Molecular characterization of surface antigen 3 (SAG3) gene of *Toxoplasma gondii* RH-IVRI strain

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Abstract Toxoplasmosis, caused by *Toxoplasma gondii*, is a zoonotic disease and is responsible for abortions, still-birth, and neonatal complications in livestock, especially in sheep, goats, and pigs. The molecular characterization of the parasite having global distribution is considered important to delineate the phylogenetic relationship among different isolates/strains of the parasite. The present communication deals with the molecular cloning and sequence analysis of the 1,158 bp entire open reading frame of surface antigen 3 (SAG3) of *T. gondii* RH strain being maintained at the Indian Veterinary Research Institute (IVRI). The sequence comparison analysis revealed 99.9 % homology with the published sequence of *T. gondii* RH strain, with a single substitution of guanine ‘G’ instead of adenine ‘A’ at the 397th position of SAG3 sequence. The substitution of single

nucleotide consequently resulted in the change of one amino acid residue of aspartic acid (D) instead of asparagine (N) present in the published sequence of RH strain. This denotes that the SAG3 gene of this RH strain has not undergone a major change in its molecular conformation even after repeated passage in mice for more than a decade at IVRI. The finding is important from the molecular phylogeny point of view.

Keywords Molecular characterization · Surface antigen 3 (SAG3) gene · *Toxoplasma gondii* · RH strain

Introduction

Toxoplasmosis, caused by heteroxenous coccidian parasite *Toxoplasma gondii*, is prevalent in a wide range of mammalian and avian hosts. Felines serve as the definitive hosts, while non-felines act as intermediate hosts. Based on serological evidences, it is speculated that one-third of the total world’s population is at risk of infection (Montoya and Liesenfeld 2004). The disease affects production of animals, influences public health throughout the globe, and is incriminated to abortion, stillbirth, and neonatal complications especially in sheep, goats, and pigs leading to considerable financial losses to the small and marginal farmers (Tenter et al. 2000; Hill and Dubey 2002; Dubey 2004). The impact of toxoplasmosis in developing countries like India is difficult to assess on account of absence of quality reagents for putative diagnosis (Dubey 1993) and the scanty epizootiological data in susceptible mammalian hosts species. Nevertheless, *T. gondii* infection in pig, sheep, goat, cat, chicken as well as humans, has been, from time to time, documented in the literature (Gautam et al.

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1979; Dubey 1987). Surface Antigens (SAG's) of *Toxoplasma* isolates are being sequenced and exploited for diagnostic purposes.

Most of the experiments on *T. gondii* are carried out using the lab-adapted 'RH' strain, which is continuously maintained in mouse peritoneal passage and also cryopreserved. It was investigated if continuous passage in mice could lead to mutation in the genes of the organism. The entire open reading frame (ORF) of surface antigen 3 (SAG3) gene of RH strain of *T. gondii* maintained at Indian Veterinary Research Institute (IVRI) (RH-IVRI) was amplified and cloned in a heterologous prokaryotic system. The cloned gene was custom sequenced and the sequence was compared with the available sequences of the same gene in the GenBank to establish the phylogenetic identity of SAG3.

Materials and methods

Propagation of *T. gondii* tachyzoites

In-bred Swiss albino adult mice, maintained on standard feed (pellets) and water ad libitum, were intra-peritoneally inoculated with 1×10^2 tachyzoites of mouse adapted RH strain of *T. gondii*, cryopreserved and maintained at the IVRI (RH-IVRI). The infected mice were daily monitored for the development of signs of infection. Those exhibiting peritonitis were euthanized and the peritoneal fluid was aspirated after inoculation of 5 ml of sterile phosphate buffered saline (PBS, pH 7.2) in the peritoneal cavity with due care of avoiding injury to visceral organs. The process was repeated until the peritoneal contents were clear. The contents were thrice washed with PBS and the live tachyzoites were counted.

