

MOLECULAR CHARACTERIZATION AND SEQUENCE PHYLOGENETIC ANALYSIS OF SURFACE ANTIGEN 3 (SAG3) GENE OF LOCAL INDIAN ISOLATES (CHENNAI AND IZATNAGAR) OF *Toxoplasma gondii*

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SUMMARY

Context and objective: The molecular characterization of local isolates of *Toxoplasma gondii* is considered significant so as to assess the homologous variations between the different loci of various strains of parasites. **Design and setting:** The present communication deals with the molecular cloning and sequence analysis of the 1158 bp entire open reading frame (ORF) of surface antigen 3 (SAG3) of two Indian *T. gondii* isolates (Chennai and Izatnagar) being maintained as cryostock at the IVRI. **Method:** The surface antigen 3 (SAG3) of two local Indian isolates were cloned and sequenced before being compared with the available published sequences. **Results:** The sequence comparison analysis revealed 99.9% homology with the standard published RH strain sequence of *T. gondii*. The strains were also compared with other established published sequences and found to be most related to the P-Br strain and CEP strain (both 99.3%), and least with PRU strain (98.4%). However, the two Indian isolates had 100% homology between them. **Conclusion:** Finally, it was concluded that the Indian isolates were closer to the RH strain than to the P-Br strain (Brazilian strain), the CEP strain and the PRU strains (USA), with respect to nucleotide homology. The two Indian isolates used in the present study are known to vary between themselves, as far as homologies related to other genes are concerned, but they were found to be 100% homologous as far as SAG3 locus is concerned. This could be attributed to the fact that this SAG3 might be a conserved locus and thereby, further detailed studies are thereby warranted to exploit the use of this particular molecule in diagnostics and immunoprophylactics. The findings are important from the point of view of molecular phylogeny.

KEYWORDS: Indian isolates; Molecular characterization; SAG3; *Toxoplasma gondii*.

INTRODUCTION

Toxoplasma gondii, an obligate intracellular coccidian parasite, has acquired utmost zoonotic relevance in the current scenario around the globe, accounting for abortions, stillbirths, and neonatal complications in livestock, especially in sheep, goats and pigs^{9,16,30}. The condition leads to life-threatening consequences both in immunocompromised human patients suffering from acquired immune deficiency syndrome (AIDS) and those with organ transplants². In India, the condition has exhibited itself as acquired ocular toxoplasmosis⁴, in immunocompetent patients, bringing about possible similarities with South American strains which are known to exhibit a high rate of ocular involvement²⁰. A third of the world's total population is thought to be at risk of infection²². Of late, different strains of *Toxoplasma gondii* are known to induce different cytokine responses⁵ and thereby vary in their pathogenesis. The surface antigens of *T. gondii* are the major targets as key molecules for immunodiagnosis as well as immunoprophylaxis because of their initial presentation to the host immune system. Surface antigen 3 (SAG3), an under-reported 43kDa glycoaminoglycan-binding protein associated with

binding of host heparin sulfate proteoglycans (HSPGs)¹⁸, shares primary structure similarity with another proven Surface antigen 1 (SAG1)⁷ protein. It was considered interesting to carry out the primer-directed amplification of the open reading frame (ORF) of surface antigen 3 (SAG3) gene of Indian isolates of *T. gondii* viz. Chennai (CHEN) and Izatnagar (IZN) isolates, maintaining them at the IVRI and cloning them in a heterologous prokaryotic system. Moreover, the two Indian isolates used in the present study are known to vary between themselves as far as homologies related to other gene loci like GRA 5²⁶, MIC 3²³ and SAG 2²⁷ are concerned, but there is no literature available as far as SAG3 homologies are concerned. In the present study, the cloned genes were custom sequenced and the information was compared with the available sequences of the same gene in the GenBank in order to establish the phylogenetic identity of the SAG3 gene among the various isolates.

METHODS

Propagation of *T. gondii* tachyzoites: Inbred Swiss albino adult mice, maintained on standard feed (pellets) and water *ad libitum*, were

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intraperitoneally infected with 100 mouse-adapted Chennai and Izatnagar *T. gondii* tachyzoite isolates that were cryopreserved and maintained at a divisional laboratory, IVRI. These two Indian isolates were originally isolated from the tested-positive blood, heart and brain tissues of free-range chickens (*Gallus domesticus*) naturally infected by *T. gondii*²³ and isolated after Cat inoculation assays. The infected mice were monitored daily for the development of signs of infection. Infected mice exhibiting peritonitis were euthanized and peritoneal lavage was aspirated following inoculation of 5 mL of sterile phosphate buffered saline (PBS, pH 7.2) in the peritoneal cavity with due care in avoiding injury to visceral organs. The contents were washed thrice with PBS (pH 7.2) and the live tachyzoites were counted.

