Indian Journal of Animal Sciences **84** (8): 824–828, August 2014/Article

Prokaryotic expression and molecular characterization of surface antigen 3 (SAG 3) protein of *Toxoplasma gondii*

VIKRANT SUDAN[1], ANUP KUMAR TEWARI[2], HARKIRAT SINGH[3] and B C SARAVANAN[4]

Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

Received: 15 October 2011; Accepted: 22 April 2014

ABSTRACT

Toxoplasmosis, caused by *Toxoplasma gondii*, is an important zoonotic disease with worldwide prevalence. In the present communication, we report molecular cloning, heterologous expression and characterization of 1158 bp complete open reading frame (ORF) of surface antigen 3 (SAG3) of *T. gondii*, RH strain, IVRI stock for the first time from India. The rSAG3 protein was expressed in *E. coli* as a 46kDa histidine-tagged fusion protein. Sequence analysis of the SAG3 coding sequence revealed 99.9% homology with the published sequence of *T. gondii* RH strain with substitution of a single base adenosine 'A' with guanosine 'G' at nucleotide position 397 in the ORF. Substitution of a single nucleotide resulted in substitution of aspartic acid (D) residue replacing asparagine (N).

Key words: Expression, Molecular characterization, RH Strain, SAG3, *Toxoplasma gondii*

Prevalence of *Toxoplasma gondii*, the causative agent of toxoplasmosis, is reported from all continents. The parasite multiplies virtually in any nucleated cell before encystment and causes disseminated tissue infection. The disease is of serious public health importance throughout the world and serological evidences suggested that about one-third of the total human population is at risk of infection (Montoya and Liesenfeld 2004). Abortion, stillbirth and neonatal complications are the common consequences of infection in small ruminants especially in sheep, goats and pigs, resulting in significant economic loss (Hill and Dubey 2002, Dubey 2004). Though the epidemiological information about the disease from India is scanty, *T. gondii* infection was reported from pig, sheep, goat, cat and chicken (Velmurugan *et al.* 2008).

Microscopical detection of the parasite in biological samples is difficult and therefore, serological tests are used for routine diagnosis of infection in the intermediate hosts. However, the native antigen based serological tests suffer from the limitations of sensitivity and specificity necessitating the need for identification of specific proteins of diagnostic relevance to development of high throughput diagnostic asaays. Surface antigen-3 (SAG3), a 43kDa glycosylphosphatidylinositol (GPI) anchored membrane bound glycoprotein, is present in both tachyzoite and bradyzoite stages of the parasite (Burg *et al*. 1988, Kazemi *et al.* 2007) and mediates the attachment of *T. gondii* to host cell-surface proteoglycans (Jacquet *et al*. 2001). SAG3 has structural similarity with surface antigen 1 (SAG1) (Cesbron-Delauw *et al.* 1994). Therefore, the protein is not only having the diagnostic potential, it has also immunoprophylactic relevance. The present communication reports cloning of the complete open reading frame (ORF) of SAG3 gene of *T. gondii*, RH strain being maintained at IVRI as cryostock, its heterologous prokaryotic expression and subsequent characterization of the recombinant protein.

Present address: [1]Assistant Professor (viks.sudan @gmail.com), Department of Veterinary Parasitology, College of Veterinary Sciences and Animal Husbandry DUVASU, [2]Principal Scientist (anupktewari@gmail.com), [4]Senior Scientist (drbcsaravanan @gmail.com), Division of Parasitology, IVRI. [3]Assistant Professor (drharkiratsingh@hotmail.com), Department of Veterinary Parasitology, GADVASU, Ludhiana.

MATERIALS AND METHODS

In vivo *propagation of* Toxoplasma gondii *tachyzoites in mice*

The *T. gondii,* RH strain tachyzoites ($1\times10^{2)}$ were inoculated in four adult Swiss albino mice of either sex, maintained on standard feed and water *ad libitum*, through intra peritoneal route . On development of peritonitis, the peritoneal exudate was aspirated aseptically from the euthanized mice. The contents were washed thrice in 5ml of sterile phosphate buffered saline (PBS, pH 7.2) and the number of live tachyzoites was counted. Specific permission for experimentation on mice was obtained from the Institute Animal Ethics Committee.

