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Cloning of Major Surface Antigen (SAG1) Gene of *Toxoplasma gondii* Tachyzoites

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Toxoplasma gondii is an obligate intracellular protozoon parasite of all the warm blooded animals. Members of feline family are the definitive hosts whereas other warm blooded animals are the intermediate hosts. Serological assays employing whole cell antigens are widely used for diagnosis of toxoplasmosis. Availability of stage specific antigen increases the specificity of a test in accurate detection of infection. This study was aimed at cloning of tachyzoites stage specific SAG1 gene of *T. gondii* to make availability of specific *Toxoplasma* antigen for accurate detection of acute infection. *T. gondii* SAG1 gene was amplified from the tachyzoite DNA using the published set of primers and amplicon was cloned in the pPROEXHTc cloning cum expression vector after RE double digestion of insert and vector. The results showed that immunodominant SAG1gene of *Toxoplasma* tachyzoites was successfully cloned in pPROEXHTc expression vector which was confirmed by PCR and sequencing for the presence of insert in the recombinant plasmid and thus it is ready for further recombinant protein expression studies.

Introduction

Toxoplasma gondii is an obligate intracellular protozoon parasite of all the warm blooded animals including birds and humans. It completes a sexual part of its life cycle in the intestinal epithelial cell of the Felidae (definitive host) and an asexual part in warm blooded (intermediate host). Human is an accidental host who contracts infection through handling cat litter and gardening, ingestion of water and vegetation contaminated with cat faeces by consumption of raw or undercooked meat containing tissue cyst and transplacentally from mother to children (Miller *et al.,* 1972). Toxoplasmosis occurs in two forms: acute infection and chronic infection. In acute infection tachyzoites multiply rapidly, reaches different organs/tissues of body via blood and lymph and convert to a slow multiplying stage bradyzoite inside tissue cyst. Toxoplasmosis is mostly detected by serological assays employing whole cell antigen in both animals and human. Early accurate diagnosis of *Toxoplasma* infection in animals and human is very important to avoid the untoward consequences from the infection. Among serological tests, ELISA has a specific value and availability of stage specific parasite

antigen increases the specificity of test compared to the whole cell lysate antigens. Therefore, many researchers have proposed the usefulness of 30 kDa *Toxoplasma* tachyzoites surface protein (SAG1)which though accounts for only 3% to 5% of the total parasite proteins but has excellent antigenicity and immunogenicity suggesting its application for diagnosis of acute acquired toxoplasmosis (Kasper, 1987). The aim of this study was to clone major surface antigen (SAG1) gene of *T. gondii* tachyzoites into pPROEXHTc bacterial expression vector for better expression of a recombinant SAG1 protein for diagnosis of toxoplasmosis by application in ELISA.

Materials and Methods

Tachyzoites

As SAG1 gene of *T. gondii* is an intron free gene hence, cloning was attempted with genomic DNA. Genomic DNA was isolated from *Toxoplasma* tachyzoites harvested from the peritoneal cavity of infected mice.

PCR reaction

Cloning was attempted in bacterial system with a published primer pair (Meng *et al.,* 2012) with *SalI* and *Xba*I restriction sites at 5` end of forward and reverse primer respectively (SAG1-F 5'-GAGTCGACATGT CGGTTTCGCTGCACCAC-3' and SAG1-R5' GCTCTAGATCACGCGACACAAGCT GCGAT-3') using pPROEXHTc cloning cum expression vector. PCR reaction was carried out in a total volume of 50 µl using 2µg DNA, 10picomol each of forward and reverse primers, 1.5 mM MgCl2, 0.2 mMdNTP, 1X PCR buffer, 1.5 unit of *Taq* DNA polymerase (Fermantas) and nuclease free water for volume make up. Amplification was performed with initial denaturation at 94ºC for 5 min, with 35 cycles of final denaturation at 94 ^{0}C for 45 sec, annealing at 61ºC for 1 minand extension at 72ºC for 1 min. Final extension was done at 72ºC for 7min and holding reaction at 4ºC.