Separation of host cell-free tachyzoites: The host cell-free tachyzoites were separated using standard protocol¹⁵. Briefly, the peritoneal fluid containing free tachyzoites and tachyzoite infected macrophages was collected in PBS (pH 7.4) and washed thrice in PBS (pH 7.4) while repeatedly centrifuging at 5000 rpm for 10 min. Following this, a final pellet was re-suspended in 5 mL of PBS (pH 7.4). The intracellular tachyzoites were separated and made free from the macrophages by passing the contents repeatedly through a 27g needle fitted in a 10 mL sterile syringe. The host cell-free tachyzoite suspension was washed with 20 mL of PBS (pH 7.4), debris was allowed to settle down in the centrifuge tube for 10 min and the supernatant was collected and, following this, passed through a pre-wetted (with PBS pH 7.4) polycarbonate membrane filter of 3 µm pore size slowly (at the rate of one mL per 2-3 min). The filtrate was centrifuged (3000 rpm for 10 min) and the tachyzoites in sediment were re-suspended in one mL of PBS (pH 7.4).

Isolation of total RNA of *T. gondii*: Total RNA was extracted directly from the purified tachyzoites using Trizol[®] reagent (Gibco BRL) while following the manufacturer's protocol. Briefly, one mL of Trizol was added to the suspension containing 5-10x10⁶ tachyzoites, repeatedly pipetted to kill the tachyzoites and following this, incubated at 30 °C for five min to dissociate nucleoprotein complexes. The suspension was vigorously shaken for 15 sec after adding 0.2 mL of chloroform and then centrifuged at 12,000g for 15 min at 4 °C. This facilitates the separation into lower organic phase and upper aqueous phase. The aqueous phase was transferred to a fresh tube, 0.5mL of the isopropyl alcohol was poured into the tube and the RNA was allowed to precipitate while keeping the tube at 15-30 °C for 10 min. The tube was centrifuged at 12,000g for 10 min at 4 °C. The RNA pellet was washed once with one mL of 75% ethanol prepared using 0.01% of diethylpyrocarbonate (DEPC) treated water. The sample was mixed by vortexing and centrifuged at 7,500 x g for five min at 4 °C. The RNA pellet was air-dried, reconstituted in 100 µL of RNA storage buffer (Ambion) and stored at -20 °C until further use. Purity and concentration of total RNA was checked by ethidium bromide stained agarose gel electrophoresis, performed at 2-3 volts/cm².

Synthesis of complimentary DNA (cDNA) by reverse transcription: cDNA was synthesized from the total RNA isolated from the *T. gondii* tachyzoites of both the isolates, using oligo dT primer while following the standard protocol²³. The cDNA, thus synthesized, was quantified using a spectrophotometer (Nanodrop[®], USA).

Polymerase chain reaction-based (PCR) amplification of the SAG3 gene of Indian isolates: The entire open reading frame (ORF) of

the SAG3 gene of *T. gondii* (CHN and IZN isolates) was PCR amplified using a pair of specific primers as described by SUDAN *et al.* 2012²⁸ (forward primer (TS3F) 5'-ATGCAGCTGTGGCGGCGCAG-3' and reverse (TS3R) 5'-TTAGGCAGCCACATGCACAAG-3'). The PCR reactions were carried out in a standard 25 µL reaction volume with initial denaturation of DNA strands at 95 °C for five min followed by 32 cycles of denaturation at 95 °C for 50 sec, primer annealing at 62 °C for 75 sec and strand elongation at 72 °C for 50 sec. Thereafter one cycle of final extension of the strands was carried out at 72 °C for 12 min. The PCR amplifications were confirmed by visualization of the product on 1.5% agarose gel stained with ethidium bromide following electrophoresis.

Molecular cloning and characterization of the SAG3 gene of Indian isolates: The amplified ORF of the SAG3 genes of Indian isolates of *T. gondii* were purified using a Qiagen Mini elute gel extraction kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's protocol. Following this, competent *Escherichia coli* DH5 cells were prepared following the standard calcium chloride treatment method²³. Ligation reaction for the cloning of SAG3 (amplified from *T. gondii* Indian isolates) into InsTAclone PCR cloning vector (Qiagen, Germany) as well as transformation of DH5 cells was carried out as per the company's protocol. The positive clones were identified by blue-white colony screening method. Further confirmation was carried out by restriction analysis of the plasmid DNA isolated from the white colonies with *PstI* and *EcoRI* as well as by colony PCR following standard protocol²⁴. The restriction digestion reaction was carried out at 37 °C for four h. The digested product as well, as the colony PCR amplified products, was visualized in the ethidium bromide-stained agarose gel following electrophoresis. The subcultures of a positive clone harboring the desired SAG3 genes of both the Indian isolates were custom DNA sequenced from the Department of Biochemistry, Delhi University.

