DOI: 10.2478/s11686-014-0288-1
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Acta Parasitologica, 2014, 59(4), 666–674; ISSN 1230-2821

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Genetic characterization of *Toxoplasma gondii* isolates from chickens in India by GRA6 gene sequence analysis

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Abstract

PCR-RFLP and nucleotide sequencing based genotyping of *Toxoplasma gondii* Indian isolates (Izatnagar and Chennai isolates and Chennai clone) vis-a vis RH-IVRI strain was conducted by targeting GRA6 as genetic marker. The 791 bp GRA6 product was PCR amplified from the genomic DNA of different *T. gondii* Indian isolates, including the RH-IVRI strain. *Tru*1I restriction endonuclease based PCR-RFLP of GRA6 sequence produced polymorphic digestion pattern that discriminated the virulent RH-IVRI strain (as type I) from the moderately virulent local isolates as type III. The PCR amplicon of *T. gondii* GRA6 from RH-IVRI strain as well as from the local isolates were cloned in cloning vector and custom sequenced. The nucleotide and deduced amino acid sequences of *T. gondii* isolates were aligned with that of the type I, II and III strains (RH, BEVERLEY, ME49, C56, TONT and NED) available in public domain and analyzed *in silico* using MEGA version 4.0 software. Nucleotide sequencing and phylogenetic analysis of GRA6 marker from the Indian isolates revealed a close genetic relationship with type III strains of *T. gondii*. Further, detection of a single nucleotide polymorphism (SNP) at positions 162 and 171 of the GRA6 marker, established the lineage of Indian isolates as type III. This is the first report on characterization of *T. gondii* lineage as type III in selective chicken population of India based on PCR-RFLP and sequence analysis of GRA6 gene.

Keywords

Toxoplasma gondii; Genotyping; PCR-RFLP; Phylogenetic analysis; GRA6; Chicken

Introduction

Toxoplasma gondii, an obligate intracellular coccidian parasite with worldwide distribution has the potential of infecting almost all warm-blooded animals (Tenter *et al*. 2000; Hill and Dubey 2002). The organism is zoonotically important and up to one-third of the human population in the world is chronically infected (Dubey and Beattie 1988; Tenter *et al*. 2000). A recent national survey revealed that 24.3% of Indian population is exposed to this parasite (Dhumne *et al*. 2007). Postnatal infection in humans occurs through ingestion of undercooked meat containing tissue cysts or through consumption of food or water contaminated with oocysts (Dubey and Beattie 1988; Dubey *et al*. 2011). The genotyping of *T. gondii* isolates from free-range chickens indicates the level of soil contamination with *T. gondii* oocysts as they feed from the ground (Sreekumar *et al*. 2003; Dubey *et al*. 2006a) and also provides information on their lineage which determines the virulence.

Recent upsurge in the research on *T. gondii* can be traced from the increasing reports of characteristic infection associated pathological syndromes in AIDS and other immunocompromised patients, raising questions about the existence of clonal lineages. Genetic variation in *T. gondii* is low and most *T. gondii* isolates from human and animal sources have been grouped into one of the three clonal lineages (types I, II, and III) by multi-locus enzyme electrophoresis, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and microsatellite typing (Darde *et al*. 1992; Howe and Sibley 1995; Ajzenberg *et al*. 2002a,b). Recently, a fourth clonal lineage, referred to as type 12, has been described in North America where it is commonly found in wildlife (Khan

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et al. 2011; Su *et al*., 2012). Several studies have shown that most of the animal isolates of *T. gondii* belong to types II, III and IV (Howe and Sibley 1995; Mondragon *et al*. 1998; Owen and Trees 1999; Jungersen *et al*. 2002; Khan *et al*. 2011; Su *et al*., 2012). Congenital toxoplasmosis is caused mostly by the mouse-avirulent genotype (Costa *et al*. 1997).

