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Molecular cloning, comparative sequence analysis and prokaryotic expression of GRA5 protein of *Toxoplasma gondii**

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ABSTRACT

Among the several target molecules for sensitive detection of *Toxoplasma gondii*, dense granule antigens are considered important as these help in growth and multiplication of the organism in the host. The communication deals with the cloning and sequence analysis of 363 bp entire open reading frame (ORF) of GRA5, a dense granule protein, from 2 Indian isolates of *T. gondii* (Izatnagar and Chennai) as well as the standard RH strain. The sequence comparison analysis revealed 100% homology between the Chennai and Izatnagar isolates, 99.2% homology of RH strain with both the Chennai and Izatnagar isolates and 100% sequence homology of RH strain of *T. gondii* with the published sequence. The GRA5 protein (mature) was subsequently expressed in prokaryotic expression system. It had molecular size of ~29 kDa and the level of expression was measured as 12% of the total protein. The concentration of the mature recombinant GRA5 protein was 92 μg/ml. Western blot with Ni-NTA anti-histidine HRPase conjugate and known positive serum confirmed the presence and purity of protein by immunoreactivity at the unique ~29 kDa region.

Key words: Cloning, Expression, GRA5, *Toxoplasma gondii*

Toxoplasmosis, caused by heteroxenous coccidian parasite *Toxoplasma gondii*, is prevalent in a wide range of mammalian and avian hosts. Based on serological evidences, it is estimated that one-third of the total world's population is at risk of infection (Montoya and Liesenfeld 2004). The disease is of great public health significance and is responsible for abortions, stillbirth and neonatal complications in livestock especially in sheep, goats and pigs leading to significant economic loss to the small and marginal farmers (Tenter *et al*. 2000, Hill and Dubey 2002, Dubey 2004). In the absence of quality diagnostic reagents for putative diagnosis (Dubey *et al*. 1993), the epidemiological information of the disease in various host species in India is scanty. Nevertheless, *T. gondii* infection has been described from pig, sheep, goat, cat, chicken as well as humans from India (Gautum *et al*. 1979, Dubey 1987).

The serological tests using the soluble native antigens of the organism suffer from the limitations of sensitivity and specificity. The dense granule proteins of *T. gondii* play an important role in remodelling the parasitophorus vacuole enabling the parasite to survive, grow and divide within it. The dense granule proteins are a mixture of at least 8 separate gene products, termed as GRA proteins (Cesbron *et al*. 1993). Secretion of dense granules occurs by fusion of the granules with the parasite surface membrane near the apical end of the parasite and release of contents into the lumen of the vacuole (Dubremetz *et al*. 1993). The dense granule proteins secreted in abundance are major components of both the vacuole surrounding tachyzoites as well as the cyst wall surrounding the more slow-growing bradyzoites and therefore, are considered important target molecules which may serve the dual purpose of conferring protection as well as for use as diagnostic antigens (Cesbron 1994). The present study reports the primer-directed amplification of the open reading frame (ORF) of GRA5 gene of 2 Indian isolates of *T. gondii*, viz. Izatnagar (IZN) and Chennai (CHEN) as well as the standard RH strain, their cloning and sequence comparison for studying the molecular homology between

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these strains/isolates and prokaryotic expression of mature GRA5 protein.

MATERIALS AND METHODS

In vivo propagation of T. gondii tachyzoites in mice: Inbred Swiss albino adult mice (of either sex), maintained on standard feed (pellets) and freshwater *ad lib.* were infected intraperitoneally with 1×10^3 tachyzoites of mouse adopted RH strain of *T. gondii* and were examined daily for development of clinical signs of infection. On development of peritonitis, the mice were euthanized and 5–10 ml of sterile phosphate buffered saline (PBS, pH 7.2) was injected aseptically in the peritoneal cavity with a 10 ml glass syringe fitted with 21 G needle and the peritoneal lavage was aspirated into the same syringe with due care of avoiding injury to other abdominal organs. The process of collection of peritoneal washings was repeated till the peritoneal lavage became clear. The contents were washed thrice in PBS (pH 7.2) and the number of live tachyzoites was counted.