Data analysis: The sequence information received was analyzed using DNASTAR and GeneTool software. The sequences, hence received sequence submitted to GenBank (Accession No.: HQ291783 & HQ291784 for Chennai and Izatnagar isolates, respectively). Moreover, these two sequences were compared with an earlier sequenced RH strain sequence (Accession No.: FJ825705) from the department along with other published sequences *viz.*, CEP (Accession No.: AF340229); P-Br (Accession No.: AY187280) and PRU (Accession No.: AF340228) from across the world through the GenBank using online Nucleotide BLAST Softwares (<http://blast.ncbi.nlm.nih.gov/>).

RESULTS

Viability of cryopreserved *T. gondii*: All the infected mice started showing characteristic signs of the disease from Day-7 Post Infection (PI). The clinical signs included raised & rough fur coat, pendulous abdomen, severe ascites, dullness, tachypnoea marked by resting on either the walls of the cages, on the nozzle of water bottle or on other resting mice with their forelegs. Microscopically, a large number of tachyzoites were detectable (either free or within the peritoneal macrophages suspended in the aspirated peritoneal fluid).

PCR amplification, molecular cloning and molecular characterization of the SAG3 gene of Indian isolates: The whole ORF of the SAG3 gene was amplified from the cDNA of Indian isolates of *T. gondii* using the specific forward and reverse primers. The amplicons

were resolved as a single band of 1158 bp (Fig. 1). It was further purified for ligation in InsTAclone PCR cloning vector. The selection of positive colonies was performed by colony PCR using the specific primers and also by restriction enzyme digestion of the recombinant plasmids with *PstI* and *EcoRI* for the release of insert. The results of restriction enzyme digestion (Fig. 2) as well as colony PCR (Fig. 3) were checked by agarose gel electrophoresis.

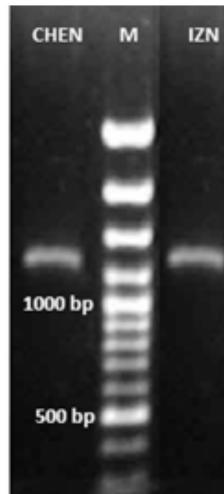


Fig. 1 - Specific PCR amplification of ORF of SAG3 gene of Indian isolates of *T. gondii* on 1.5% agarose gel. Lane CHEN: Amplicon of 1158 bp from *T. gondii* Chennai isolate; Lane M: Marker 100 bp DNA ladder plus; Lane IZN: Amplicon of 1158 bp from *T. gondii* Izatnagar isolate.

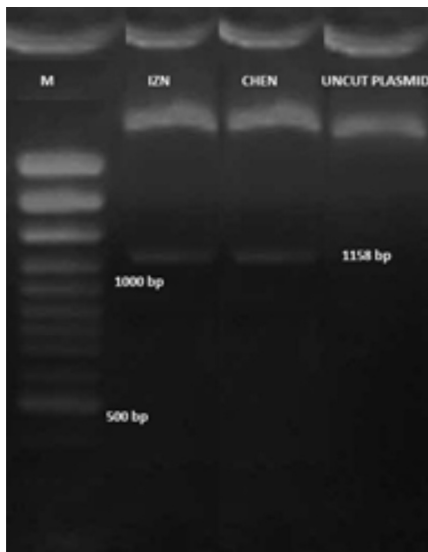


Fig. 2 - Release of SAG3 insert by restriction digestion of insTA cloning vector of the two Indian isolates on 1.5% agarose gel. Lane M: Marker 100 bp DNA ladder plus (MBI Fermentas); Lane IZN: Insert release after *PstI* and *EcoRI* digestion of vector containing Izatnagar isolate; Lane CHEN: Insert release after *PstI* and *EcoRI* digestion of vector containing Chennai isolate; Lane Uncut Plasmid: Undigested recombinant insTA cloning vector.

