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Molecular cloning and prokaryotic expression of truncated surface antigen protein (SAG1) of *Toxoplasma gondii*

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ABSTRACT

The WTO guidelines on control strategies, especially of food-borne diseases, insist on mandatory systematic serological investigations of the causative agent(s) at the farm level and in slaughtered animals for serodetection purposes. Amongst the several target molecules for sensitive detection of *Toxoplasma gondii*, surface antigens are considered important as these are always exposed to host's cellular immune response. The communication deals with the molecular cloning, prokaryotic expression and purification of SAG1, a surface antigen protein, from standard RH strain of *T. gondii*. Accordingly, the SAG1 protein (mature) was subsequently expressed in prokaryotic expression system. It had molecular size of ~47 kDa and the level of expression was measured as 42% of the total protein. The concentration of the mature recombinant SAG1 protein was 0.678 mg/ml. Western blot with Ni-NTA anti-histidine HRPase conjugate confirmed the presence and purity of protein by immunoreactivity at the unique ~47 kDa region.

Key words: Cloning, Expression, SAG1, *Toxoplasma gondii*

The economic impact of toxoplasmosis in countries like India is difficult to assess, as there is limited information available on the prevalence in various reared livestock species (Singh *et al.* 2014). Diagnosis of infection mainly relies on serological detection of *Toxoplasma* specific IgG and IgM molecules. For a high throughput sero-surveillance of toxoplasmosis, a purified recombinant protein based test is more relevant and is perhaps the need of the hour. The surface antigens of *T. gondii* are key molecules for immunodiagnosis as well as immunoprophylaxis because of their initial presentation to the host immune system. The present communication reports the primer-directed amplification of the open reading frame (ORF) of SAG1 gene of *T. gondii*, RH strain being maintained at IVRI as cryostock, its heterologous prokaryotic expression and subsequent characterization of the recombinant protein.

MATERIALS AND METHODS

In vivo propagation of T. gondii *tachyzoites in mice:* The animal experimentations were conducted in compliance with the ethical considerations and guidelines issued by CPCSEA and/or Institutional Animal Ethics Committee (IAEC) on laboratory animals. Tachyzoites of *T. gondii* RH strain (1ml vial) maintained as cryostock in the Protozoology Laboratory, Division of Parasitology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India were propagated in Swiss-albino mice and used in the study (Sudan *et al.* 2014b).

Isolation of host cell- free tachyzoites: The host cell-free tachyzoites were isolated following the method of Gross *et al.* (1991). Briefly, the peritoneal lavage containing tachyzoites, was collected aseptically on development of peritonitis following experimental infection and washed thrice in PBS (pH 7.4). The intracellular tachyzoites were released by mechanical rupture of the parasitized macrophages by passing through a 27G needle. The cell debris were pelleted by spinning the contents at 5000rpm for 10 min and the tachyzoites were isolated by passing the supernatant through a polycarbonate membrane filter (3µm pore size), pre-wetted with PBS (pH 7.4). Finally the purified tachyzoites were pelleted by centrifugation at 3000 rpm for 10 min and then re-suspended in 1 ml of PBS (pH 7.4).

Extraction of total RNA of T. gondii *and synthesis of complimentary DNA (cDNA) by reverse transcription:* Total RNA was extracted from the purified tachyzoites (1×10^7) using Trizol reagent following the manufacturer's recommendations. The RNA pellet was washed once with1 ml of 75% ethanol prepared using 0.01%

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diethylpyrocarbonate (DEPC) treated water, air-dried and preserved in 100µl of RNA storage buffer at –20°C until further use. The cDNA was synthesized from the total RNA using oligo dT primer following standard protocol (Sambrook *et al.* 1989) and the concentration of the synthesized cDNA was quantified using spectrophotometer. The freshly synthesized cDNA was used as template for PCR amplification of SAG1 sequence.

Bacterial strains and growth conditions: Escherichia coli BL21(DE3) pLysS cells were used as prokaryotic bacterial host for experiments pertaining to plasmid DNA manipulation. It was grown in either Luria Bertani (LB) broth or LB agar, supplemented with ampicillin (100 mg/ml) and chloramphenicol (32 mg/ml).