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 pellated 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 pellated 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 (10×10^6) using Trizol reagent following the manufacturer's recommendations. The RNA pellet was washed once with 1 ml of 75% ethanol prepared using 0.01% 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 SAG3 sequence.

Polymerase chain reaction (PCR) based amplification of SAG 3

The complete ORF of SAG 3 was PCR amplified using a pair of self designed primers [forward primer (TS3F) 52 -ATGCAGCTGTGGCGGCGCAG-32 and reverse (TS3R) 52 -TTAGGCAGCCACATGCACAAG-32]. The PCR reaction was carried out in 25µl reaction volume for 32 cycles with primer annealing temperature of 62°C for 75 sec. The amplification was confirmed by visualization of the specific 1158 bp product in 1.5% agarose gel stained with ethidium bromide.

Molecular cloning and characterization of SAG3

The PCR product was purified using Qiagen Mini elute gel extraction kit and ligated into InsTA clone PCR cloning vector. DH5α cells were transformed and the positive clones were identified by blue-white colony screening method. Further confirmation of the positive clones was done by restriction analysis using Pst I and *Eco*RI as well as by colony PCR following standard protocol (Sambrook *et al.* 1989). A subculture of positive clone was custom sequenced for nucleotides coding SAG3 from the Department of Biochemistry, Delhi University. The nucleotide sequence was analyzed by DNASTAR and GeneTool software.

Amplification of the mature SAG 3 gene of T. gondii *(RH strain) for directional cloning*

For directional cloning, a restriction site for *Hind III* was added to the reverse primer. The PCR amplification was achieved using purified SAG 3 PCR amplicon (1: 5 diluted) as template and *Pfu* polymerase. The thermal cycling conditions remaining the same as described in the previous section. The amplification was confirmed by agarose gel electrophoresis.

Prokaryotic expression of mature recombinant SAG3 protein (mSAG 3)

pPROExHT[a] expression vector was chosen for expression of rSAG3. To facilitate directional cloning, both the PCR product and the pPROExHT[a] expression vector were double digested with *Eco*RI and *Hin*d III restriction enzymes simultaneously in a 50 µl reaction mixture at 37°C for 4 h. The purified products were ligated at 4°C for 16 h. The competent *E. coli* DH5α cells were transformed with the recombinant pPROExHT[a] expression vector. Five positive colonies were grown in 5 ml of LB broth containing ampicillin (100µg/ml) at 37°C under constant shaking. The overnight grown culture was further grown in 10 milliliters of fresh LB broth at 37°C with constant shaking until mid-log phase and then induced with 1mM IPTG, at 37°C with constant shaking at 140 rpm for 6 h. One milliliter of the induced culture was collected at hourly interval starting from 3 h of induction onwards. The *E. coli* cultures collected at hourly intervals of induction, were pellated, lysed and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% gel) along with the uninduced controls under denaturating conditions at 90V for 2–3 h (Laemmli 1970). The gel was stained using Coomassie brilliant blue R-250.

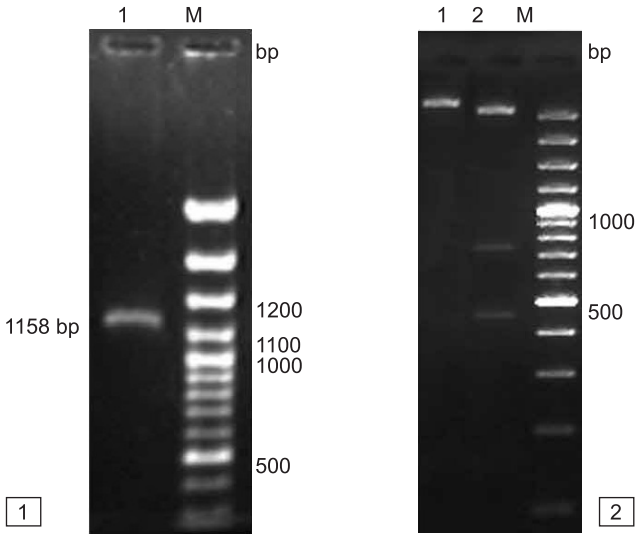
Purification of recombinant His6-tagged fusion protein