The clonal population structure of *T. gondii* in North America, Europe and Africa comprises three predominant lineages (types I, II and III) as defined by multi-locus enzyme electrophoresis (MLEE), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or microsatellite analysis (Darde *et al*. 1992; Howe and Sibley 1995; Ajzenberg *et al*. 2002a; Velmurugan *et al*. 2008). However, *T. gondii* isolates from South America are diverse and largely distinct from those of North America and Europe (Ajzenberg *et al*. 2004; Khan *et al*. 2006; Lehmann *et al*. 2006; Pena *et al*. 2008). In Asia there are few reports from Japan (Smith and Frenkel 2003), Korea (Quan *et al*. 2008), Vietnam (Dubey *et al*. 2007b), Iran (Zia-Ali *et al*. 2007) and China (Dubey *et al*. 2007c; Zhou *et al*. 2009) indicating the existence of limited diversity among the isolates. There is a solitary report from India on a RFLP based genotyping targeting the SAG 2 locus of *T. gondii* isolates (Sreekumar *et al*. 2003). However, the overall diversity of the *T. gondii* population might be much higher than speculated. Recent genotyping studies on *T. gondii* strains isolated from wild animals or human patients belonging to different geographical regions revealed high frequency of non-archetypal genotypes. Further, most genotyping studies had relied on a few bi-allelic markers and therefore, the resolution and discriminative power of identifying parasite isolates were quite low (Su *et al*. 2006).

The present communication deals with molecular genotyping of *T. gondii* from chicken population of India based on PCR-RFLP and sequence analysis of GRA6 gene.

Materials and Methods

Toxoplasma gondii **isolates**

The *T. gondii* RH-IVRI strain maintained in the protozoology laboratory of IVRI for more than a decade in liquid nitrogen as cryostocks and the tachyzoites were proliferated in experimental mice when required. The local Indian isolates were collected from backyard chicken of Chennai (Tamil Nadu) and Izatnagar (Uttar Pradesh). The chicken hearts were fed to cats and oocysts isolated. Subsequently experimental mice were infected and the tachyzoites were isolated following standard protocol. A clone of *T. gondii* (Chennai clone), expanded from a single tachyzoite of a Chennai isolate was also included for genotyping.

Experimental animals

Laboratory bred adult Swiss albino mice of either sex were maintained on standard feed (pellets) and water ad libitum. *Toxoplasma* infection free status of the mice was ensured by IFAT.

Propagation of T. gondii tachyzoites

The four different isolates of *T. gondii*, *viz.* mouse adopted RH-IVRI strain, Izatnagar and Chennai isolates, and Chennai clone were propagated in four different groups of mice, each with four animals, by intra-peritoneal inoculation of viable tachyzoites of *T. gondii* ($n = 1\times10^2$). The infected mice were monitored daily and on development of peritonitis, the mice were euthanized and the peritoneal fluid was aspirated. The peritoneal cavity was inoculated with 5 ml of sterile phosphate buffered saline (PBS, pH 7.2) avoiding injury to visceral organs. The washing process was repeated till the peritoneal contents were clear. The tachyzoites were pelleted by light centrifugation and washed thrice with PBS (pH 7.2) and the number of live tachyzoites were enumerated.

Separation of host cell-free tachyzoites and extraction of genomic DNA of T. gondii

The homogenous population of different isolates of *T. gondii* tachyzoites free from host cell contamination was achieved following standard protocol (Gross *et al*. 1991). The genomic DNA was extracted using Wizard® Genomic DNA purification kit (Promega, USA).

Oligonucleotide primers

A pair of PCR primers, described earlier by Fazaeli *et al.* (2000), specific for a 791 bp coding sequence of *T. gondii* dense granule protein 6 (GRA6) was custom synthesized and used in the present study. The nucleotide sequence of the forward and the reverse primers were GRA6F 5'-GTAGCGTGCTTGTTGGCGAC-3' and GRA6R 5'-TACAAGACATA GAGTGCCCC-3', respectively.