Separation of host cell-free tachyzoites

The host cell-free tachyzoites were separated using standard protocol (Gross et al. 1991) as below. The peritoneal fluid containing free tachyzoites and tachyzoite infected macrophages was collected in PBS (pH 7.2) and washed thrice in PBS while repeatedly centrifuging at 5,000 rpm (Remi R8C) for 10 min and thereafter, final pellet was re-suspended in 5 ml of PBS. 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, debris was allowed to settle down in the centrifuge tube for 10 min and the supernatant was collected and thereafter passed through a polycarbonate membrane filter of 3 μ m pore size slowly (at the rate of 1 ml per 2–3 min). The membrane

was pre-wetted with PBS. The filtrate was again centrifuged at 3,000 rpm for 10 min and the tachyzoites in sediment were re-suspended in 1 ml of PBS.

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. Trizol (1 ml) was added to the suspension containing about 10^7 tachyzoites, repeatedly pipetted to kill the tachyzoites and thereafter, incubated at 30 °C for 5 min to dissociate nucleoprotein complexes. The suspension was vigorously shaken for 15 s after adding 0.2 ml of chloroform and then centrifuged at $12,000 \times g$ for 15 min at 4 °C. The upper aqueous phase was transferred to a fresh tube, 0.5 ml of the iso-propyl alcohol added and the RNA was allowed to precipitate at 15–30 °C for 10 min. The tube was centrifuged at $12,000 \times g$ for 10 min at 4 °C. The RNA pellet was washed once with 1 ml of 75 % ethanol prepared using 0.01 % diethylpyrocarbonate (DEPC) treated water. The sample was mixed by vortexing and centrifuged at $7,500 \times g$ for 5 min at 4 °C. The RNA pellet was air-dried, reconstituted in 100 μ l 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 v/cm².

Synthesis of complimentary DNA (cDNA) by reverse transcription

cDNA was synthesized from the total RNA isolated from the *T. gondii* tachyzoites, using oligo dT primer while following the standard protocol (Gautam et al. 1979). The cDNA thus synthesized was quantified using spectrophotometer (Nanodrop[®], USA).

Polymerase chain reaction (PCR) based amplification of SAG3

The entire ORF of SAG3 gene of *T. gondii* RH-IVRI strain was PCR amplified using a pair of specific primers [forward primer (TS3F) 5'-ATGCAGCTGTGGCGGCGCAG-3' and reverse (TS3R) 5'-TTAGGCAGCCACATGCACAAG-3'] (Kazemi et al. 2007). The PCR was carried out in a standard 25 μ l reaction volume with initial denaturation of DNA strands at 95 °C for 5 min followed by 32 cycles of denaturation at 95 °C for 50 s, primer annealing at 62 °C for 75 s and strand elongation at 72 °C for 50 s. Thereafter one cycle of final extension of the strands was given at 72 °C for 12 min. The PCR amplification was confirmed by visualization of the product on 1.5 % agarose gel stained with ethidium bromide following electrophoresis.

Molecular cloning and characterization of SAG3

The amplified ORF of SAG3 gene of *T. gondii* was purified using Qiagen Mini elute gel extraction kit (Qiagen GmbH, Hilden, Germany) following manufacturer's protocol. Thereafter, competent *Escherichia coli* DH5 α cells were prepared following the standard calcium chloride treatment method (Sambrook et al. 1989). Ligation reaction for cloning of amplified SAG3 into InsTAclone PCR cloning vector (Qiagen, Germany) as well as transformation of DH5 α cells was carried out as per manufacturer's protocol. The positive clones were identified by blue-white colony screening method. Further confirmation was done by colony PCR following standard protocol (Sambrook et al. 1989) as well as restriction analysis of the plasmid DNA isolated from the white colonies with *Pst*I and *Eco*RI. The restriction digestion reaction was carried out at 37 °C for 4 h. The digested products as well as the colony PCR amplified products were visualized in the ethidium bromide stained agarose gel following electrophoresis. A subculture of positive clone harboring the desired SAG3 gene of *T. gondii* RH-IVRI strain was custom DNA sequenced from the Department of Biochemistry, Delhi University. The sequence information received was analyzed using DNASTAR and GeneTool software.

