



Construction and Sequencing of Dense Granular14 (*GRA14*) Gene of *Toxoplasma gondii* (RH) in Expression Prokaryotic Plasmid PET32a: A Preliminary Study in Vaccine Production

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Abstract

Introduction: *Toxoplasma gondii* is an obligatory intracellular protozoan parasite, which infects human beings. Since the current antigens used for diagnosis or vaccination are contaminated with non-parasitic material in which the parasite is grown, it is tried to produce recombinant antigens to design vaccines against toxoplasmosis, or make diagnostic kits. Choosing the type of antigen to produce recombinant vaccine or diagnostic kits is considerably important. The dense granule protein 14 (*GRA14*) gene is one of the excretory-secretory antigens of *Toxoplasma* which seems to be an appropriate candidate in production of recombinant vaccines and diagnostic kits. The current study aimed to clone *GRA14* gene of *T. gondii* (RH) in a cloning vector for further production of dense granular proteins. **Materials and Methods:** Genomic DNA was isolated from tachyzoite of parasite by phenol chloroform method and gene fragment was amplified by polymerase chain reaction (PCR). The PCR products were ligated into restriction enzymes sites of pTG19-T cloning vector. Then transformed into *Escherichia coli* Top10 strain and screened by IPTG and X-Gal. Then recombinant plasmid confirmed by the colony-PCR and restriction enzyme digestion using *SacI* and *NotI* was done followed by sequencing. After isolation of this gene from pTG19-T, it was subcloned into a prokaryotic expression plasmid (pET32a). The pET32a - *GRA14* constructs were analyzed by PCR, restriction analysis and sequencing.

Results: Evaluation of PCR products by agarose gel electrophoresis and analysis of nucleotide sequencing of 1227 bp gene encoding the protein *GRA14*, revealed the complete homology with the recorded sequences in the gene bank. After enzyme restriction and electrophoresis a fragment about 1227 bp was separated from pET32a.

Conclusions: The result of this study showed that recombinant *GRA14 Toxoplasma* was constructed successfully and ready for future study which seems like the antigen is a suitable candidate to produce recombinant vaccine and diagnostic kit.

Keywords: *Toxoplasma gondii*; Dense Granule Antigen 14; Cloning, Subcloning

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Introduction

Toxoplasma gondii is an obligatory intracellular protozoan that causes toxoplasmosis disease. The life cycle of parasite is completed by cat and warm-blooded animals.¹ In acute form of disease the parasite reproduced inside the host blood and other organ's cells and in chronic form of disease the parasite transforms to cysts.¹ *Toxoplasma* may cause different clinical forms such as asymptomatic or sever symptoms like abortion, congenital defects and death.² In spite of current strategies in vaccine production that are based on using living or non-living agents, it seems necessary using novel methods such as molecular methods to improve vaccine production and inhibiting of activation risk of organisms.³⁻⁵ The novel

strategies in production of vaccine are based on production of recombinant antigens of microorganisms.⁶ Natural antigens of toxoplasma are used to identify parasite antibodies in commercial kits, and considering that the parasite is cultivated in living cell, the possible risk for contamination of parasite antigens with host-cell antigens may lead to diagnostic deviations and also the quality of this kind of antigens are not stable.⁷ Considering that the production of these recombinant antigens are possible and such antigens do not have deficiencies listed for natural antigens, so nowadays the production of these types of antigens are considered.⁸ Since the current antigens that are used for diagnosis or vaccination are contaminated with nonparasitic material in which the parasite is grown, a

lot of efforts are made to produce recombinant antigens to design vaccine against toxoplasmosis or make diagnostic kits.⁹ Choosing the type of antigen to produce recombinant vaccine or diagnostic kits is considerably important because it should not be immunogenic, trigger of cellular immunity response, proliferator of T lymphocytes and also be presented in most of the parasite life-cycle.¹⁰ Identification of a potential target gene or protein is an important step in developing a diagnostic method to detect a pathological agent.