For bulk production of the protein, positive colonies were grown in 100 ml Luria Bertani broth in presence of ampicillin (100 µg/mL) with vigorous shaking at 37°C. The culture was induced with IPTG and incubated for 6 h for expression of proteins. Purification of expressed His-tagged fusion proteins was achieved using Ni-affinity chromatography following the manufacturer's protocol. The purity of the recombinant proteins was confirmed by SDS-PAGE analysis. The expressed His-tagged rSAG3 fusion protein was renatured by sequential dialysis following standard protocol and 30 ml of dialyzed sample was concentrated to one-third of its original volume in a dialysis sac (cutoff 10 kDa) using solid PEG-20,000.The identity of the purified rSAG3 was checked by western blot analysis following standard protocol using Ni-NTA anti-histidine HRPase conjugate (1: 1000 dilution) at 37°C for 1 h. The membrane was developed with diaminobenzidine (DAB) substrate.

RESULTS AND DISCUSSION

Observations on infectivity of cryopreserved T. gondii: Severe ascites, dullness and tachypnoea were characteristic clinical signs in the infected mice developed between days 5 to 7 post infection (PI). Abundant tachyzoites, either free or within the macrophages were isolated from the peritoneal fluid.

Amplification, molecular cloning and characterization of SAG3 gene: The complete ORF of SAG3 was amplified and resolved as a single band of 1158 bp size (Fig. 1). The selective identification of the positive colonies was made by restriction digestion analysis of the recombinant plasmids using *Pst* I and *Eco*RI (Fig. 2). The nucleotide sequence revealed 99.9% homology of cloned SAG3 sequence with that of published standard sequence (Fig. 3). A comparison of the nucleotide sequence with the published sequences of *T. gondii* strains revealed that RH strain used in the present study was more closely related to CEP strain (99.4%) than to P-Br strain (99.1%). A greater degree of non-similarity was observed in sequence comparison with the Prugniaud strain (97.6%) of *T. gondii*. The sequence information was submitted to GenBank (accession number HM585285). A phylogenetic tree of SAG 3 RH stain maintained at IVRI using bootstrap method revealed nucleotide and/ or amino acid homology with other referral stains (Fig. 4). The SAG3 gene sequence was rich in GC content as the guanine and cytosine (G+C) content was determined as 57.43% and the adenine and thymine (A+T) determined as 42.57%. Substitution of a single nucleotide



Figs. 1–2. Specific PCR amplification of ORF of SAG3 gene of *T. gondii*; Lane M: Marker 100 bp DNA ladder plus, Lane 1: Amplicon of 1158 bp from *T. gondii*, **2.** Release of SAG3 insert by restriction digestion of insTA cloning vector, Lane M: Marker 100 bp DNA ladder plus (MBI Fermentas), Lane 1: Undigested recombinant insTA cloning vector, Lane 2: Insert release after *PstI* and *Eco*RI digestion

Percent Identity

Divergence	1	2	3	4	5		
1		99.4	99.9	99.1	97.6	1	SAG 3 RH strain
2	0.6		99.3	98.5	97.4	2	SAG 3 CEP strain
3	0.1	0.7		99.1	97.5	3	SAG 3 IVRI
4	0.9	1.5	1.0		96.6	4	SAG 3 P-Br strain
5	2.4	2.5	2.5	3.3		5	SAG 3 Prugniaud strain
	1	2	3	4	5		

Fig 3. Sequence pair distances of SAG 3 RH Clustal V (Weighted).

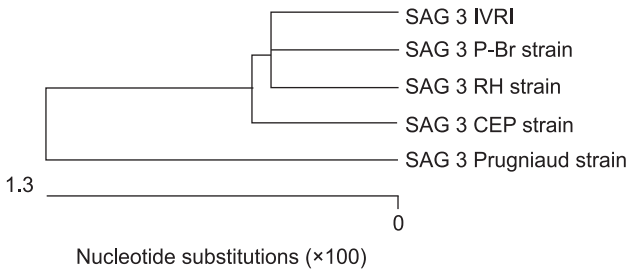


Fig. 4. Phylogenetic tree of nucleotide sequence of SAG 3 RH Clustal V (Weighted).

of G instead of A at 397th position of the SAG3 nucleotide sequence of RH strain maintained at IVRI was the reason for 99.9% sequence homology with the published sequence of RH strain. The substitution of a single nucleotide resulted in change in the deduced amino acid sequence at position 133 as aspartic acid (D) instead of asparagine (N).