Polymerase chain reaction

The PCR assay for amplification of GRA6 coding sequence from different isolates of *T. gondii* was laboratory standardized in 25 µl reaction volume containing 10 ng of genomic DNA, 10 pmol of each primer (GRA6F and GRA6R), 200 µmol of each dNTP and 1.0 U *Taq* DNA polymerase (MBI Fermentas Life Sciences, Lithuania) in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% w/v gelatin. The final volume was made to 25 µl with nuclease-free water (MBI Fermentas Life Sciences, Lithuania). The reactions were performed on a thermocycler (Gene Amp PCR System -2400, Perkin Elmer, USA) with a preheated lid. The cycling conditions were standardized as an initial denaturation of strands for 5 min at 95°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing of primers at 60°C for 1 min and extension of strands at 72°C

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for 1 min. A final extension of the synthesized strands was given at 72°C for 7 min. The PCR amplification was confirmed by running the product on an ethidium bromide stained 1.4% agarose gel and visualization of the amplicon on a transilluminator under UV light.

PCR restriction fragment length polymorphism (PCR-RFLP) analysis

For genotyping the different isolates, a conventional PCR-RFLP assay was performed to digest the 791 bp PCR amplified GRA6 product using *Tru*1I endonucleases (Fermentas, Germany). The digestion reaction was set up in 40 µl volumes in 200 µl PCR tubes. Ten microlitres of the PCR product was used for each digestion. The digestion mixture consisted of 4 µl of the 10× buffer, 10 µl PCR product and 1 µl (10 U) of the restriction enzyme made up to 40 µl with nuclease free water. The digestion mix was incubated at 65°C for 3 h. The digested products were separated by electrophoresis at 3v/cm for 60 min in an ethidium bromide stained 1.5% agarose gel and visualized on a transilluminator under UV light.

Cloning and nucleotide sequencing of PCR amplified product

The 791 bp PCR amplified products from different strain/isolates were purified using gel extraction kit (Qiagen, Germany). The purified GRA6 amplicon was cloned in pDRIVE cloning vector (Qiagen, Germany) following standard protocol. Competent *Escherichia coli* (DH5α) cells were transformed with the recombinant plasmid construct containing GRA6 insert and plated on Luria–Bertani agar medium containing ampicillin (50 µg ml^{-1}), X-gal (80 µg ml^{-1}) and IPTG (50 µ mole). The recombinant clones were selected initially by blue–white colony screening method. A white colony was picked up and sub-cultured for 8h in LB medium. The recombinant plasmids were extracted using mini-prep plasmid DNA isolation kit (Fermentas, USA) and the insert was released by restriction digestion with *EcoR* I.

The white colonies were further tested by colony PCR to confirm the presence of specific insert. A confirmed positive clone from each strain/isolate was custom sequenced for nucleotides from the Department of Biochemistry, South Campus, Delhi University, New Delhi. Sequence information was submitted for accession number in primary bioinformatics web servers.

Sequence alignment and phylogenetic analysis

The sequence alignment and analysis were done with the MegAlign component of the DNAstar programme (Version 5.0 DNASTAR, Madision, Wis.). Phylogenies were constructed by neighbor-joining using Kimura 2-parameter model using homogeneous pattern among lineages and tested by bootstrap with 1000 replicates, using MEGA version 4.0 (Tamura *et al*. 2007). The GenBank accession number, host, virulence in mice and origin of the GRA6 gene sequence used for sequence alignment and phylogenetic tree analysis are presented in Table I. Pair wise distance of *T. gondii* strains from GenBank and Indian isolates of *T. gondii* was calculated using Kimura 2-parameter model using homogeneous pattern among lineages and tested by bootstrap with 1000 replicates, using MEGA version 4.0 (Tamura *et al*. 2007).