Separation of host cell- free tachyzoites: The host cell-free tachyzoites were separated as per Gross *et al.* (1991). Briefly, the peritoneal lavage containing free tachyzoites, as well as, infected macrophages was collected in PBS (pH 7.4), as described above and washed thrice in PBS (pH 7.4) by centrifuging each at 5000 rpm for 10 min. The final pellet was re-suspended in 10 ml of PBS (pH 7.4). The intracellular tachyzoites were rendered host cell-free by rupturing the intact parasitized macrophages mechanically by passing the contents through a 27 G needle fitted in a 10 ml sterile syringe. The resultant tachyzoite suspension was washed again by re-suspending in 20 ml of PBS (pH 7.4) and the debris was allowed to settle down in the centrifuge tube for 10 min. The supernatant was collected carefully and passed slowly at the rate of 1 ml per 2–3 min through a polycarbonate membrane filter of 3 mm pore size, pre-wetted with PBS (pH 7.4). The filtered suspension was centrifuged at 3000 rpm for 10 min to sediment the tachyzoites. The supernatant was completely removed and the sedimented tachyzoites were re-suspended in 1 ml of PBS (pH 7.4).

Isolation of total RNA of T. gondii: Total RNA was extracted directly from the purified tachyzoites using reagent following the manufacturer's recommendations. In brief, 1 ml of Trizol reagent was added to $5–10\times10^6$ tachyzoites, which were subsequently lysed by repetitive pipetting. The mixture was incubated at 30°C for 5 min for complete dissociation of nucleoprotein complexes. This was vigorously shaken for 15 sec after adding 0.2 ml of chloroform and then centrifuged at 12000 g for 15 min at 4°C which permitted separation of the phases into lower organic phase and upper aqueous phase. The aqueous phase was transferred to a fresh tube from which the RNA was precipitated by keeping the tube at 15–30°C for 10 min following addition of 0.5 ml of isopropyl alcohol. Then the content was centrifuged at 12 000 g for 10 min at 4°C. The supernatant was discarded and 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 × g for 5 min at 4°C and then the RNA pellet was air-dried and re-dissolved in 100 ml of RNA storage buffer 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/cm^2.

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 following Sambrook *et al*. (1989). The cDNA thus synthesized was quantified using spectrophotometer.

Polymerase chain reaction (PCR) based amplification of GRA5: The entire open reading frame (ORF) of GRA5 gene of *T. gondii* (RH strain) was PCR amplified using forward primer (Tg-GRA5-F: 5'-ATGGCGTCTGTAAAACGCG-3') and reverse primer (Tg-GRA5-R: 5'-TTACTCTTCCTCGGCAACTTC-3'). The PCR reaction was carried out in a standard 25 ml reaction with initial denaturation of DNA strands at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 51°C for 45 sec and strand elongation at 72°C for 45 sec. Thereafter one cycle of final extension of the strands was given at 72°C for 10 min. The PCR amplification was confirmed by visualization of the product on 1.5% agarose gel stained with ethidium bromide following electrophoresis. Similarly, the GRA5 sequence of IZN and CHEN isolates of *T. gondii* were also PCR amplified for sequence comparison.

Molecular cloning and characterization of GRA5: The amplified ORFs of GRA5 gene of *T. gondii* RH strain, IZN and CHEN isolates were purified using gel extraction kit following manufacturer's protocol. Thereafter competent *Escherichia coli* DH5a cells were prepared following the standard calcium chloride treatment method (Sambrook *et al.* 1989). Ligation reaction for cloning of GRA5 (amplified from *T. gondii* RH strain, IZN isolate and CHEN isolate) into pGEM-T Easy cloning vector as well as transformation of DH5a cells was carried out following standard protocol. The positive clones were identified by blue-white colony screening method. Further confirmation was done by restriction analysis of the plasmid DNA isolated from the white colonies with *Eco*RI as well as by colony PCR following standard protocol (Sambrook *et al.* 1989). The restriction digestion reaction was carried out at 37°C for 4 h. The digested product 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 GRA5 genes from various isolates was custom DNA sequenced from the Department of Biochemistry, Delhi University. The sequence information received was analyzed using DNASTAR and GeneTool software.