Directional cloning of SAG3 gene coding for the mature protein for expression

The SAG3 gene sequence coding for the mature protein (1158 bp) was PCR amplified using the expression primers containing the restriction sites for *Eco*RI *and Hin*d III at 52 end. The amplicon was purified and was double digested with *Eco*RI *and Hin*d III at 37°C for 4 h. The purified digested product was used for ligation in the pPROExHT[a] expression vector after confirmation by the Colony PCR as well as RE analysis. Three-dimensional model of the computer simulated recombinant SAG3 had a single domain. The protein conformation of mSAG3 consisted of beta sheets with intermittent alpha chains. The template was monomer in nature**.**

Prokaryotic expression of mature recombinant SAG3 protein (mSAG3) in pPROExHT[a] expression vector

Purified SAG3 sequence was ligated to *Eco*RI *and Hin*d III double digested pPROExHT[a] expression vector overnight at 4°C and thereafter DH5α cells of *E. coli* were transformed and were grown in presence of ampicillin. A high level expression of mSAG3 was achieved following 6 h of induction of the culture with 1mM IPTG and the level of expression was recorded at 18% of the total bacterial protein and visualized by SDS-PAGE as a 46 kDa polypeptide (Fig. 5).

Purification of recombinant mature SAG3 protein

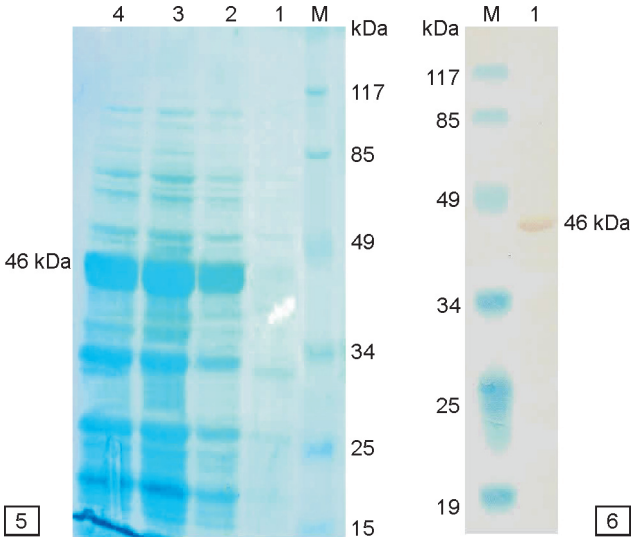
The protein was purified using Ni-NTA agarose beads, where the N-terminal histidine -tag attached to the recombinant protein facilitated competitive recovery at low pH. Nearly 6–7.5 g of pellet was obtained from centrifugation of 1 liter of culture which was further lysed to isolate the recombinant protein. The co-expression of the His-tag of the vector along with the mSAG3 sequence facilitated purification of the expressed protein to its homogeneity. Purification of the fusion protein was done under denaturing conditions using 8M urea to lyse and

recover the cytoplasmic contents into the lysis buffer supernatant. The purity of the fusion protein was checked by SDS-PAGE analysis. The protein was resolved at ~46 kDa. The addition of histidine residues may be the apparent reason for increasd molecular size of the protein. The refolding of the eluted protein was achieved by dialysis against decreasing molar concentrations of urea and finally in PBS (pH 7.4). Subsequently, the purified protein was dialysed against decreasing concentration of urea for proper refolding and checked by SDS-PAGE. The concentration of the recombinant mSAG3 protein was 112 µg/ml.

Western blot analysis of expressed histidine-tagged recombinant mature SAG3 protein

The specific reactivity of the recombinant mSAG3 protein was checked by western blotting. Ni-NTA anti-histidine HRPase conjugate when used for specific reactivity to the recombinant His-tagged protein, confirmed the presence and purity of histidine tagged protein with immunoreactivity at the unique ~46 kDa region specific for mature SAG3 on the nitrocellulose membrane (Fig. 6). The immuno-blot analysis using specific Ni-NTA HRP conjugate, that binds specifically with the histidine tagged protein confirmed that the recombinant protein was expressed and purified efficiently.