PCR amplification of SAG1 gene: For amplification of the SAG1 gene, the self designed PCR primers incorporating suitable restriction sites based on the information generated by molecular cloning and comparative sequence analysis of the entire open reading frame (ORF) of SAG1 gene of the parasite (Velmurugan *et al.*2008) were used. A truncated 958 bp SAG1 fragment was PCR amplified using the primer pair containing the forward primer 5/-GGTTGTATGTCGGTTTCGCTGCAC-3/ and a reverse primer 5/-CGTCAAGCTTCAGCCGAT TTTGCTGAC-3/. The reverse primer contained a *Hind III* restriction site to facilitate splicing of the amplified PCR fragment into the corresponding enzyme sites of the expression plasmid vector, pET-32b(+). The PCR conditions were optimized in 25 µl reaction volume using 30 ng cDNA; 20 pmol each forward and reverse primer; *Pfu* polymerase buffer containing 1.5 mM $MgCl_2$; 200 µM dNTP mix and 1U *Pfu* polymerase with the cycling conditions as initial denaturation at 94°C for 5min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min. Thereafter, 1 cycle of final elongation was given at 72°C for 10min. The amplification was confirmed by visualization of the specific 958 bp product in relation to a marker under UV light following electrophoresis on 1.25% agarose gel stained with ethidium bromide. Purification of the PCR product was done using commercially available gel extraction kit (Qiagen, Germany), following the manufacturer protocol.

Construction and transformation of recombinant plasmid: The purified DNA fragment (958 bp) was double digested with *Bam*HI and *Hin*dIII at 37ºC for 4 h and ligated to the corresponding cloning sites of pET-32b(+) vector (following standard protocol (Sambrook *et al.* 2000). The *E. coli* BL21(DE3) pLysS were transformed with recombinant plasmid and plated on LB agar containing ampicillin. Confirmation of the recombinant clones was done by colony PCR, as well as, restriction enzyme analysis for release of insert following standard protocols (Sambrook *et al.*2000).

Expression of recombinant plasmids: Five random positive recombinant clones were selected for induction. The colonies were grown overnight in 5 ml of LB broth containing ampicillin at 37°C in a shaking water bath. The overnight culture (100 microlitres) was further grown in 10 ml of fresh LB broth at 37ºC with constant shaking until mid-log phase (approx. 4 h when the OD reached to 0.6) and 1ml of the culture was collected from each tube and kept as an uninduced control. To the rest of the culture, isopropyl-ß-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1mM and incubated at 37ºC with constant shaking at 140 rpm. One milliliter of the induced culture was collected at hourly interval starting from 3 h onwards and the cells were pelleted by centrifugation at 13,800 × g in refrigerated centrifuge kept at 4°C.

Protein harvest and purification of recombinant His6–tagged fusion protein: Purification of expressed His-tagged thioredoxin fusion proteins was carried out using Ni-affinity chromatography following the manufacturer's protocol. In brief, cells from 1L of the induced culture were pelleted and the pellet was resuspended in 15 ml of lysis buffer containing 8M urea (pH 8.0). The cell suspension was kept at room temperature for 2 h on rotatory shaker with intermittent vortexing. Following lysis, the debris was pelleted by centrifugation for 10 min at 7,600 × g and the clear supernatant was transferred to a clean tube. Then 800 µl of Ni-NTA agarose slurry containing 15 mM imidazole and 20 mM ß-mercaptoethanol was mixed thoroughly with the supernatant and kept on a rotatory shaker for 1 h with intermittent mixing. The lysate-resin mixture was then loaded on to an empty 5 ml polypropylene equilibrated with 1X Tris-phosphate buffer (pH 8.0). The flow-through passed through the column was collected in the tube and the column was washed with 15 ml of wash buffer (pH 7.0) to which 5 mM imidazole (pH 7.0) was added and finally eluted as 500 µl fractions with 4 ml of elution buffer (pH 4.2–4.5). For renaturation, the His-tagged thioredoxin fused rSAG1 was diluted to a final concentration of 100 mg/ml in dilution buffer (pH 8.0) containing 50 mM sodium dihydrogen phosphate and 100 mM sodium chloride. The diluted sample was dialyzed first against buffer I (urea 0.5 M, Tris–HCl (pH 8.0) 20 mM, EDTA 1 mM) followed by buffer II (Tris–HCl (pH 8.3) 20 mM, EDTA 1 mM, reduced glutathione 2 mM, oxidized glutathione 0.2 mM) at 4°C for 24 h. About 30 ml of dialyzed sample was concentrated in a dialysis sac (10 kDa cutoff) using solid PEG–20,000 to one-third of its original volume. The resultant rSAG1 was designated as refolded rSAG1. Any debris formed during the renaturation was removed by centrifugation at 8200 x g for 10 min in refrigerated centrifuge. Concentration of the purified recombinant protein was assayed by modified Lowry protein assay kit (Pierce, USA) according to the manufacturer's protocol. The protein was aliquoted in 0.5 ml volume and stored at –20°C after addition of antiprotease mix till use.