Electrophoresis

Amplified product was subjected to1% agarose gel electrophoresis. Amplicon band size of 1011bp was cut under UV trans-illuminator and was purified using gel extraction and PCR purification kit (Invitrogen Cat.NoK220001).

Gene cloning

Cloning was performed as per the standard protocol (Sambrookand Russell, 2001). Briefly, recovered product (insert) was double RE digested and pPROEXHTc cloning cum expression vector was linearized with *Sal* I and *Xba* I Fast Digest restriction enzymes (Fermantas) at 37^{0}C for 2 hours.

Digested products were gel purified after gel electrophoresis and ligated with T_4 DNA ligase enzyme at 22ºC overnight. The ligation reaction was confirmed by gel electrophoresis of 2 µl of ligation reaction. Remainder of the ligated product was used to transformDH5-α *E. coli* competent cell by heat shock at 42ºC for 45 sec. Transformed cells were plated onto the selective LB agar plates containing 100µg/ml ampicillin.

Ampicillin resistant bacterial colonies produced were confirmed by colony PCR for the presence of SAG1insert.Clone confirmed by PCR was ass cultured in LB ampicillin broth to harvest recombinant plasmid using plasmid DNA miniprep purification kit (HiMedia). Isolated recombinant plasmids were sequenced in both the directions for further confirmation of presence of SAG1insert and its amino acid sequence was compared with protein blast software in Gene Bank data base.

Results and Discussion

PCR successfully amplified SAG1 gene of 1011bp (Fig. 1). RE digestion of gene (insert) and pPROEXHTc expression vector by *Sal*I and *Xba*I restriction enzymes showed single linearized band for both insert and vector (Fig. 2). Upon gel purification, RE digested products yielded single desired bands without any non-specific bands (Fig. 3). Further, 1% gel electrophoresis of 2µl of ligated product confirmed the ligation with a band size heavier than the linearized vector size (> 4780 bp) (Fig. 4). Transformation of DH5 alpha *E. coli* cells, produced many tiny round clones on LB ampicillin agar plate (Fig. 5). Colony PCR of clones showed the desired band size of 1011bp (Fig. 6) and sequencing of recombinant plasmid revealed 97%-99%identity with other reference sequences for *T. gondii* SAG1 gene in the Gene Bank (Fig. 7).

There is a widespread distribution of *Toxoplasma* infection in a variety of warm blooded animals. Ingestion of environmentally robust stages (sporozoites in oocysts) or eating raw or undercooked meat or meat products containing tissue stages (tachyzoites or bradyzoites in tissue cysts), are the main transmission routes for *T. gondii* to humans (EFSA, 2007). Some authors assume that about 50% of all human toxoplasmosis cases are related to foodborne infection (Slifko *et al.,* 2000), and retrospective epidemiological analyses of human toxoplasmosis outbreaks suggest that many are associated with consumption of raw or undercooked meat or other edible parts of animals. *T.gondii* leads to dangerous manifestation in humans and economic loss in animal industry. This parasite is routinely detected by whole cell lysate based serological assays which lacks sensitivity and specificity, therefore, specific antigen is very essential in early accurate diagnosis of the infection. In this study for availability of tachyzoite stage specific antigen (P30), SAG1 gene of *Toxoplasma* tachyzoites surface was cloned into pPROEXHTc bacterial expression vector for diagnosis of *T. gondii* by ELISA. Previously cloning of SAG1 was attempted by many researchers for expression in bacterial host system using different vectors. Most of these vectors used the T7 promoter, which originates from bacteriophage T7 and the *E. coli lac* promoter promoters in *E. coli* as well as its modified form *lac*UV5. Depending upon the vector promoter wide variation in the level of expression was observed.