Results

Polymerase chain reaction and PCR-RFLP

PCR amplification of a single fragment of 791 bp specific for *T. gondii* GRA6 gene resulted at annealing temperature of 60°C from the whole genomic DNA from the different strain/isolates.

Restriction digestion of the 791 bp PCR amplicon with *Tru*1I (*Mse*I) restriction enzyme produced polymorphic digestion pattern that discriminated the virulent RH-IVRI strain as type I from the local isolates of moderate or intermediate vir-

Table I. GenBank accession number, host and origin of the GRA6 gene sequence of *Toxoplasma gondii* strains used in phylogenetic tree analysis

Strain	Origin	Virulence in mice[a]	Host	Accession no.	References for virulence phenotypes
Chennai	India	I	Chicken	JN649065	Sreekumar (2001)
Chennai clone	India	I	Chicken	JN649066	Sreekumar (2001)
Izatnagar	India	I	Chicken	JN649064	Sreekumar (2001)
RH-IVRI	USA	V	Human	JN649063	Sreekumar (2001)
RH	USA	V	Human	AF239283	Fazaeli *et al.* (2000)
Beverley	England	A	Rabbit	AF239284	Fazaeli *et al.* (2000)
ME49	USA	A	Sheep	AF239285	Fazaeli *et al.* (2000)
NED	France	A	Human	AF239286	Fazaeli *et al.* (2000)
TONT	France	V	Human	AF239292	Fazaeli *et al.* (2000)
C56	USA	I	Chicken	DQ512729	Fazaeli *et al.* (2000)

[a] V: virulent in mice; A: avirulent; I: intermediately virulent

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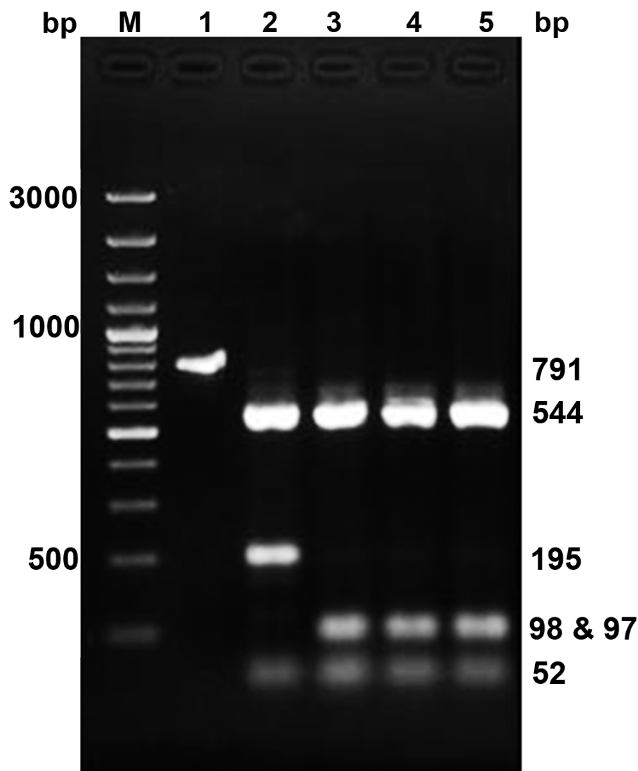

Fig. 1. Agarose (1.4%) gel showing the PCR-RFLP of GRA6 amplicon with *Tru*1I endonuclease. Lane M indicating 100 bp Plus DNA ladder. Lane 1indicates uncut PCR product. Lane 2 – 5 indicates RFLP pattern of *Toxoplasma gondii*, type I (RH), Izatnagar isolate, Chennai isolate and Chennai clone respectively

ulence as type III. Restriction digestion of the PCR amplification products from the local isolates with *Tru*1I produced four distinct DNA fragments of 544 bp, 98 bp, 97 bp and 52 bp, respectively while three digestion products of 544 bp, 195 bp and 52 bp were visualized from the RH-IVRI strain (Fig. 1).