Amplification of the mature GRA5 gene of T. gondii *(RH*

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strain) for directional cloning: To amplify the GRA5 gene sequence containing specific restriction sites to facilitate its release, a forward primer containing restriction site for *Nco*I (*Nco*I-Tg-M-GRA5-F: 5' CAGGATCCATGGGTTCAAC GCGTGACGTA 3') and reverse primer containing *Xho*I restriction site (*Xho*I-Tg-F-GRA5-R: 5'-TGGATC CTCGAGCTCTTCCTCGGCAAC TTC -3') at the 5' end were designed. The PCR reaction was carried out using 1:5 diluted purified GRA5 amplicon as template and *Pfu* polymerase in the reaction mixture. The cycling conditions were same as described in the previous section with the exception of annealing temperature which was 54°C for 1 min. Following PCR amplification, the apparent confirmation of the product was done on the basis of its size in the gel.

Prokaryotic expression of mature recombinant GRA5 protein (mGRA5) in pET32a expression vector: Purification of the PCR product containing mature GRA5 gene of *T. gondii* (RH strain) was achieved using gel extraction kit following manufacturer's protocol. For expression of the gene in a prokaryotic expression system, pET32a expression vector under the influence of T7 promoter was chosen. In order to clone the gene for expression, the mGRA5 gene as well as the pET32a expression vector was double digested with *Nco*I and *Xho*I restriction enzymes simultaneously in a 50 ml reaction to facilitate directional cloning. The reaction was carried out at 37°C for 4 h and digestion was checked by electrophoresis in 1.25% agarose gel. Following digestion, both the expression vector and mGRA5 gene were purified using gel extraction kit following manufacturer's protocol. Directional cloning of the mGRA5 gene into pET32a expression vector was carried out by performing a ligation reaction at 4°C for 16 h. The transformation of *E. coli* BL21 (DE3) cells, grown in LB broth containing chloramphenicol (34 mg/ml), was carried out with the recombinant plasmid. Confirmation of the recombinant clones was done by colony PCR as well as restriction enzyme analysis with *Nco*I and *Xho*I. Five positive colonies, harbouring the desired mGRA5 gene were chosen from the master plate for induction. The colonies were picked up with a sterile toothpick and inoculated in 5 ml of LB broth containing ampicillin (100 mg/ml) and chloramphenicol (34 mg/ml) and grown overnight at 37°C with constant shaking at 140 rpm. Ten milliliters of fresh LB broth was inoculated with 100 ml of the overnight grown culture and further incubated at 37°C with constant shaking until mid-log phase (approx. 4 h). One milliliter of the culture was collected from each tube and kept as an uninduced control. To the rest of the culture, IPTG was added at a final concentration of 1 mM and kept at 37°C with constant shaking at 140 rpm. One milliliter of the induced culture was collected every hour starting from 3 h onwards. All the cultures collected were pelleted by centrifugation at 13,000 rpm and kept at –20°C till further use. The pellets of recombinant *E. coli* cells collected at different hourly intervals of induction, as well as, the uninduced controls were subjected to electrophoresis by SDS-PAGE (12% gel) under denaturating conditions at 90V for 2–3 h (Laemmli 1970). The gel was stained using Coomassie brilliant blue R–250.