Dense granule antigens (GRAs) which are involved in parasite survival and virulence are known to be potential and useful diagnostic markers.¹¹ They are found abundantly in both tachyzoites and bradyzoites and make up most of the circulating antigens in the blood stream of an infected host which can be detected as early as a few hours post infection (acute phase).^{11,12} Dense granule (GRA) proteins are proteins with high immunogenicity¹³ and are also able to induce strong antibody responses during acute infection. GRA proteins were also found during the chronic stage of *T. gondii* infection.^{11,13} As a result, the immunogenicity and prolonged expression of GRA proteins make them one of the promising candidates for recombinant protein production. Therefore have higher potential to develop tools for laboratory diagnosis of toxoplasmosis.^{13,14} Hence, GRA proteins have been suggested as potential targets to diagnose *T. gondii* infection,^{12,15-18} and hereby continuous study of the proteins are essential. GRA14 a novel dense granule protein, which is secreted into the vacuole and traffics both the PVM and IVN.¹⁹ GRA14 is targeted to membranous structures within the vacuole known as the intravascular network and to the vacuolar membrane surrounding the parasite.¹⁹ It has an unexpected topology in the PVM with its C terminus facing the host cytoplasm and its N terminus facing the vacuolar lumen.²⁰ GRA14 can be transferred between vacuoles during infection; a process is called intervacuolar transport.¹⁹ GRA14 colocalizes with other GRA proteins on PVM extensions, where GRA14-positive extensions were found to connect neighboring PVs.¹⁹

GRA14 protein has 409 amino acids and its gene is about 1227 bp and without introns.²¹ Regardless of the possible roles of GRA14, there is no study to clone and subclone GRA14 *T. gondii* in prokaryotes vector.²² In this work we describe the cloning of a new gene encoding a novel, GRA14 *T. gondii* for the first time in prokaryotic expression vector for further production of Dense Granular proteins. It could be used to reach completely expressed GRA14 and constructing of the vaccine or diagnostic kit.

Materials and Methods

Chemicals, Reagent and Kits

The polymerase chain reaction (PCR) cloning kit was purchased from Fermentas (Germany) and other kits were purchased from Qiagen (Germany). Restriction endonucleases were purchased from Fermentas, USA, also T4 DNA ligase, Taq polymerase, dNTP and protein weight markers were provided from the Roche Company (Germany). 5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal) and Isopropyl- β -D-thiogalactopyranoside (IPTG) were purchased from the Sigma-Aldrich Corporation (Germany).

The *Escherichia coli* strain TOP10 (Invitrogen), plasmid pTG19-T (Vivantis, Canada) and pET32a(+) prokaryotes expression vector were provided from Invitrogen (Invitrogen, Carlsbad, USA). All chemicals were obtained from Merck (Germany), Sigma-Aldrich (Germany) and HiMedia Corporations (India).

Production of *Toxoplasma gondii* Tachyzoites

The RH strain of *T. gondii* was provided from parasitology department of Public Health Faculty, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Tachyzoites were harvested from the peritoneal cavity of BALB/c mice 3-4 day after intraperitoneal (ip) injection with 1×10^5 of parasite suspension in sterile phosphate-buffered saline (PBS; pH 7.4) containing 100 IU/mL penicillin and 100 mg/mL streptomycin^{23,24} for lysate preparation and DNA extraction and stored at -70°C .

DNA Extraction and PCR Amplification of GRA14 Gene

Standard methods were used for DNA extraction.^{25,26} Genomic DNA of *T. gondii* RH strain was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA extraction products were electrophoresed in 1% agarose gel and then photographed. A pair of primers based on the full protein-coding region of the *T. gondii* GRA14 gene sequence (Accession number FJ015061.1) was designed by the DNASIS primer designer software (version 2.6) with SacI and NotI restriction sites at 5' end of forward and reverse primers, respectively.

The sequences were GRA14F:5'-GAGCTCATGCAGGCGATAGCG-3' and GRA14R:5'-GCGGCC GCCTATTCGCTTGGTCTCTG -3' (Bioneer, South Korea). Also, the PCR reaction was performed in a total volume of 50 μL containing: 100 ng DNA, 1 μL forward and reverse primers at 25 pM, 50 mM MgCl_2 , 200 μM dNTP, 10X PCR buffer, and 2.5 U Taq polymerase (Germany).

The PCR conditions were as follows: initial denaturation at 94°C for 5 minutes; denaturation at 94°C for 1 minute, annealing at 62°C for 30 seconds and extension at 72°C for 45 seconds, which were followed by 30 cycles and a final extension at 72°C for 5 minutes in a thermal cycler. The PCR products were analyzed by electrophoresis on 1% agarose gel against a standard DNA Ladder (Fermentas, Germany), in 1X TBE buffer and visualized using the ethidium bromide staining on UV transilluminator. The PCR products were then purified from the agarose gel using QIAquick gel extraction kit (Qiagen, Germany) according to the manufacturer's recommendations and then DNA concentration was measured by spectrometry at OD260 and OD280 using Nanodrop 2000 (Thermo Scientific, USA). The purified DNA was ligated into pTG19-t vector by using T/A PCR product cloning kit (Fermentas, Germany).