Nucleotide sequencing and phylogenetic analysis

PCR product of 791 bp *T. gondii* GRA6 gene marker amplified from different strain/isolates (GenBank accession numbers RH-India-JN649063; Chennai-JN649065; Chennai clone-JN649066 and Izatnagar-JN649064) was sequenced for nucleotides from both directions. The nucleotide sequences revealed high percentage of sequence similarity with the published sequences of *T. gondii* GRA6 and thereby established its specificity. Pair wise nucleotide distance of the GRA6 gene was calculated on the basis of 740 nucleotides (28-767 of 791nts). Indian isolates of *T. gondii* were grouped into type III strains (C56, NED and TONT) of *T. gondii* (Table II). Phylogenetic tree was constructed with the *T. gondii* Indian isolates and other valid type I (RH), II (Beverley and ME49) and III (NED, TONT and C56) of *T. gondii* strains (Fig. 2). The Indian isolates of *T. gondii* were clustered with the type III strains (NED, TONT and C56) of *T. gondii*. The nucleotide analysis of GRA6 gene showed 99.9 to 100% homology of the Indian isolates with the type III strains (NED, TONT and C56) of *T. gondii*.

The alignment of the sequences amplified from the local isolates and the three reference strains (type I, II and III) showed 20 polymorphic positions at the nucleotide level (Table

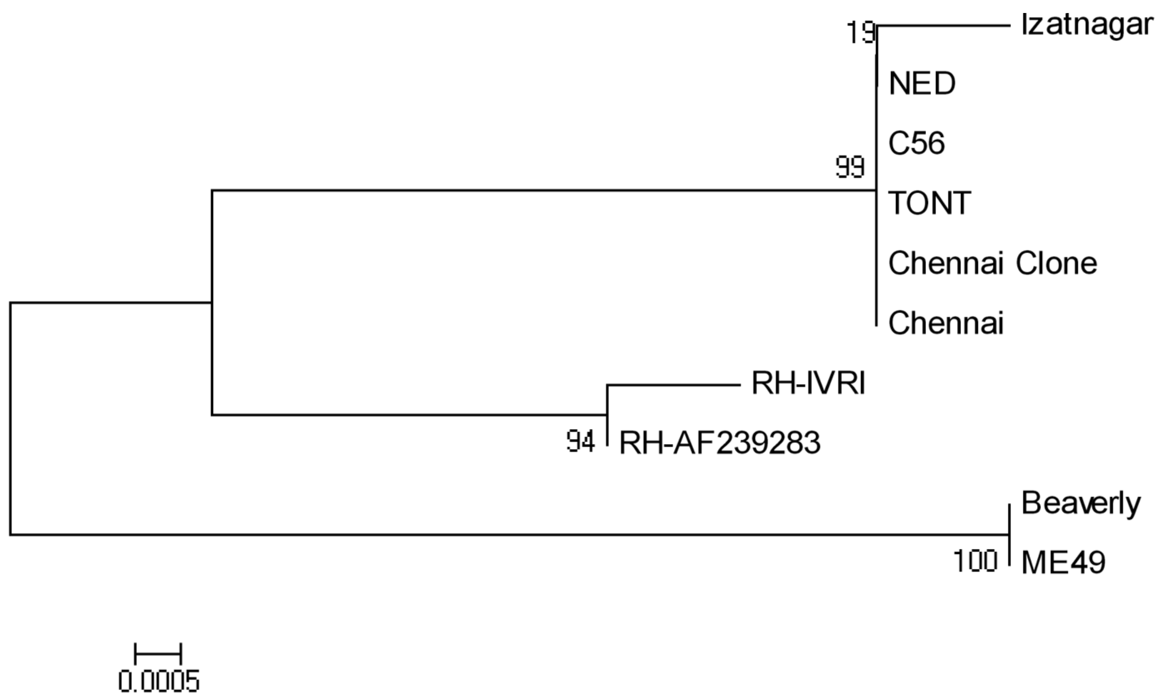

Fig. 2. Phylogenetic analysis by neighbour-joining using Kimura 2–parameter on GRA6 sequences of *T. gondii* reference strains and representative Indian isolates. Numbers at nodes represent percentage occurrence of clades in 1000 bootstrap replications of data