Purification of mature recombinant GRA5 protein: The induced cells were grown in 1 liter of bulk culture. The cells were pelleted and resuspended in 10 ml of lysis buffer containing 8 M urea (pH 8.0) and incubated at room temperature for 2 h on rotatory shaker with intermittent vortexing. Following lysis, the debris was pelleted by centrifugation for 10 min at 10,000 rpm and the clear supernatant was transferred to a clean tube. To the supernatant 800 ml of Ni-NTA agarose slurry was added and mixed thoroughly and kept on a rotatory shaker for 1 h with intermittent stirring. The lysate-resin mixture was loaded on an empty 5 ml polypropylene column (Qiagen) and equilibrated with 1X Tris-phosphate buffer (pH 8.0). The flow-through of the column was collected in a separate tube and the column was subsequently washed with 15 ml of wash buffer (pH 7.0) containing 5 mM imidazole (pH 7.0). Finally the bound histidine-rich recombinant proteins were eluted as 500 ml fractions with 4 ml of elution buffer (pH 4.2–4.5).

Renaturation of the protein: Renaturation of the recombinant protein was achieved by dialysis of the eluted product against decreasing molar concentration of urea. The protein was dialysed against tris-saline-EDTA (TSE) buffer (pH 7.2) containing 6 M and 4 M urea for 3 h in each concentration with periodic mixing and against 2 M urea at 4°C overnight. Thereafter, the protein was kept in TSE buffer for 3 h at 4°C followed by TSE:PBS (50:50 v/v) buffer for 3 h and then only in PBS (pH 7.2) for 3 h with three changes of the buffer at hourly interval. Any debris formed during the renaturation was removed by centrifugation at 10,000 rpm for 10 min in refrigerated centrifuge. The purity of the protein was checked by electrophoresis on SDS-PAGE using 12% gel. Finally, the protein was aliquoted in 0.5 ml volume and stored at 4°C in the presence of anti-proteases till use. Following purification, the concentration of the recombinant protein was assayed using modified protein assay kit following the manufacturer's instruction.

Western blot analysis of the recombinant mGRA5 protein: The specific immunoreactivity of the expressed recombinant mGRA5 protein was checked by western blotting following standard protocol (Towbin *et al.* 1979). About 500 ng of the purified recombinant protein was loaded and ran on SDS-PAGE using 12% gel. The resolved protein was subsequently electrophoretically transferred to a nitrocellulose membrane using a blotting apparatus (BIO-RAD) at 80 mA constant current for 2.5 h. Successful transfer of the protein to the membrane was confirmed by staining the membrane with Ponceau-S stain. Following electro-transfer of the protein, the unbound surface of the membrane was blocked with 3% non-fat milk powder in PBS (pH 7.4) at 4°C overnight. The membrane was washed with PBS-Tween 20 (0.05%) (PBST),

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three times for 5 min, Ni-NTA anti-histidine HRPase conjugate was added at 1:1000 dilution and incubated at 37°C for 1 h. The membrane was washed 3 times with PBST and subsequently developed with DAB tablets as substrate.