Cloning of GRA14 Gene

The PCR product was cloned into the pTG19 vector via T/A cloning method using the PCR cloning kit (Fermentas, Germany) according to the manufacturer's instructions. Then the pTGRA14 was transformed in *E. coli* TOP10 competent

cells (Invitrogen) by calcium chloride method and dispensed on Luria-Bertani (LB) medium (HiMedia, Mumbai, India) agar plates containing Ampicillin (50 mg/L). Bacterial colonies were screened by agar plate containing X-gal (Sigma, USA) and IPTG (Sigma, USA) to discriminate between recombinant (white) and non-recombinant (blue) plasmid containing ones.^{25,26} Recombinant plasmids were detected by blue/white colonies screening method and were confirmed by rusconis test which was applied by Rast et al.²⁷ For the rusconis test a white colony was picked up and mixed with 12 μ L rusconis solution, centrifuged at 12000 rpm for 2 minutes. Eight microliters of supernatant was electrophoresed in 1% agarose and visualized after staining with ethidium bromide.²⁷ After selecting recombinant clones, the plasmid DNA was extracted from cells cultured overnight with shaking at 250 rpm at 37°C by using the Miniprep plasmid purification kit (Bioneer, Germany) following the manufacturer's instructions. The selected recombinant plasmids were verified by PCR, restriction digestion with SacI and NotI restriction enzymes (Fermentas, USA) and sequenced.

Construction of the Recombinant Expression Plasmid

The pTG19 vector carrying the *GRA14* gene and the eukaryotic expression vector pET32a (+) (Invitrogen, USA) were digested by SacI and NotI restriction enzymes. After thermal inactivation of the restriction enzymes and analysis by LMP agarose gel electrophoresis, the linearized pET32a (+) plasmid and the *GRA14* gene were extracted from the 1% agarose gel by agarose gel DNA extraction kit (Bioneer, Germany). The purified linear vector and insert were subjected to ligation reaction using T4 DNA ligase (Roche, Germany), generating the pET32a-*GRA14* plasmid. *E. coli* TOP10 competent cells were transformed with 2 μ L of ligation product.

Transformed *E. coli* cells were selected on LB medium agar plates containing ampicillin (50 mg/L). Several colonies were assayed by colony PCR and Rusconis using specific primers.^{25,27} After selecting recombinant clones, the plasmid DNA was extracted from the cells cultured overnight by using the Miniprep plasmid isolation kit (Bioneer, Germany) and confirmed by PCR, restriction-enzyme digestion, followed by DNA sequencing.

Ethical Considerations

Current study does not contain any studies with human participants or animals.

Results

Cloning and Verification of pTG19-*GRA14* Transformation

Genomic DNA of RH strain of *T. gondii* was subjected to PCR to amplify *GRA14* gene fragment. PCR products revealed a 1227 bp band on agarose gel (Figure 1).

After ligation of gene fragments to plasmids, transformation of plasmids into competent *E. coli* cells and screening of colonies consisted of recombinant plasmid.

Both white and blue bacterial colonies appeared on the ampicillin-LB agar medium following an overnight cultivation of TOP10 competent cells in exposure to the constructed pTG19-*GRA14* recombinant plasmids. However, more than

90% of the colonies were white, which indicated successful transformation of pTG19-*GRA14* recombinant plasmids into the TOP10 hosts. Several transformed colonies were analyzed by colony PCR with the *SAG3* gene specific primers and rusconis (Figure 2).

In addition, agarose gel electrophoresis analysis on plasmids extracted from blue and white colonies exhibited 1227 bp bands of *GRA14* gene, which indicated that white colonies had received pTG19-*GRA14* recombinant plasmids through successful transformation (Figure 2).

The recombinant plasmid was analyzed by restriction enzyme and rusconis also the resulting fragments were compared with molecular weight marker. Results of agarose gel electrophoresis analysis on constructed pTG19-*GRA14* recombinant plasmids following restriction enzymatic digestion revealed two different bands, the first band, which was heavier, indicating linear plasmids and the second, a lighter 1227 bp band, indicating the *GRA14* gene (Figure 3). These findings confirmed that the *GRA14* gene was successfully cloned into pTG19 plasmids.