Data analysis: The nucleotide sequence revealed 99.9% (Fig.4) sequence homology of SAG3 ORF between the Indian isolates with

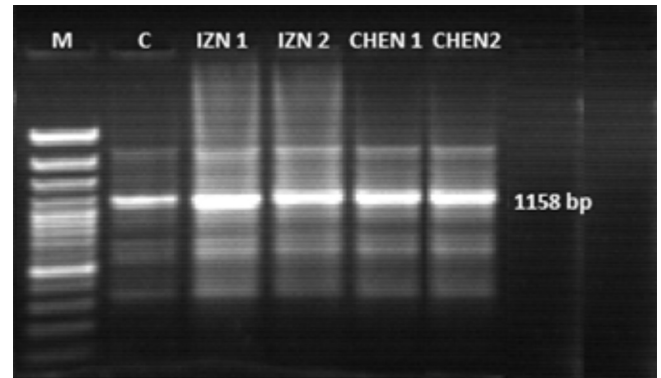


Fig. 3 - Colony PCR confirming the amplifications of 1158 bp specific SAG3 amplicons of Indian isolates on 1.5% agarose gel. Lane M: Marker 100 bp DNA ladder plus; Lane C: Positive control DNA of *T. gondii*; Lane IZN 1,2: Amplicon of 1158 bp from *T. gondii* Izatnagar isolate; Lane CHEN 1,2: Amplicon of 1158 bp from *T. gondii* Chennai isolate.

that of the earlier sequenced RH strain sequence. A comparison of the nucleotide sequence of *T. gondii* Indian isolates revealed 100% homology between the Chennai and the Izatnagar isolates. Furthermore, there is a 99.3% identity with P-Br and the CEP SAG3 sequence and 98.4% with PRU. A phylogenetic association, for analyzing the identity between strains and testing the robustness of the association, was done using the online bootstrap method (<http://blast.ncbi.nlm.nih.gov/>) to delineate its relationship with other referral stains (Fig. 5).

		Percent Identity						
		1	2	3	4	5	6	
Divergence	1	■	99.4	99.9	99.9	99.4	97.7	1
	2	0.6	■	99.3	99.3	98.8	97.6	2
	3	0.1	0.7	■	100.0	99.3	97.6	3
	4	0.1	0.7	0.0	■	99.3	97.6	4
	5	0.9	1.5	1.0	1.0	■	97.0	5
	6	2.4	2.5	2.5	2.5	3.3	■	6
		1	2	3	4	5	6	

Fig. 4 - Sequence pair distances of SAG3 Clustal V (weighted).

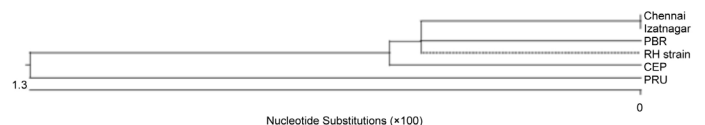


Fig. 5 - Phylogenetic tree of nucleotide sequence of SAG3 Clustal V (weighted).

The Adenine and Thymine (A+T) content of the SAG3 gene of both the Indian isolates was found to be 42.57%, whereas the Guanine and Cytosine (G+C) content was 57.43%. The nucleotide homology was found to be 99.9% with the earlier sequenced RH strain. There was a substitution of a single nucleotide of A instead of G at the 397th position of the SAG3 nucleotide sequence of both the Indian isolates. The nucleotide substitution resulted in the change of a single nucleotide residue in the deduced amino acid sequence at the 133rd position as asparagine (N) instead of aspartic acid (D). As a whole, Indian isolates were closer to the RH strain than to the P-Br strain (Brazilian strain) and CEP strain and PRU strains (USA), with respect to the nucleotide homology.

DISCUSSION

The significance of toxoplasmosis has increased particularly in immune compromised and/or HIV/AIDS patients, with an alarming prevalence in developing countries such as India. The presence of brain cysts is often associated with various psychiatric disorders and behavioral alterations²⁹ such as schizophrenia^{8,32} alongside other brain pathologies and ocular involvements²⁵ in both immunocompromised and immunocompetent individuals¹¹. In order to precisely define the magnitude of the disease, it was of interest to investigate the genetic diversity of the pathogen among the *T. gondii* strains using advanced biotechnological approaches.

Surface antigen 3 (SAG3), a 43kDa glycoprotein, is a glycosylphosphatidylinositol-anchored (GPI) membrane-bound protein in the developmental stages of the pathogen (tachyzoites & bradyzoites) parasite^{6,19}. The protein was earlier identified as P₄₃. It was cloned and sequenced for the first time by CESBRON-DELAUW *et al.* in 1994⁷ followed by FUX *et al.* in 2003¹³. SAG3 has primary structure similarity with Surface antigen 1 (SAG1)⁷. SAG3 is a glycoaminoglycan-binding protein associated with binding of host heparin sulfate proteoglycans (HSPGs)¹⁸. The SAG3-HSPGs interaction facilitates the parasite's attachment to target cells. Furthermore, it has been shown that 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 mice¹⁰. In immunoprophylactic application, rSAG3 conferred partial protection in mice, which was mediated through Th1 type immune response²¹. However, molecular characterization of the SAG3 gene of *T. gondii* of Indian isolates has not been attempted so far. The present study reports the molecular characterization of the surface antigen 3 (SAG3) gene of *T. gondii* of Indian isolates and ascertains its molecular homology with some other strains of the same parasites that are prevalent across the globe.