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Table II. Pairwise distance of *T. gondii* from GenBank and Indian isolates of *T. gondii* by MEGA4 (Kimura 2- parameter)

No.	Strain/isolate of *T. gondii*	1	2	3	4	5	6	7	8	9	10
1	**Chennai JN649065**										
2	**Chennai clone JN649066**	0.000									
3	**Izatnagar JN649064**	0.001	0.001								
4	**RH-IVRI JN649063**	0.013	0.013	0.014							
5	**RH AF239283**	0.012	0.012	0.013	0.001						
6	**Beverley AF239284**	0.020	0.020	0.022	0.019	0.017					
7	**ME49 AF239285**	0.020	0.020	0.022	0.019	0.017	0.000				
8	**NED AF239286**	0.000	0.000	0.001	0.013	0.012	0.020	0.020			
9	**TONT AF239292**	0.000	0.000	0.001	0.013	0.012	0.020	0.020	0.000		
10	**C56 DQ512729**	0.000	0.000	0.001	0.013	0.012	0.020	0.020	0.000	0.000	

III). Two deletions of 15 bp and 3 bp were observed in the sequence of the type II strains. However, changes of only one nucleotide at position 39 was detected in Izatnagar isolate and at position 637 in Chennai clone in respect to NED, TONT and C56 (type III) strains. Seventeen of 20 nucleotide substitutions resulted in changes at amino acid level (Table IV). Six amino acids (Gly, Tyr, Gly, Gly, Arg, and Ala) were deleted in the type II strains. Among the local isolates, substitution of a single amino acid, isoleucine substituted with threonine was observed at position 6 in the Izatnagar isolate in comparison to Chennai, Chennai clone and the other type III strains *T. gondii*.

The two single nucleotide polymorphisms (SNP) at positions 162 and 171 of the GRA6 gene allowed the differentiation of type I, II and III. The SNP detected at position 162 in the local isolates was adenine (a) instead of guanine (g). Presence of adenine (a) at position 162 was specific for type III lineage (Table III). Change in the corresponding amino acid was aspartic acid (D) which was observed in type III, instead of glycine (G) found in type I & II (Table IV). Another SNP at position 171 in the nucleotide sequence (Table III) of local isolates was adenine (a) instead of guanine (g) resulting in the change of corresponding amino acid lysine (K) instead of arginine (R) (Table IV). Presence of arginine (R) at position 171 was specific for type II lineage. A third specific polymorphism in the nucleotide sequence was observed at position 106 as thymine in the local isolates which was specific for their type III lineage. However, this replacement of a single nucleotide did not result in a change at amino acid level. Nucleotide sequence and phylogenetic

Table III. Nucleotide polymorphisms of the GRA6 gene coding region within *Toxoplasma gondii* strains