SDS-PAGE and Western blotting: The purity and immunoreactivity, of the expressed protein was checked by SDS-PAGE using 12% gel under denaturing conditions at 90V for 2–3 h and then electro-transferred to nitrocellulose membrane following standard protocol. All the reagents required for performing SDS-PAGE and

Western blotting were procured commercially. The protein (500 ng) was probed for immunoreactivity using hyperimmune serum raised in rabbit and known reference positive and negative goat sera (kindly provided by Dr J P Dubey, USDA, Beltsville) at 1:100 dilution at 37°C for 1 h after overnight blocking of the nitrocellulose membrane with 3% non-fat milk powder at 4°C. Following stringent washing, with PBS-Tween 20 (PBS-T) the membrane was incubated with anti-rabbit and anti-goat HRPO conjugate at 1: 1000 dilution at 37°C for 1 h and then developed with diaminobenzidine tetrahydrochloride (DAB) solution.

RESULTS AND DISCUSSION

Observations on infectivity of cryopreserved T. gondii: Characteristic clinical signs of acute toxoplasmosis in rats as described by Sudan *et al.* (2014a, 2014b) were observed in infected mice. These symptoms appeared between days 5 to 7 post infection (PI) and included severe ascites, dullness and tachypnoea. Abundant tachyzoites, either free or within the macrophages were isolated from the peritonea fluid.

Directional cloning and expression of recombinant protein: The 958 bp SAG1 gene segment coding for the mature protein was PCR amplified (Fig. 1 (a)). The PCR product was purified and double digested with *Bam*HI and *Hin*dIII at 37°C for 4 h, further purified and eluted in 30 µl of elution buffer. Two hundred nanogram of the digested product was used for ligation. Overnight grown *E. coli* BL21(DE3) pLysS cells were transformed with recombinant pET-32b(+) plasmids and plated in presence of ampicillin and chloramphenicol. The white colonies were selectively isolated. Positive colonies were identified by colony PCR (Fig. 1 (b)) and restriction enzyme analysis. A high level expression of SAG1 protein was achieved after 8 h of induction with IPTG. The expression was confirmed by SDS-PAGE analysis and the recombinant SAG1 was resolved as a distinct band of ~47 kDa (Fig. 2 (a)). Level of expression of a histidine tagged recombinant fusion protein was measured as 42% of the total cell protein.

Purification and Western blot analysis of expressed histidine-tagged protein: One litre of bulk culture yielded nearly 5–6.5 g of cell pellet which was lysed in 15 ml of lysis buffer containing 8M urea. Affinity purification using Ni-NTA agarose slurry containing 15 mM imidazole and 20 mM ß-mercaptoethanol yielded histidine-tagged recombinant fusion protein. The recombinant protein was washed in buffer containing 5 mM imidazole. The protein was dialysed against decreasing concentration of urea for 3 to 4h and finally against PBS for 2–3 h at 4°C for refolding. SDS-PAGE analysis resolved the recombinant protein as a clear band corresponding to the molecular weight of ~47 kDa. The concentration of the recombinant protein was measured as 0.678 mg/ml by modified lowry protein assay kit. The specific reactivity and purity of the recombinant protein was checked by western blot analysis using hyperimmune as well as the known positive serum (Fig. 2 (b)).

Detection of the parasite in the intermediate hosts through parasitological techniques is difficult as none of the biological stages are demonstrable in the blood and/ or natural excretions or secretions. Hence, the diagnosis of toxoplasmosis mainly relies on serological methods (Pfrepper *et al.* 2005). ELISA is perhaps the most relied test for diagnosis of toxoplasmosis. Native protein based ELISA suffers from the limitations of specificity and hence the application of purified recombinant proteins is considered to be of utmost relevance for high throughput applications. Amongst the various proteins tested for their diagnostic capabilities, the cell surface proteins have gained the focus owing to the fact that these proteins are easily accessible to the host immune system and hence can act as