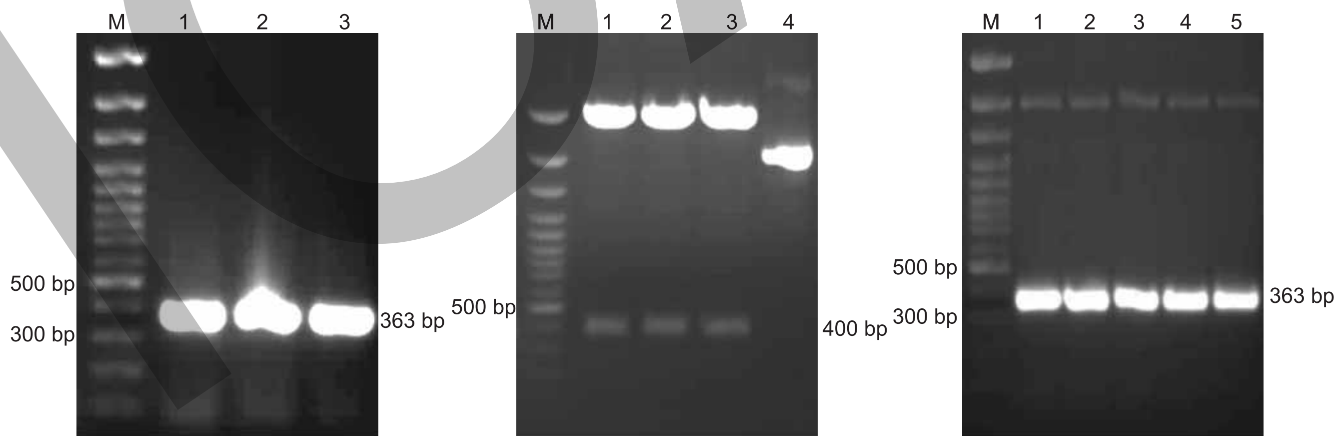
The immunoreactivity of the recombinant mGRA5 was further analyzed by western blot using known positive and known negative goat sera at 1:100 dilutions. Anti-goat HRPO conjugate was used in 1: 1000 dilutions and the membrane was incubated at 37°C for 1 h. Following stringent washing with PBS-T, the membrane was developed with DAB solution.

RESULTS AND DISCUSSION

With the objective of exploring the use of molecular parasite targets as future tools for diagnosis and prophylaxis, the present study is focused on cloning and expression of GRA5 gene product of *T. gondii*. The gene coding for GRA5 was cloned and expressed in prokaryotic expression system. The reason for selecting this molecular target was based on its importance in host cell invasion, maintenance of the parasitophorous vacuole and survival of the parasite after cellular invasion (Charif *et al*. 1990, Achbarou *et al*. 1991, Dubremetz and Schwartz-man 1993, Metsis *et al*. 1995, Carruthers *et al*. 1999). Additionally, GRA5 protein is present in all stages of the parasite (Tilley *et al*. 1997), thereby precluding stage to stage variation which qualifies the protein molecule, a suitable immunoprophylactic target as well as its application in serological tests for specific immunodiagnosis (Zenner *et al*. 1999, Igarashi *et al*. 2008a and b, Holec-Gasior and Kur 2010).

Observations on infectivity of cryopreserved T. gondii: Between day 4 to 7 post-infection (PI), all the mice started showing symptoms of infection. The clinical signs included raised and rough hair coat, development of severe ascites characterized by pendulous abdomen, dullness, tachypnoea marked by resting with forelegs either on walls of the cages or on the nozzle of water bottle or on other resting mice. Large numbers of tachyzoites were obtained from the peritoneal fluid of the experimentally infected mice.

Amplification, molecular cloning and characterization of GRA5 gene: The whole ORF of GRA5 gene was amplified from cDNA of RH strain of the parasite using the specific forward and reverse primers. The amplicon was resolved as a single band of 363 bp size (Fig.1) which was further purified for ligation in pGEM-T Easy cloning vector. To study the intron-less nature of the gene, amplification was also done from the whole genomic DNA of *T. gondii* (RH strain). The similar sized amplification products confirmed the intron-less nature of the gene. GRA5 gene sequence (363 bp) amplified from the genomic DNA of both CHEN and IZN isolates (Fig. 1) of *T. gondii* was also cloned into pGEM-T easy cloning vector. The selection of positive colonies containing the insert for all the three isolates was done by restriction enzyme digestion of recombinant plasmids with *Eco*RI for the release of insert and also by colony PCR with the specific primers. The results of restriction enzyme digestion as well as colony PCR were checked by agarose gel electrophoresis (Figs 2, 3). The positive clones were selected and custom sequenced for nucleotides. The nucleotide sequence revealed 100% homology of ORF of GRA5 between the CHEN and IZN isolates of *T. gondii*.