Fig.1 Amplification of SAG1 gene of *T. gondii* **lane 1:** 100bp ladder, **lane 2:** 1011bp amplified gene, **lane 3:** NTC

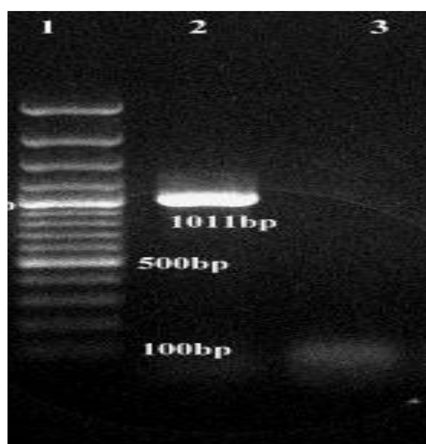

Fig.2 RE digestion of insert (SAG1) and linearization of pPROEXHTc expression vector **lane 1:** RE digested insert ; **lane 2:** RE digested linearized pPROExHTc vector; **lane 3:** undigested pPROExHTc vector; **lane 4:**100bp ladder

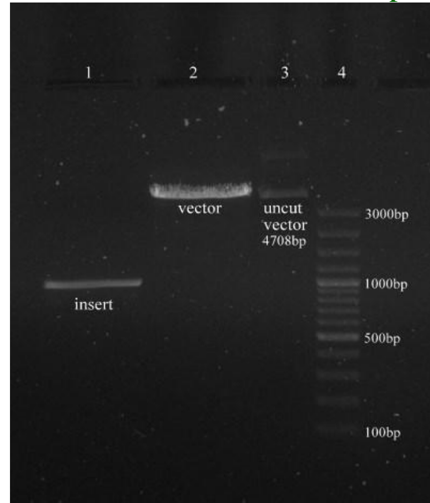

Fig.3 Gel purification of RE digested products **lane 1:** purified digested vector, **lane 2:** 100bp ladder, **lane 3:** 1011bp purified digested insert

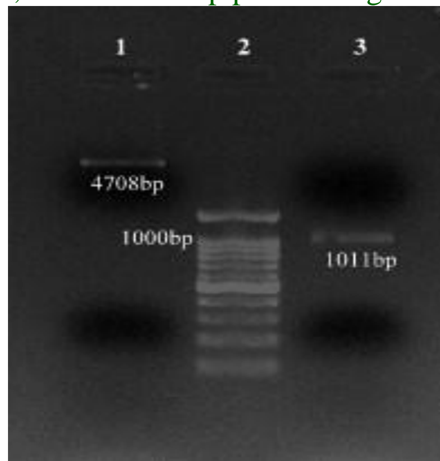

Fig.4 Confirmation of ligation by 1% gel electrophoresis **lane 1:** unligatedlinear pPROExHTc vector, **lane 2, 4 and 5:** ligated products, **lane 3**:100bp ladder

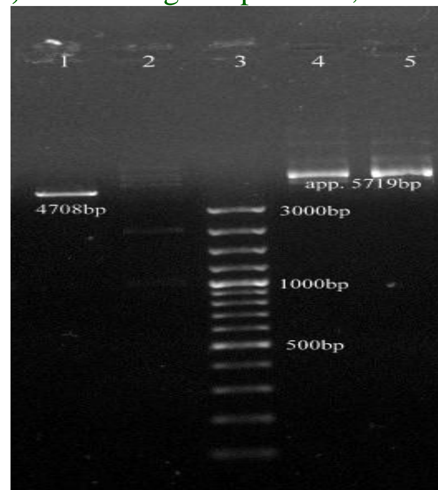

Fig.5 Clones on LB-ampicillin agar plate

Fig.6 Confirmation of recombinant clones by colony PCR **lane 1 to lane 7: P**ositive recombinant plasmids with 1011bp insert, **lane 8:** 100bp ladder