Strains	Positions of variable nucleotides																				
	39	41	71	106	162	171	304	336	561	576	596	614	635–649	650-652	654	660	677	690	692	702	711
RH -AF239283	T	C	G	C	G	A	A	T	A	A	G	G	TACGGAGGCAGAGGT	GAA	G	C	C	A	C	T	A
RH-IVRI	.	.	.	.	.	.	.	.	.	.	A	.	....	...	.	.	.	.	.	.	.
BEVERLEY	.	T	T	.	.	G	T	C	.	G	.	.	–––––––––––––––	–––	A	G	.	G	A	A	T
ME49	.	T	T	.	.	G	T	C	.	G	.	.	–––––––––––––––	–––	A	G	.	G	A	A	T
NED	.	.	T	T	A	.	T	.	C	.	.	C	....	...	.	G	G	.	.	.	.
TONT	.	.	T	T	A	.	T	.	C	.	.	C	....	...	.	G	G	.	.	.	.
C56	.	.	T	T	A	.	T	.	C	.	.	C	....	...	.	G	G	.	.	.	.
IZATNAGAR	C	.	T	T	A	.	T	.	C	.	.	C	....	...	.	G	G	.	.	.	.
CHENNAI	.	.	T	T	A	.	T	.	C	.	.	C	....	...	.	G	G	.	.	.	.
CHENNAI-C	.	.	T	T	A	.	T	.	C	.	.	C	..T............	...	.	G	G	.	.	.	.

Periods (.) indicates identical nucleotides related to the sequence of RH-AF239283 (first row), and Dashes (–) indicate deletions

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Table IV. Amino acid changes of the GRA6 gene coding region within *Toxoplasma gondii* strains

StrainsL	Positions of variable nucleotides																
	39	41	71	162	171	304	336	561	576	596	614	635–649	660	677	690	692	702	711
	Corresponding deduced amino acids																	
RH -AF239283	I	H	V	G	K	E	V	Q	D	G	G	G Y G G R	A	P	E	R	V	Y
RH-IVRI	.	.	.	.	.	.	.	.	.	S	.	. . . . .	.	.	.	.	.	.
BEVERLEY	.	Y	L	.	R	D	A	.	G	.	.	– – – – –	–	.	G	S	E	F
ME49	.	Y	L	.	R	D	A	.	G	.	.	– – – – –	–	.	G	S	E	F
NED	.	.	L	D	.	D	.	P	.	.	R	. . . . .	G	A	.	.	.	.
TONT	.	.	L	D	.	D	.	P	.	.	R	. . . . .	G	A	.	.	.	.
C56	.	.	L	D	.	D	.	P	.	.	R	. . . . .	G	A	.	.	.	.
IZATNAGAR	T	.	L	D	.	D	.	P	.	.	R	. . . . .	G	A	.	.	.	.
CHENNAI	.	.	L	D	.	D	.	P	.	.	R	. . . . .	G	A	.	.	.	.
CHENNAI-C	.	.	L	D	.	D	.	P	.	.	R	. . . . .	G	A	.	.	.	.

Periods (.) indicates identical amino acids related to the sequence of RH-AF239283 (first row), and Dashes (-) indicate deletions

analysis of GRA6 marker revealed the lineage of the Indian isolates of *T. gondii* as type III (Fig. 2).

Discussion

Several molecules that are stored within dense core granules of *Toxoplasma* are secreted into the parasitophorous vacuole (PV) following invasion (Cesbron-Delauw, 1994; Coppens *et al*. 1999). The dense granule proteins are responsible for intracellular survival of the parasites and are immunogenic (Decoster *et al*. 1988; Lecordier *et al*. 1995). The GRA6 gene exists as a single-copy (Lecordier *et al*. 1995) and is highly polymorphic (Fazaeli *et al*. 2000). Therefore, the coding region of GRA6 gene was chosen as marker for polymorphism analysis as well as to determine the lineage of *T. gondii* isolates associated with infection in chickens in India.

