Accession No.S^{Υ}^{Υ}^{Υ},¹), Sequence analysis of the SAG¹ region revealed high homology of ⁹A[?] with RH strain (GenBank Accession No. AY^Y¹V^VA^{Υ},¹) and ZS¹ Strain (GenBank Accession No. S^V^{Υ}^{Υ}^{Υ},¹).

DISCUSSION

Genetic typing methods of *T. gondii* strains have been extensively perfected in recent years. From a technical point of view, many tools usable for genetic studied on single-copy loci have been used: RFLP, PCR-RFLP, sequencing, random amplified polymorphic DNA PCR (RAPD-PCR) and isoenzyme analysis. Most of these studies were performed on a small sampling of stocks and described the use of only one locus, mainly SAG^Y locus, for genetic typing (Ajzenberg *et al* ^Y··^Yb). We describe the cloning and sequence analysis of

the gene which encodes the major surface antigen (SAG¹ or P^{π}) of *T. gondii*. Results showed that the P^{π} · gene is a single copy, contains no introns and can extract it from genomic DNA of tachyzoite stage. Results also showed that SAG¹ gene is cloned in PTZ^{Δ}/R/T plasmid, forming pT-SAG¹ recombinant plasmid and *E. coli* TG¹ strain is the best host for pT-SAG¹ transformation.

Burg *et al* ($\uparrow\uparrow\uparrow\uparrow\uparrow$), Hunter *et al* ($\uparrow\uparrow\uparrow\uparrow\uparrow$) and Kimbita *et al* ($\uparrow\cdot\cdot\uparrow$) also showed that SAG¹ is stage specific, being detected only in the tachyzoite stage, but absent in the sporozoite and bradyzoite stages and this antigen is abundant and homogeneously distributed on the surface of both extracellular and intracellular tachyzoites. Kimbita *et al* ($\uparrow\cdot\cdot\uparrow$) and Biemans *et al* ($\uparrow\uparrow\uparrow\uparrow$) also resulted that the gene encoding SAG¹ occurs as a single copy, without introns. Results also showed that according to the ligation patterns (figure 1); digestion products may have two different electrophoresis patterns (figure 2,1 and 2,7) and different ligation patterns have no effect on cloning and sequencing.

Sequence analysis of SAG¹ gene cloned into $pTZ \Delta VR/T$ shows that the sequence has $\vee \cdot \cdot /$ identity with P-Br strain, P strain and C Strain and high homology of 41% with RH strain and ZSV strain. This result shows that SAG dimorphism and chromosomal localization are windows through which are population biology of T. gondii can be observed and is similar to reports of other researchers (Letscher-Bru et al Y ... ", Burg et al (9AA) about highly conserved of SAG¹ sequence in T. gondii strains and Lekutis et al $(\uparrow \cdots \uparrow)$ that believed that in addition to developmentally regulated differences in SAG expression, there is measurable allelic variation between the three prototypic strains of T. gondii. Interestingly, just to alleles were identify at the SAG¹ and SAG⁷A luci when Type I, II and III strains were compared. In fact, most SAGs are dimorphic.

Burg et al (19AA) showed that comparison of nearfull length cDNA to genomic DNA by sequence and restriction mapping (as well as full length protection of the Δ' end of P^{μ} . mRNA with genomic DNA) demonstrate that the P^{r} , gene contains no introns and northern blot analysis shows that the P^{π} mRNA is about 12... nucleotides in length and accumulates to very high levels and predicted size for P^{π} . primary translation product deduced from the cDNA sequence is $\forall \mathsf{P}, \mathsf{V}$ kDa also showed that there are two potential methionines for SAG'; although translational machinery most often utilizes the methionine codon it encounters, some data suggest that the second methionine codon of P^{τ} is used to initiate translation. Since a signal sequence of γ amino acids is unprecedented, this potential signal sequence cleavage site would also suggest the second methionine codon as the initiator of the primary translation product (with a signal peptide of r, amino acids). Therefore, the amplified qr, bp DNA segment in this work utilizes the second methionine codon of Pr, and it is used to initiate translation.

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