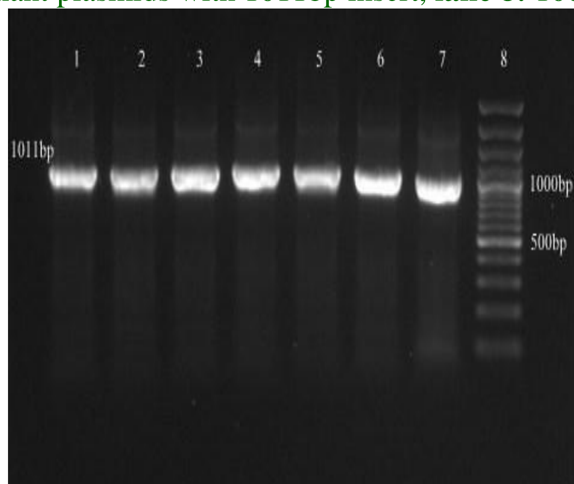

Fig.7 Amino acid sequence of SAG1(in this study) compared by amino acid sequence of SAG1 accession number ACT64638.1

```
Query  1    MSFLRCGVMASDPPLVANQVVTCPDKKSTAAVILTPTENHFTLKCPKTALTEPPTLAYSP  60
            MSFLRCGVMASDPPLVANQVVTCPDKKSTAAVILTPTENHFTLKCPKTALTEPPTLAYSP
Sbjct  38   MSFLRCGVMASDPPLVANQVVTCPDKKSTAAVILTPTENHFTLKCPKTALTEPPTLAYSP  97

Query  61   NRQICPAGTTSSCTSKAVTLSSLIPEAEDGWWTGDSASLDTAGIKLTVPIEKFPVTTQTF  120
            NRQICPAGTTSSCTSKAVTLSSLIPEAED WWTGDSASLDTAGIKLTVPIEKFPVTTQTF
Sbjct  98   NRQICPAGTTSSCTSKAVTLSSLIPEAEDSWWTGDSASLDTAGIKLTVPIEKFPVTTQTF  157

Query  121  VVGCIKGDDAQSCMVTVTVQARASSVVNNVARCSYGADSTLGPVKLSAEGPTTMTLVCGK  180
            VVGCIKGDDAQSCMVTVTVQARASSVVNNVARCSYGADSTLGPVKLSAEGPTTMTLVCGK
Sbjct  158  VVGCIKGDDAQSCMVTVTVQARASSVVNNVARCSYGADSTLGPVKLSAEGPTTMTLVCGK  217

Query  181  DGVKVPQDNNQYCSGTTLTGCNEKSFKDILPKLTENPWQGNASSDKGATLTIKKEAFPAE  240
            DGVKVPQDNNQYCSGTTLTGCNEKSFKDILPKLTENPWQGNASSDKGATLTIKKEAFPAE
Sbjct  218  DGVKVPQDNNQYCSGTTLTGCNEKSFKDILPKLTENPWQGNASSDKGATLTIKKEAFPAE  277

Query  241  SKSVIIGCTGGSPEKHHCTVKLEFAGAAGSAKSAAGTASHVSIL  284
            SKSVIIGCTGGSPEKHHCTVKLEFAGAAGSAKSAAGTASHVSI
Sbjct  278  SKSVIIGCTGGSPEKHHCTVKLEFAGAAGSAKSAAGTASHVSIF  321
```

In this study cloning was performed with an aim to clone a SAG1 insert into a pPROEXHTc expression vector with synthetic *trc* promoter for better expression of recombinant protein for further studies. Some researchers conducted the epidemiological studies with this antigen and others used this antigen for vaccination (Liu *et al.,* 2006; Caetano *et al.,* 2006; Siachoque *et al.,* 2006 and Mevelec *et al.,* 2005). Dubremetz *et al.,* (1985) demonstrated that P30 antigen express on the surface of intra and extra cellular tachyzoites hence, suggesting the role of SAG1 in detection of active infection. It was suggested that P30 is the most immunogenic constituent of tachyzoites and that a single region of this molecule contains most of the immunogenic activity (Rodriguez *et al.,* 1985).

Others used this antigen for diagnosis of congenital toxoplasmosis (Buffolano *et al.,* 2005). In conclusion the 30 kDa protein gene of *Toxoplasma* tachyzoites surface antigen (SAG1) was cloned in pPROEXHTc bacterial expression vector and is ready for further protein express studies for diagnosis of toxoplasmosis using recombinant ELISA.

Author's contribution

DGK PhD scholar conducted the work. JPSG guided and supervised the work.

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Conflict of interest

The authors declare that they have no competing interests.

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