Worldwide, only one valid species of *Toxoplasma* exists. However, based on molecular genotyping studies, varied fundamental clonal population isolates of *T. gondii* have been recognized. The molecular diversity in the distinct and/or related *Toxoplasma* stabilates is routinely evaluated by sequence-based analysis among the different isolates. Recently, different strains of *Toxoplasma gondii* have been known to induce varying levels of cytokine responses⁵ and thereby vary in their pathogenesis, hence the study of the phylogeny has gained ultra importance owing to the variation in pathogenesis at the strain levels. Moreover, the two Indian isolates used in the present study are known to vary between themselves as far as homologies related to other gene loci like GRA 5²⁶, MIC 3²³ and SAG 2²⁷ are concerned but they were found to be 100% homologous as far as SAG3 locus is concerned. This could be attributed to the fact that this SAG3 might be a conserved locus and thereby, further detailed studies are thereby warranted to exploit the use of this particular molecule in diagnostics and immunoprophylactics.

Interestingly, differences at the lineages sequence level of DNA among the predominant clones are less than 2%¹⁴. Transmission of the parasite through carnivorous and scavenging among intermediate hosts, bypassing sexual recombination events in the definitive host, *i.e.* cats^{17,30}, parthenogenetic formation of oocysts by many unfertilized macrogametes of the parasite in the small intestine of cats¹², and simultaneous infection with different strains of *T. gondii* are some plausible reasons that can explain the existence of the clonal population structure in *T. gondii*³.

CONCLUSION

In the present study, the SAG3 gene of *T. gondii* was cloned, sequenced and aligned, before being compared with various published strains and the homologies between the two Indian isolates were found both with one another and with other strains across the globe. The two Indian isolates used in the present study are known to vary between themselves as far as homologies related to other genes are concerned but they were found to be 100% homologous as far as SAG3 locus is concerned. This could be attributed to the fact that this SAG3 might be a conserved locus and therefore, further detailed studies are thereby warranted to exploit the use of this particular molecule in diagnostics and immunoprophylactics. The findings are important from the point of view of molecular phylogeny.

RESUMO

Caracterização molecular e análise filogenética de seqüências do antígeno de superfície 3 (SAG3) em isolados indianos (CHENNAI E IZATNAGAR) de *Toxoplasma gondii*

Contexto e objetivo. A caracterização molecular de isolados indianos de *Toxoplasma gondii* é importante para a investigação de variações genéticas existentes entre cepas do parasito em diferentes locos gênicos. **Delineamento e disposição.** A presente comunicação realizou a clonagem e o sequenciamento dos 1158 pares de base correspondendo à totalidade do quadro de leitura do antígeno de superfície 3 (SAG3) de *Toxoplasma gondii* em dois isolados indianos (Chennai e Izatnagar) mantidos em um biorrepositório localizado em IVRI. **Método.** As seqüências do SAG3 dos dois isolados indianos foram clonadas, sequenciadas e posteriormente comparadas com seqüências SAG3 de *Toxoplasma gondii* disponíveis em publicações. **Resultados.** A comparação das seqüências revelou 99,9% de homologia com a cepa RH padrão; 99,3% de homologia com as cepas P-Br e CEP; e 98,4% de homologia com a cepa PRU. Os dois isolados indianos eram 100% idênticos no que diz respeito à seqüência SAG3. **Conclusão.** Concluiu-se que os isolados indianos são filogeneticamente mais próximos da cepa RH em relação à cepa brasileira P-Br, ou às cepas CEP e PRU (USA). No entanto, a análise de outros genes de *Toxoplasma gondii* destes dois isolados indianos mostrou diferenças na composição de nucleotídeos, ao contrário do que foi encontrado para o locus SAG3. Estes resultados poderiam ser atribuídos ao fato do locus SAG3 ser altamente conservado, necessitando de estudos adicionais para determinar se SAG3 poderia ser utilizado no diagnóstico da toxoplasmose. No entanto, estes resultados são importantes do ponto de vista da filogenia molecular.

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