Figs 1–3. **1.** Specific PCR amplification of ORF of GRA5 gene of *T. gondii;* Lane M: Marker 100 bp DNA ladder plus (MBI Fermentas); Lane 1: Amplicon of 363 bp from *T. gondii* (RH strain); Lane 2: Amplicon of 363 bp from *T. gondii* (IZN isolate); Lane 3: Amplicon of 363 bp from *T. gondii* (CHEN isolate). **2.** Release of GRA5 insert by restriction digestion of pGEM-T Easy cloning vector; Lane M: Marker 100 bp DNA ladder plus (MBI Fermentas); Lane 1: Insert release by *Eco*RI digestion in *T. gondii* (RH strain); Lane 2: Insert release by *Eco*RI digestion in *T. gondii* (IZN isolate); Lane 3: Insert release by *Eco*RI digestion in *T. gondii* (CHEN isolate); Lane 4: Undigested recombinant pGEMT Easy vector. **3.** Colony PCR from transformed colonies of GRA5; Lane M: Marker 100 bp DNA ladder plus (MBI Fermentas); Lane 1,2: GRA5 amplicon from *T. gondii* (RH strain); Lane 3,4: GRA5 amplicon from *T. gondii* (IZN isolate); Lane 5: GRA5 amplicon from *T. gondii* (CHEN isolate)

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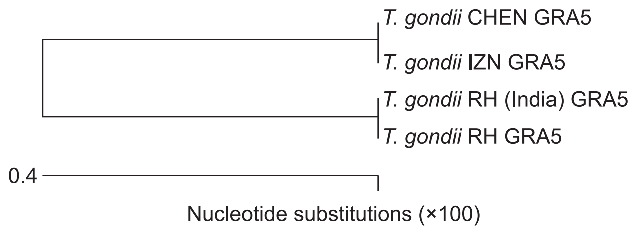


Fig 4. Sequence pair distances of *T. gondii* GRA5 ORF by Clustal W method.

Percent identity (rows: Divergence)

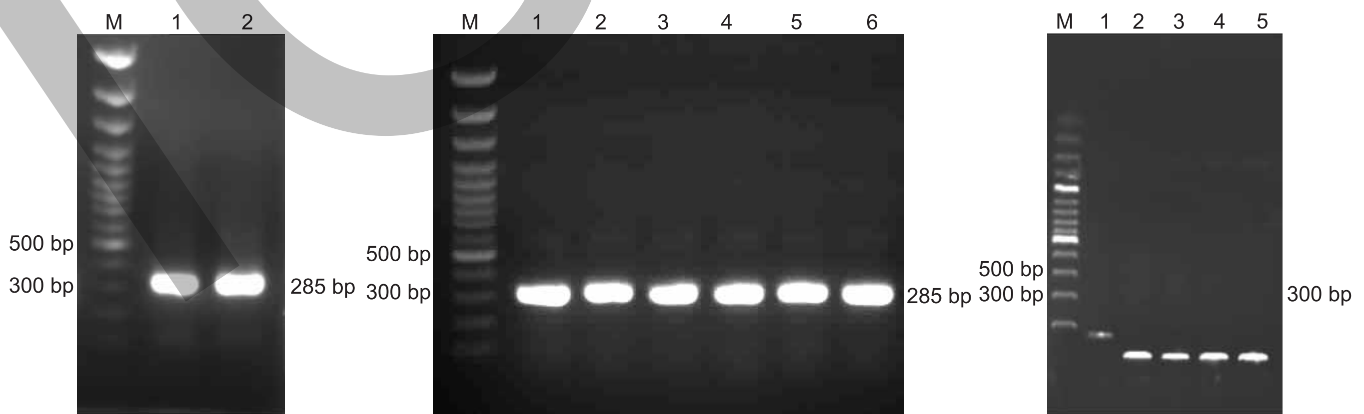
	1	2	3	4		
1		100	99.2	99.2	1	*T. gondii* CHEN GRA5
2	0		99.2	99.2	2	*T. gondii* IZN GRA5
3	0.8	0.8		100	3	*T. gondii* RH (India) GRA5
4	0.8	0.8	0		4	*T. gondii* RH GRA5
	1	2	3	4		

Fig 5. Phylogenetic tree of *T. gondii* GRA5 ORF by Clustal W method.