T. gondii is an intracellular parasite distributed worldwide with a potential to infect almost any vertebrate species. Though the parasite is apparently innocuous, the infection has devastating consequences in pregnant and immunocompromised individuals. SAG3 belongs to surface antigen gene (SAG) family of *T. gondii* and is evenly distributed on the cell membrane of tachyzoites as well as bradyzoites through a GPI-anchor. Therefore, SAG3 satisfies the criteria for a relevant diagnostic molecule for *T. gondii*. The protein plays an active role in invasion of the parasite to the host cell. Recent data show that SAG3 has affinity for sulfated proteoglycans and specific interaction of SAG3 with cellular proteoglycans mediates the adsorption of *T. gondii* to host cells (Jacquet *et al*. 2001). Targeted disruption of the GPI-anchored surface antigen SAG3 gene in *T. gondii* resulted in decreased host cell adhesion and virulence of the parasite for mice (Dzierszinski *et al*. 2000) and thereby rSAG3 conferred partial protection in mice which was mediated through Th1 type cellular immune response (Lee *et al.* 2007).



Figs 5–6. **5.** SDS page (12% gel) analysis of SAG3. Lane M: Prestained molecular weight marker; lane 1: 0 h; lane 2: 4 h; lane 3: 6 h; lane 4: 8 h. **6.** Western blot analysis of affinity purified recombinant SAG3 using anti-His antibody.Lane M: Prestained molecular weight marker; lane 1: blotted rSAG3 recombinant protein.

We achieved a high level of expression SAG3 gene in *E. coli* and the recombinant SAG3 protein was produced in abundant quantities to facilitate further studies on its diagnostic/prophylactic application. Total RNA was extracted from mouse amplified tachyzoites. The Trizol reagent, a monophasic solution of phenol and guanidine isothiocyanate, was used to isolate RNA. The coding sequence of SAG3 was amplified from a cDNA template synthesized from the toral RNA extracted from the tachyzoites. The 1158 bp coding sequence for SAG3 was PCR amplified using the expression primers containing the restriction sites for *Eco*RI *and Hin*d III at 52 end. The amplicon was purified and was double digested with *Eco*RI *and Hin*d III at 37°C for 4 h. The purified digested product was ligated in pPROExHTa expression vector after confirmation by the Colony PCR as well as RE analysis.

Though only single valid species of *T. gondii* exists world over, molecular genotyping studies revealed the existence of varied fundamental clonal populations of *T. gondii*. Despite of high genetic similarity marked differences in many phylogenetic characteristics have been reported in different strains of *T. gondii* (Dlugonska 2008). This explains the high virulence of the RH strain for mice, while the same does not hold true for the local isolates. The molecular diversity in the distinct and/ or related *Toxoplasma* stabilates is routinely evaluated by sequence based analysis amongst the different isolates. However, maximum difference at DNA sequence level among the predominant clonal lineages was found to be less than 2% (Grigg *et al*. 2001). The existence of clonal population structure in *T. gondii* (Ajzenberg *et al*. 2004) has been attributed to the transmission of the parasite through carnivorism bypassing sexual recombination events (Howe and Sibley 1995, Su *et al*. 2003), parthenogenetic formation of oocysts by many unfertilised macrogametes of the parasite in the small intestine of definitive host, *i.e.* cats (Ferguson 2002) and a probability of simultaneous infection with more than one strains of *T. gondii*. So far as the molecular conservation of the SAG3 sequence of *T. gondii*, RH strain, IVRI stock is concerned, it showed a more close relationship to CEP (99.4%) and P-Br (99.1%) strains. However a greater degree of non-similarity was observed while comparing the SAG3 nucleotide sequence with Prugniaud strain (97.6%) of *T. gondii*.

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 SAG3 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 SAG3 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 46 kDa polypeptide. The high level expression ensures its application in versatile high throughput diagnostic assays. Since SAG3 molecules are present on the surface membrane of both bradyzoite as well as tachyzoite stages, in sharp contrast to the SAG1 molecules which are present on the surface membrane of tachyzoites alone, it has the added advantage of more efficient diagnosis of acute and chronic infections over SAG1 molecules. The subsequent renaturation of the protein to its conformational integrity further ensured the suitability for prophylactic applications.

This is the first report on molecular cloning, sequence analysis and prokaryotic expression of SAG3 protein of *T. gondii* from standard RH stain from India. It would be interesting to further investigate the role of SAG3 molecule in the specific diagnosis and immune protection of the disease in different mammalian intermediate hosts including humans.