Results

Infection of mice

All the mice infected with RH-IVRI strain of *T. gondii* started showing characteristic signs of the disease from day 5 to 7 post infection (PI). The clinical signs included raised and rough hair coat, pendulous abdomen, severe ascites, dullness, and tachypnoea marked by resting with fore legs on walls of the cages, the nozzle of water bottle or on other resting mice. Microscopically, large number of tachyzoites was detectable (either free or within the peritoneal macrophages suspended in the aspirated peritoneal fluid).

PCR amplification, molecular cloning, and molecular characterization of SAG3 gene

The whole ORF of SAG3 gene was amplified from cDNA of RH-IVRI strain using the specific forward and reverse primers. The amplicons were resolved as a single band of 1,158 bp (Fig. 1). It was further purified for ligation in InsTAclone PCR cloning vector. The selection of positive colonies was done by colony PCR using the specific primers and also by restriction enzyme digestion of the recombinant plasmids with *Pst*I and *Eco*RI for the release of insert. The results of restriction enzyme digestion as

Fig. 1 Specific PCR amplification of ORF of SAG3 gene of RH-IVRI isolate of *T. gondii*. Lane M: Marker 100 bp DNA ladder plus. Lane 1: Amplicon of 1,158 bp from *T. gondii*

well as colony PCR were checked by agarose gel electrophoresis. The positive clones were selected and custom sequenced for nucleotides and the obtained sequence submitted to GenBank (Accession No. HM585285).

The nucleotide sequence revealed 99.9 and 99.1 % (Fig. 2) sequence homology of SAG3 ORF between the RH-IVRI strain of *T. gondii* with that of the published sequences *T. gondii* RH-USA (GenBank Accession No. AF340227) and RH-Iran strains (GenBank Accession No. JF312642), respectively. A comparison of the nucleotide sequence of SAG3 with that of other documented sequences of *T. gondii* strains revealed that RH-IVRI strain is closely related to *T. gondii* CEP strain (type III) with 99.3 % sequence homology and *T. gondii* P-Br strain (a natural recombinant strain of type I and III) with 99.2 % sequence homology. However, it showed a greater degree of non-similarity with *T. gondii* Prugniaud strain (type II) with 97.6 % nucleotide sequence homology. The deduced amino acid sequence analysis revealed that *T. gondii* RH-IVRI strain had 99.7 % amino acid sequence homology with the *T. gondii* RH-USA strain, 98.4 % homology with *T. gondii* CEP and P-Br strains, 97.9 % homology with *T. gondii* RH-Iran strain and 95.6 % homology with Prugniaud strain (Fig. 3). A phylogenetic tree for nucleotides and amino acids of SAG3 RH-IVRI strain has been constructed using bootstrap method to delineate its relationship with other referral stains (Figs. 4, 5).

Discussion

World over, only one valid species of *Toxoplasma* exists. However, based on molecular genotyping studies, varied

Fig. 2 Sequence pair distances of SAG3 Clustal V (weighted)

		Percent Identity						Divergence	
		1	2	3	4	5	6		
1			98.8	97.6	99.4	99.3	99.8	1	SAG3-CEP AF340229
2	1.5			97.0	99.3	99.2	98.5	2	SAG3-P-Br AY187280
3	2.5	3.3			97.7	97.6	97.4	3	SAG3-Prugniaud AF340228
4	0.6	0.9	2.4			99.9	99.2	4	SAG3-RH AF340227 USA
5	0.7	1.0	2.5	0.1			99.1	5	SAG3-RH HM585285 IVRI
6	0.2	1.7	2.6	0.8	0.9			6	SAG3-RH JF312642 Iran
	1	2	3	4	5	6			

Fig. 3 Deduced amino acid pair distances of SAG3 Clustal V (PAM 250)

		Percent Identity						Divergence	
		1	2	3	4	5	6		
1			97.4	96.1	98.7	98.4	99.5	1	SAG3-CEP AF340229
2	2.6			94.5	98.7	98.4	96.9	2	SAG3-P-Br AY187280
3	4.0	5.7			95.8	95.6	95.6	3	SAG3-Prugniaud AF340228
4	1.3	1.3	4.3			99.7	98.2	4	SAG3-RH AF340227 USA
5	1.6	1.6	4.6	0.3			97.9	5	SAG3-RH HM585285 IVRI
6	0.5	3.2	4.6	1.8	2.1			6	SAG3-RH JF312642 Iran
	1	2	3	4	5	6			

Fig. 4 Phylogenetic tree of nucleotide sequence of SAG3 Clustal V (weighted) **Fig. 5** Phylogenetic tree of deduced amino acid of SAG3 Clustal V (PAM 250)

fundamental clonal populations 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.