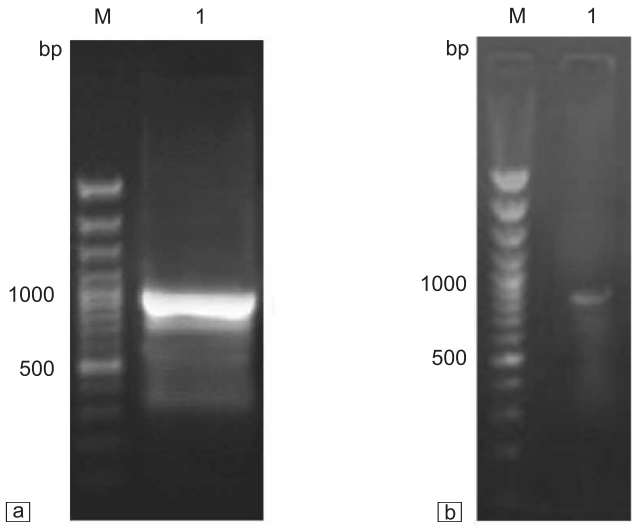

Fig 1(a-b). a PCR amplification of SAG1 gene of *T. gondii.* Lane M, marker 100 bp DNA ladder plus. Lane 1, desired amplicon. b. Colony PCR of transformed colonies of SAG1 gene of *T. gondii.* Lane M, marker 100 bp DNA ladder plus; lane 1: amplicon from colony PCR.

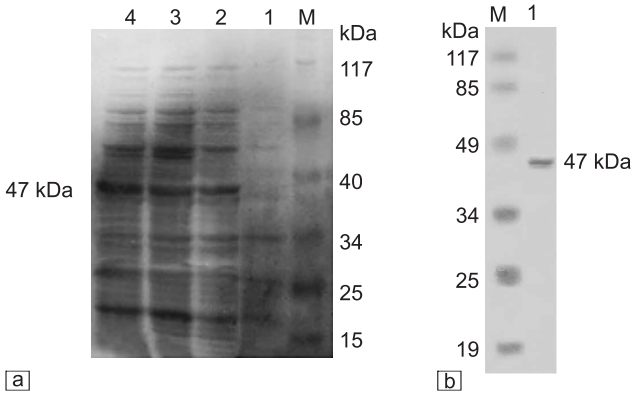

Fig. 2. a) SDS-PAGE analysis of expression of recombinant SAG1 protein. Lane M, molecular weight marker, pre-stained (Biomatik); lane 1, uninduced control;lane 2, 4h post induction; lane 3, 6h post induction; lane 4, 8h post induction. b) Western blot analysis of purified recombinant truncated SAG1 protein. Lane M, Molecular weight marker, pre-stained;lane 1, Strong reactivity with known positive sera.

immunogenic proteins in detection of infection induced antibodies (Sudan *et al.*2012, 2014c).

In the present study, the rSAG1 was expressed in the prokaryotic expression system as insoluble His-tag-thioredoxin fusion protein. Howewer, Sawicka *et al.* (2003) isolated soluble fractions of protein without thioredoxin fusion in much higher expression levels. But, the immunogenic nature of mature rSAG1 protein is questionable when it is denatured owing to its conformational nature thereby, inflicting its immunogenicity and the immunogenicity of this protein is entirely based on the correct folding (Chen *et al.* 2001). Renaturation of protein was therefore, needed to make it immunogenic. Mature SAG1 protein has 12 cysteine residues, which forms six disulfide bonds (Chen *et al.* 2001) and as is the case with disulfide-bonded proteins, inclusion body formation are more common, wherein the protein is present in the expression vector's cytosol and the reducing cellular compartment is not favourable for the formation of disulfide bonds subsequently, leading to aggregation of improperly folded protein (Lilie *et al.*1998). Thioredoxin is a small (11.7 kDa, 109 amino acids), inherently thermally stable protein localized on the cytoplasmic face of adhesion zones between the inner and outer cell membrane. These two properties viz., theromostability and its precise location can be exploited by retaining it as a fusion partner for rapid purification. So the choice of expression of SAG1 protein as insoluble His-tag-thioredoxin fusion protein was thereby felt to get an optimum level of conformation contributing to effective immunogence.

The present study has generated important data for expression of mature rSAG3 in heterologous host system. The co-expression of the His-tag of the vector along with the SAG1 sequence facilitated purification of the expressed protein to homogeneity. The addition of extra histidine residues as well as few residues from the vector has contributed to increased molecular size of the protein. The immuno-blot analysis using specific Ni-NTA HRP conjugate, however, confirmed the expression and efficient purification of the recombinant protein. A high level expression of SAG1 was achieved following 6 h of induction with 1mM IPTG. The level of expression was recorded as 18% of the total bacterial protein and visualized by SDS-PAGE as a 47 kDa polypeptide. The high level expression ensures its application in versatile high throughput diagnostic assays. Since SAG1 molecules are present on the surface membrane of tachyzoite stages it has the added advantage of more efficient diagnosis of acute and chronic infections. The subsequent renaturation of the protein to its conformational integrity further ensured the suitability for prophylactic applications. It would be interesting to further investigate the role of SAG1 molecule in the specific diagnosis and immune protection of the disease in different mammalian intermediate hosts including humans.

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