Subcloning and Gene Sequencing of *GRA14* Gene

After the confirmation of a recombinant pTG19-*GRA14* plasmid, the recombinant plasmid was isolated from white

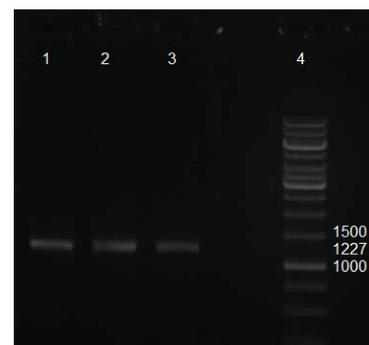


Figure 1. PCR Product on 1% Agarose Gel Electrophoresis. Lanes 1, 2, 3: *GRA14* gene with 1127 bp amplified by PCR; Lane 4: DNA ladder (1 kbp).

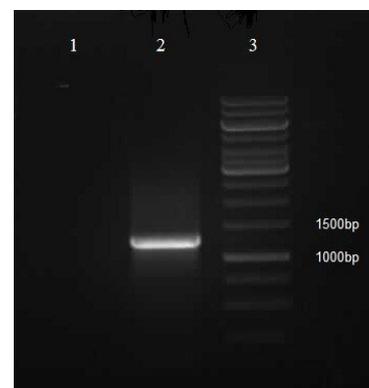


Figure 2. Electrophoresis of PCR Product of pT-*GRA14* Plasmid. Lane 1: PCR product of non-recombinant pTG19 plasmid extracted from blue colonies (without band); Lane 2: PCR product of recombinant pT-*GRA14* plasmid showing an approximate 1227 bp band; Lane 3: DNA ladder (1 kbp).

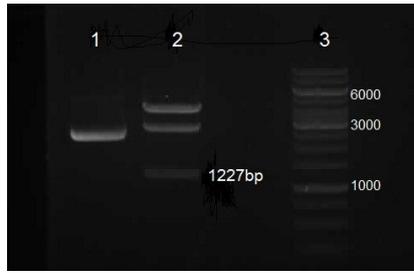


Figure 3. Gel Electrophoresis Analysis Cleavage of GRA14 Fragment From Recombinant pT-GRA14 Plasmid. Lane 1: recombinant pT-GRA14 non cleavage; Lane 2: recombinant pT-GRA14 plasmid subjected to restriction cleavage showing a 1227 bp GRA14 fragment. Lane 3: DNA ladder (1kbp).

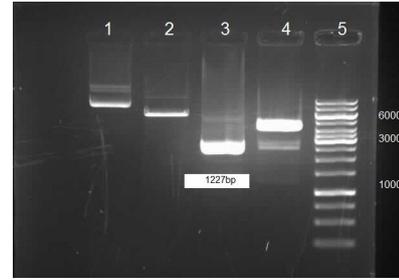


Figure 4. Gel Electrophoresis Analysis Cleavage of GRA14 Fragment From Recombinant pET-GRA14 Plasmids. Lane 1: non-recombinant plasmids; lane 2: recombinant plasmid; Lane 3: recombinant plasmid linear cleavage with SacI; Lane 4: recombinant pET-GRA14 plasmid subjected to restriction cleavage with SacI and NotI showing a 1227 bp GRA14 fragment; Lane 5: DNA ladder (1 kbp).

colonies and digested by SacI and NotI and released fragment (*GRA14* gene) subcloned into SacI and NotI digested pET32a expression plasmid. After transformation of *E. coli* Top10, recombinant plasmids were analyzed using the colony PCR and PCR on the isolated plasmids, rusconis, restriction digestion (Figure 4) and sequencing (Figure 5).

The recombinant plasmid (pET32a-GRA14) was sequenced (Figure 5). The sequencing result was confirmed by comparing with the databases and using basic local alignment search tool (BLAST) Software on NCBI website. The results showed a 100% homology with me49 and veg strains and 99% homology with RH strain GRA14 sequence (ref. No XM_002366572, LN714495 ,and FJ015061). The new gene sequence obtained from GRA14 was recorded with accession number of MH213492 in GenBank. These findings indicated that the reaction conditions were optimized and the designed primers were specific for amplification of the *GRA14* gene and had been successfully cloned into pET32a recombinant plasmids.

Discussion

Toxoplasma gondii is an obligatory intracellular parasite which has a complicated life cycle and attacks almost all nucleated cells. The high incidence and severe or lethal damages of toxoplasmosis clearly indicate the need for the development of a more effective vaccine.²⁸ Moreover, in recent years, researchers are trying to improve the serological diagnosis of toxoplasmosis using recombinant proteins.