Similarly, 100% sequence homology of GRA5 ORF between the RH strain of the parasite was observed with that of the published sequence. The nucleotide sequence of RH strain showed 99.2% homology with CHEN and IZN isolates (Fig. 4) with changes at nucleotide position 104, 124 and 137. Similarly, deduced amino acid sequence analysis revealed 100% homology between the local isolates, as well as, between RH strain and published sequence of the parasite. The deduced amino acid sequence of RH strain showed 97.5% homology with CHEN and IZN isolates with substitutions at positions 35, 42 and 46, respectively. Based on this information a phylogenetic tree using bootstrap method was constructed (Fig. 5) which clearly shows that CHEN and IZN isolates are phylogenetically closely related to each other and the same holds true for RH strain of the parasite used in the present study as well as the published sequence. Despite the high genetic similarity observed between different strains of *T. gondii*, marked differences in many phylogenetic characteristics including virulence have been reported (Dlugonska 2008) that might hold true here as RH strain is highly virulent while the same does not hold true for the local isolates. The sequence information was submitted to GenBank under these accession numbers (EU918733, EU918735, and EU918734).

Directional cloning of GRA5 gene coding for the mature protein for expression: The GRA5 gene sequence coding for the mature protein (285 bp) was PCR amplified (Fig. 6) using the expression primers having restriction sites for *Nco*I and *Xho*I. The restriction enzymes *Nco*I and *Xho*I were incorporated in the expression primers since the sequence information of the gene revealed the absence of the sites for either of these restriction enzymes. The amplicon was purified and was double digested with *Nco*I and *Xho*I at 37°C for 4 h. The purified digested product was used for ligation in the pET32a expression vector.

Prokaryotic expression of mature recombinant GRA5 protein (mGRA5) in pET32a expression vector: The *Nco*I and *Xho*I double digested pET32a expression vector was used for overnight ligation with purified mature GRA5 at 4°C. Transformed BL21 (DE3) cells of *E. coli* were grown in presence of antibiotics, ampicillin and chloramphenicol. Following an overnight incubation, colony PCR (Fig. 7) as well as restriction enzyme analysis (Fig. 8) confirmed the presence of 285 bp mature GRA5 gene-specific product. A moderate level of expression of mGRA5 was achieved following 8 h of induction of the culture with 1 mM IPTG and the level of expression was recorded at 12% of the total



Figs 6–8. **6.** Specific PCR amplification of mature GRA5 of *T. gondii;* Lane M: Marker 100 bp DNA ladder plus (MBI Fermentas); Lane 1, 2: Amplicon of 285 bp from *T. gondii* (RH strain). **7.** Colony PCR from transformed colonies of mature GRA5; Lane M: Marker 100 bp DNA ladder plus (MBI Fermentas); Lane 1 to 6: mature GRA5 amplicon from colony PCR. **8.** Restriction digestion of recombinant pET32a plasmid of mature GRA5; Lane M: Marker 100 bp DNA ladder plus (MBI Fermentas); Lane 1: Uncut recombinant pET32-a plasmid; Lane 2: Non-recombinant colony; Lane 3–5: Insert release with *Nco*I and *Xho*I digestion.