The 791 bp coding sequence of dense granule protein 6 (GRA6) was PCR amplified from the genomic DNA of *T. gondii* RH-IVRI strain as well as the Indian isolates of *T. gondii*. In order to determine the lineage of *T. gondii* based on GRA6 marker, the PCR products were digested with *Tru*1I endonuclease, an isoschizomer of *Mse*I, to generate the restriction pattern suggestive of a specific lineage. The *Tru*1I restriction endonuclease based PCR-RFLP produced polymorphic digestion pattern from the GRA6 sequence characterized by the presence of additional 98 and 97 bp fragments and absence of 195 bp fragment in the Indian isolates in comparison to the typical digestion fingerprint of GRA6 gene sequence from the *T. gondii* RH-IVRI strain. This discriminated between the virulent RH strain (type I) from the moderately virulent Indian isolates as type III (Fig. 1). Single nucleotide polymorphisms (SNP) in the GRA6 sequence was important for a PCR restriction fragment length polymorphism (PCR-RFLP) using a single endonuclease (*Mse* I) to differentiate the *T. gondii* genotypes into type I, II, and III lineages (Fazaeli *et al*. 2000). GRA6 gene was used as a PCR-RFLP marker to genotype the *T. gondii* isolates (Fazaeli *et al*. 2000; Miller *et al*. 2004; Lin *et al*. 2005; Khan *et al*. 2005, 2006; Petersen *et al*. 2006; Dubey *et al*. 2006b, 2007a, 2011; Belfort-Neto *et al*. 2007; Velmurugan *et al*. 2009; More *et al*. 2010; Ragozo *et al*. 2010). The Indian isolates were of moderate mice virulence and a SAG2 gene based restriction fragment length polymorphisms study predicted their type III lineage (Sreekumar 2001; Sreekumar *et al*. 2003).

In silico analysis of the nucleotide sequence of GRA6, its subsequent phylogenetic analysis and pairwise distance calculations revealed the closest genetic relationship of the Indian isolates of *T. gondii* with that of type III strains. The GRA6 nucleotide sequences of the two Indian isolates and one of its clone with characteristics of III genotype showed complete sequence identity with that of the C56, NED and TONT strains (typical strain type III) except a single nucleotide substitution at position 39 in Izatnagar isolate and at position 637 in Chennai clone. However, the corresponding change at deduced amino acid level was evident in Izatnagar isolate only. Further, based on a single nucleotide polymorphisms (SNP) located at positions 162 and 171 of the GRA6 gene, the Indian isolates were typed as type III.

On the basis of sequence information of GRA6 gene, Fazeli *et al*. (2000), Zakemi *et al*. (2006) and Sousa *et al*. (2009) detected a high degree of polymorphism among the *T. gondii*

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isolates. Similarly, a pyrosequencing based genotyping of *T. gondii* isolates detected two SNPs located at positions 162 and 171 of the GRA6 gene, which allowed the differentiation of the isolates as types I, II & III (Edvinson *et al*. 2007).

Though a few reports on molecular characterization of some structural genes of *T. gondii* exists (Singh *et al*. 2011a & b; Sudan *et al*. 2012), reports on molecular typing of *T. gondii* is sparse from India and a solitary report on genotyping of Indian isolates of *T. gondii* exists (Sreekumar *et al*. 2003), targeting the *T. gondii* SAG 2 locus from free-range chickens (*Gallus domesticus*). Two isolates from South and Central India were characterized as type II and five as type III.

The three clonal genetic lineages of *T.* gondii, *viz.* type I, type II and type III are predominant in Western Europe and the United States (Howe and Sibley 1995; Howe *et al*. 1997). However, recent surveys from South American localities and some other parts of world revealed the existence of non-archetypal genotypes (Soares *et al*. 2011). Further, while genotyping the Brazilian strains of *T. gondii*, Pena *et al*. (2008) identified a highly reticulated phylogenetic structure, suggesting the important role of recombination in strain diversification of *T. gondii* in South America.

PCR-RFLP and sequencing data provided evidence of the genetic lineage of the local isolates (Izatnagar, Chennai, and Chennai clone) of *T. gondii* as type III. The molecular typing further confirmed the comparative low mouse virulence of the local isolates of *T. gondii.*

Acknowledgments. Authors acknowledge the Director, Indian Veterinary Research Institute, Izatnagar for the facilities provided.

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Received: August 28, 2013
Revised: June 4, 2014
Accepted for publication: June 10, 2014

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