Therefore, prompt diagnosis, effective treatment and prevention of this parasite is necessary to minimize the harm of this infection in humans and animals.²⁹ Because of diagnostic problems and ineffective therapeutic approaches, vaccine development might be one of the important ways for prevention of toxoplasmosis. Therefore, it is critical to generate specific antigens. Recombination is an easy method to access this specific protein.^{30,31} In recent years, many progresses have been achieved in identifying candidate antigens of vaccine which are capable to inducing protective immune response. A number of these antigens were expressed in prokaryotic organisms and after purifying used in diagnostic studies.^{32,33}

GRA proteins of *T. gondii* were first described as secreted antigens, since they were secreted following incubation of

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GGGGGGGCAACTGTGCGCTGATCGAGTTGTAGCTGGCTTTCTCATTTTTTCGTTTCTGCTTAAACGAGTGAAGCAGTGGCTGC
AGCTGCCAGTTTGGAGCAGACAATTCCTATTCTGTCCAACATCAACCCGACGAGGAAGGCATCTCCGGACACAGAAACCCCA
GACTGCCCAACTCTCAGCAGCTATTGTGCCGTAAGCTATCTAGGAGATGGTTTGTCACTACTCTCTGGAGTCTTAGGGCT
TTACCCCTCGATGTTGCAATTGGAACTTAACTAGTCTCGGAAATACCAACTGTGGCAGGTTTGTTCAAAATATATGTGCTGG
CGGCCAGCTATCTCGATCCCTCAGAGACCGGCAACGGCGTGAAGAAGATTCTAATCGCTCGATGACCCAAAATGAA
GAAGGATTTATAACAGATCTGTTGAAGTCCGCTCTGAGGTTCAAGAACTGTGAGCCGTTTCTTGGTTCTGTGCGCTGCAC
TTGCTCTTTGGACATCAATGGTCTCCATGAGGCGGTTGACGCTAGTCTCCCGGTGACAAAAGCAGTGGTCATGCTGACTTACA
TCTAGTGAGTGTCTACCCGCCGAACAACCGGATCTTCTCTCTCTTCTTATCTGCAAGATGCCGTTGGGAGGTTCAAG
ATCATGGAGGACCATGTGCCCTCTGTTGCTGGTGGACAGGAGGAAAATGTTATAAACAGTCAACCACAGGGAACGGGA
AACCTGCACAGGCGGTAGTGAGAGGGGGCATAAGAATGCTACAATCTGGGACCTCCGAAACACCAAGCTGCGGAGGACG
TGGTGAAGGTTATTTAAAGTGGCTGCTCGCCGCTTAAACGATGGCACTGCTGAAATATGGTACGCTCTGTGCGCGCTTT
CTCGAGAGAAGCGGATGCGCAAGGCGGAGGTGGGACAGTGGAGACTTTGGAGAAAAGGGCGCTCAAAAGGAGACGTGT
CAACTTCTGATGATATGCCAGAGAGCCGCCGCCCTACGTTCCGCCCATGTATCCGTTTCCGCCGAACCTGAACACAGAATG
GGCTGTTACTACGAACTCTTCAGGAGGTTAT
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Figure 5. Sequencing Result of the *Toxoplasma gondii* GRA14 Fragment.

the parasites with serum; it was found that these substances, as regulated secretory proteins, are exported through the Golgi apparatus in response to specific stimulation.³⁴ Widely detected and continuously expressed in hosts infected with *T. gondii*, this has made them as appropriate candidates for tracking the infection and pertinent events. Serological assay is actually the most popular laboratory diagnostic tool for toxoplasmosis designed based on detection of *T. gondii* GRA proteins.³⁵ The present study, for the first time showed clone and subclone *GRA14* gene in pTG19 and pET32a prokaryotes expression vector were provided.