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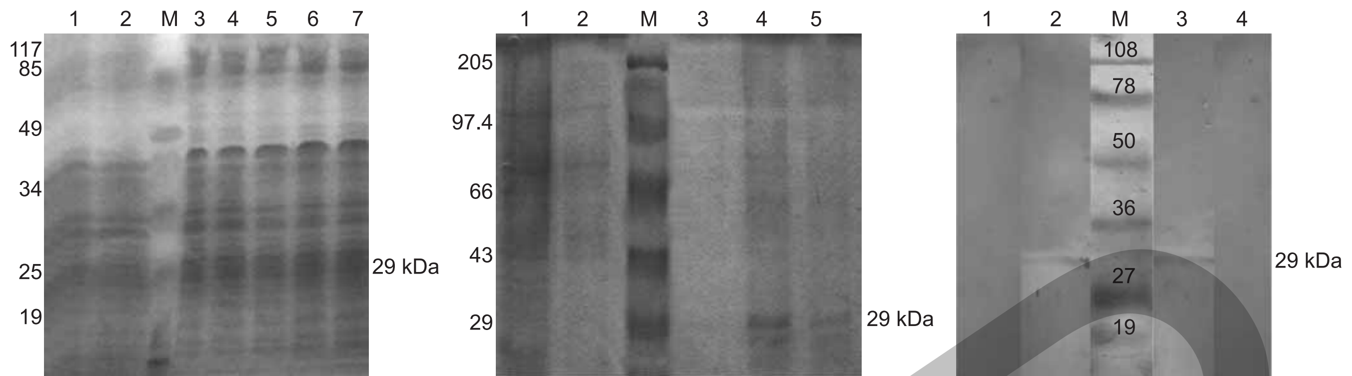

Figs 9–11. **9.** SDS-PAGE analysis of expression of recombinant mGRA5 protein; Lane 1, 2: Un-induced control; Lane M: Molecular weight marker, pre-stained (MBI Fermentas); Lane 3: 2 h post induction; Lane 4: 4 h post induction; Lane 5: 6 h post induction; Lane 6: 8 h post induction; Lane 7: 10 h post induction. **10.** SDS-PAGE analysis of the purified mature rGRA5 fusion protein; Lane 1: Flow through from Ni-NTA agarose column; Lane 2: First wash from Ni-NTA agarose column; Lane M: Molecular weight marker (Bangalore Genei); Lane 3: Elute 1 from Ni-NTA agarose column; Lane 4: Elute 2 from Ni NTA agarose column; Lane 5: Elute 3 from Ni NTA agarose column. **11.** Western-blot analysis of the purified mature rGRA5 fusion protein; Lane 1: No reactivity with known positive sera; Lane 2: Strong reactivity with known negative sera; Lane M: Molecular weight marker (Biomatik); Lane 3: Strong reactivity with Ni-NTA HRP conjugate; Lane 4: No reactivity in Ni-NTA HRP conjugate control.

bacterial protein and visualized by SDS-PAGE as a 29 kDa fusion polypeptide (Fig. 9).

Purification of recombinant mature GRA5 protein: The protein was purified using Ni-NTA agarose beads, where the 6x His-tag bound to the Ni-NTA column facilitated competitive recovery at low pH. Nearly 5–6.5 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 thioredoxin tag; S-tag and His-tag of the vector along with the mGRA5 sequence resulted in expression of the protein as a fusion protein which facilitated the purification of the protein to its homogeneity. Purification of the fusion protein was done under denaturing conditions using 8 M 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 ~29 kDa (Fig. 10). 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 3 to 4 h and finally against PBS (pH 7.2) for 2–3 h at 4°C for proper refolding and checked by SDS-PAGE. The concentration of the recombinant mGRA5 protein was 92 mg/ml.

Western blot analysis of expressed histidine-tagged recombinant mature GRA5 protein: The specific reactivity of the recombinant mGRA5 protein was checked by western blotting. Ni-NTA anti-histidine HRPase conjugate as well as known positive serum, 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 ~29 kDa region specific for mature GRA5 on the nitrocellulose membrane (Fig. 11). 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. Similarly, known positive and negative sera were used to confirm the recombinant protein.

The present study has generated important data for expression of mature rGRA5 in heterologous host system for its subsequent application in diagnostic/prophylactic purposes. This is the first report on molecular cloning, sequence analysis and prokaryotic expression of GRA5 protein of *T. gondii* from local isolates as well as the standard RH strain from India.

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