Interestingly, difference at the lineages of DNA among the predominant clones is $<2\%$ (Grigg et al. 2001). Transmission of the parasite through carnivorous/cannibalism and scavenging among intermediate hosts,

bypassing sexual recombination events in the definitive host, i.e., cats (Howe and Sibley 1995; Su et al. 2003), parthenogenetic formation of oocysts by unfertilised macrogametes of the parasite in the small intestine of cats (Ferguson 2002) and simultaneous infection with different strains of *T. gondii* are some plausible reasons which explain the existence of clonal population structure in *T. gondii* (Ajzenberg et al. 2004).

Surface antigen 3 (SAG3), a 43 kDa glycoprotein, is glycosylphosphatidylinositol (GPI) anchored membrane bound protein on the developmental stages of the pathogen (tachyzoites and bradyzoites) (Burg et al. 1988; Kazemi et al. 2007). The protein was earlier identified as P43. It was cloned and sequenced for the first time by Cesbron-Delauw et al. (1994) followed by Fux et al. (2003). SAG3 is a glycoaminoglycan-binding protein associated with binding of host heparin sulfate proteoglycans (HSPGs). The SAG3–HSPGs interaction facilitates the parasite attachment to the host cell-surface proteoglycans (Jacquet et al. 2001). Further, SAG3 has primary structure similarity with surface antigen 1 (SAG1) (Cesbron-Delauw et al. 1994). However, in India, molecular characterization of SAG3 gene of *T. gondii* seems not attempted so far. The present study reports the molecular characterization of SAG3 gene of a lab adapted strain of *T. gondii* (RH-IVRI) and to ascertain its molecular homology with some other strains of the same parasite prevalent across the globe.

The adenine and thymine (A + T) content of the SAG3 gene of *T. gondii* RH-IVRI strain was analyzed to be 42.57 %, whereas the guanine and cytosine (G + C) content was 57.43 %. The nucleotide homology of *T. gondii* RH-IVRI strain was found to be 99.9 and 99.1 % with that of *T. gondii* RH-USA and Iran strains, respectively. There was a substitution of a single nucleotide of G instead of A at position 397 of the SAG3 ORF of *T. gondii* RH-IVRI strain in comparison to RH-USA strain and RH-Iran strain. The nucleotide substitution resulted in change of a single nucleotide residue in the deduced amino acid sequence at position 133 as aspartic acid (D) instead of asparagine (N) resulting in deduced amino acid homology of 99.7 % with RH-USA strain. The IVRI-RH strain showed deduced amino acid homology of 97.9 % with the RH-Iran strain due to nucleotide substitutions at nine different places in the SAG3 ORF of *T. gondii* RH-Iran strain in comparison to RH-IVRI strain. Seven nucleotide substitutions at positions 48 (G to A), 231 (T to A), 587 (T to C), 1,037 (T to C), 1,046 (G to A), 1,053 (A to C), 1,061 (G to C) resulted in change of amino acid codons at position 16 (arginine (R) to lysine (K)), 77 (asparagine (N) to lysine (K)), 196 (valine (V) to alanine (A)), 346 (lysine (K) to serine (S)), 349 (glycine (G) to glutamate (E)), 351 (glutamate (E) to aspartic acid (D)), and 354 (serine (S) to threonine (T)), respectively, in the SAG3 ORF of *T. gondii* RH-Iran strain.

The SAG3 sequence based phylogenetic trees of nucleotides and deduced amino acids were prepared separately. It is very clear in its depiction of the differences in the sequence homology of SAG3 of *T. gondii* strains across the world. In conclusion, RH strain is more closely related to CEP strain (USA strain) and P-Br strain (Brazilian strain) in comparison with Prugniaud strain (USA strain), in respect of the nucleotide homology.

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