The novel dense granule protein GRA14 was secreted into the vacuole, and can be transferred intravascular during infection. Also, GRA14 has an unexpected topology in parasitophorous vacuole membrane. The findings show that C terminus of GRA14, facing the host cytoplasm and its N terminus facing the vacuolar lumen. Regardless of the possible roles of GRA14, there is no study to evaluate the potential of GRA14 as a vaccine candidate against *T. gondii*.^{22,35} Therefore, we constructed the recombinant DNA vaccine encoding the GRA14 antigen of *T. gondii* and evaluated its ability to induce immune response and protective effect in BALB/c mice against acute toxoplasmosis. Several antigens of *T. gondii* have been used in different vaccination studies and varying results have been reported.³⁶

Guo et al cloned a 760bp *ROP1* gene fragment into pBV220 expression plasmid and assessed the immune responses

of Balb/c mice vaccinated by this plasmid.³⁷ Meeka et al cloned fragments of ROP1 and SAG1 genes into pRP261 expression plasmid and used the resulting expressed proteins to serological diagnosis of toxoplasmosis in patients suffering from acute and chronic toxoplasmosis.³⁸

The successful use of recombinant antigenic proteins for the detection of anti-*T. gondii*-specific antibodies has been reported by a number of authors.³⁹ The recombinant antigens were assessed either separately or by combining several proteins to increase diagnostic sensitivity.³⁹ Jalallou et al⁴⁰ expressed SAG1 in pET32a and used rSAG1 for detection *T. gondii* specific IgG in human sera by ELISA. Chahed Bel-Ochi et al synthesized and evaluated a whole sequence of the recombinant *T. gondii* SAG1 antigen for screening of Toxoplasma IgG antibodies. They amplified PCR product and cloned it into the expression vector plasmid pET22b.⁴¹ Igarashi et al cloned and expressed entire ROP2, GRA5 and GRA7 open reading frames of *T. gondii* RH strain into pET102/D-TOPO⁺ vector which contains thioredoxin and polyhistidine tags at the C and N terminal ends and are expressed in *E. coli* BL21 (DE3).⁴² Mohabati et al developed a recombinant ROP1 protein and assessed its antigenicity against human infected sera by Western blot assay. A 1234 bp DNA fragment of *ROP1* gene from *T. gondii* RH strain was amplified by PCR cloned into pTZ57R/T vector and used pET-15b for its expression.⁴³ Eslamirad et al cloned a 760 bp *ROP1* gene from *T. gondii* RH strain into pTZ57R vector and then sub-cloned it in eukaryotic expression vector, pcDNA3.⁴⁴ In another study, Eslamirad et al cloned an 1183 bp ROP1 fragment into pTZ57R/T cloning vector and sub-cloned into pET32a expression plasmid. Wang et al cloned and expressed recombinant proteins GRA1 and GRA7 from *T. gondii* GT1 strain to use in serodiagnosis of toxoplasmosis in dogs by indirect ELISA. The GRA1 and GRA7 DNA fragments were amplified, and cloned into expression vector pET-28a to construct recombinant plasmids pET28- GRA1 and pET28-GRA7, which were confirmed by restriction enzymes and sequencing, and were transformed into *Escherichia coli* BL21 (DE3).⁴⁵ Sadeghiani et al developed recombinant GRA7 antigen and evaluated antigenicity of purified protein in immunoblot using human sera. They used genomic DNA from tachyzoites of *T. gondii*, RH strain for DNA extraction and amplified a 670 bp fragment encoding amino acids 18 to 236 by PCR. They utilized pTZ57R/T as cloning vector and pET-28b as expression vector.⁴⁶ Hruzik et al recognized TgSUB1 as a potential marker for serodiagnosis of acute toxoplasmosis and used recombinant TgSUB1 in a line blot assay. They isolated RNA from tachyzoites of *T. gondii* strain NTE and used it for cDNA synthesis. The C-terminal fragment of the *SUB1* gene (nucleotide positions 1657 to 2316) on the basis of the antigenic index containing amino acid residues 552 to 777 was prepared by PCR amplification and then 660 bp PCR product was cloned into pQE-30 expression vector.⁴⁷ In the performed study, the 1227 bp fragment of *T. gondii* GRA14 gene was cloned into pET32a expression plasmid and ready to produce protein and using of that in diagnostic studies, recombinant vaccine production and immunologic surveys in animal models.

Conclusions

We successfully cloned *GRA14* gene of *T. gondii* into pTG19 and pET32a plasmid, which was confirmed following restriction enzymatic digestion and gene sequencing. The cloned gene can either be subsequently to produce large amount of the GRA14 recombinant protein for developing serological diagnostic assays, or be used as protein vaccine to stimulate immune responses against *T. gondii*. However, further studies need to be carried out in order to produce recombinant GRA14 protein and to evaluate its immunoreactivity and immunogenicity, as well.

Authors' Contributions

All authors contributed equally to this research.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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