ACKNOWLEDGEMENTS

The authors acknowledge the Director, Indian Veterinary Research Institute, Izatnagar, India for providing necessary facilities.

REFERENCES

Ajzenberg D, Ban˜ulsb A L, Suc C, Dume'trea A, Demard M, Carmed B and Darde´a M L. 2004. Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. *International Journal of Parasitology* **34**: 1185.

Burg J L, Perelman D, Kasper L H, Ware P L and Boothroyd J C.1988. Molecular analysis for gene encoding the major surface antigens of *Toxoplasma gondii***.** *Journal of Immunology* **141**: 3584.

Cesbron-Delauw M F, Tomavo S, Beauchamps P, Fourmaux M P,Camus D, Capron A and Dubermetz J F. 1994. Similarities between the primary structures of two distinct major surface protein *of Toxoplasma gondii. Journal of Biological Chemistry* **269**: 16217.

D³ugoñska H. 2008. *Toxoplasma gondii*—known and unknown parasite. *Wiadomosci Parazytologiczne* **54**: 199–204.

Dubey J P. 2004.Toxoplasmosis- a waterborne zoonosis. *Veterinary Parasitology* **126**: 57.

Dubey J P. 1993. *Toxoplasma, Neospora, Sarcocystis*, and other tissue cyst forming coccidia of humans and animals. Kreier JP, editor.2nd ed. *Parasitic Protozoa* **6**: 1– 158.

Ferguson D J. 2002. *Toxoplasma gondii* and sex: essential or optional extra? *Trends in Parasitology* **18**: 355.

Fux B, Rodrigues C V, Portela R W, Silva N M, Su C, Sibley D, Vitor R W A and Gazzinelli R T. 2003. Role of cytokines and major histocompatibility complex restriction in mouse resistance to infection with a natural recombinant strain (Type I-III) of *Toxoplasma gondii*. *Infecion and Immunity* **71**: 6392.

Gautam O P, Chhabra M B, Bhardwaj R M, Gupta S L and Kalita C C.1979. Current status of toxoplasmosis research in India. *The First All India Seminar on Toxoplasmosis.* Department of Veterinary Medicine, HAU.

Grigg M E, Bonnefoy S, Hehl A B, Suzuki Y and Boothroyd J C. 2001. Success and virulence in *Toxoplasma* as the result of sexual recombination between two distinct ancestries. *Science* **294**: 161.

Gross U, Müller W A, Knapp S. and Heesemann J. 1991. Identification of a virulence-associated antigen of *Toxoplasma gondii* by a mouse monoclonal antibody. *Infection and Immunity* **59**: 4511.

Hill D and Dubey J P. 2002. *Toxoplasma gondii*: Transmission, diagnosis and prevention. *Clinical Microbiology and Infection* **8**: 634.

Howe D K and Sibley L D. 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *Journal of Infectious Diseases* **172**: 1561.

Jacquet A, Coulon L, Neve J D, Daminet V, Haumont M, Garcia L, Bollin A, Jurado M and Biemans R. 2001. The surface antigen SAG3 mediates the attachment of *Toxoplasma gondii* to cell-surface proteoglycans. *Molecular and Biochemical Parasitology* **116**: 35.

Kazemi B, Maghen L, Bandehpour M, Shahabi S and Haghighi. A. 2007. Gene cloning of P43 surface protein of *Toxoplasma gondii* tachyzoite and bradyzoite (SAG3). *Gene Therapy and Molecular Biology* **11**: 113.

Montoya J G and Liesenfeld O.2004. Toxoplasmosis. *Lancet* **363**: 1965.

Sambrook J, Fritch E F and Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold spring harbor Laboratory press.

Su C, Evans D, Cole R H, Kissinger J C, Ajioka J W and Sibley L D. 2003. Recent expansion of *Toxoplasma* through enhanced oral transmission. *Science* **299**: 414.

Velmurugan G V, Tewari A K, Rao J R, Baidya S, Udaya K M and Mishra A K. 2008. High level expression of SAG1 and GRA7 gene of *Toxoplasma gondii* (Izatnagar isolate) and their application in serodiagnosis of goat toxoplasmosis. *Veterinary Parasitology* **154**: 185–92.