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Research article

Comparative study on *Toxoplasma gondii* dense granule protein 7, peroxiredoxin 1 and 3 based on bioinformatic analysis tools

Ragab M. Fereig[1*] **and Hanan H. Abdelbaky**[2]

[1] Department of Animal Medicine, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt
[2] Doctor of Veterinary Science, Veterinary clinic, Qena, Egypt

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***Corresponding authors:**
Ragab M. Fereig
ragab.feraeg2@vet.svu.edu.eg

Abstract

Toxoplasmosis remains a devastating protozoan disease induced by *Toxoplasma gondii* (*T. gondii*) that induces extreme hazards in both medical and veterinary fields. Our previous studies revealed the high immunogenicity and antigenicity of *T. gondii* peroxiredoxin (TgPrx) 1, 3, and TgGRA7. Herein, the comparison of TgPrx1, TgPrx3, and TgGRA7 was conducted using bioinformatics analysis tools. In this computational comparison, the physico-chemical, morphometric, immunogenic, and antigenic properties were analyzed. Analyses of complete coding sequences showed the probability of signal peptides and transmembrane domains only in the case of TgGRA7. NetPhos server-based prediction revealed 23, 11, and 39 phosphorylation sites in TgGRA7, TgPrx1, and TgPrx3 proteins, respectively. The secondary structure of TgGRA7, TgPrx1, and TgPrx3 proteins were analyzed by PSIPRED servers. The percentage of the random coil and alpha-helix amino acids was higher in TgGRA7 (99.15%), followed by TgPrx3 (85.87%) and TgPrx1 (77.55%). The antigenic epitopes of the protein were predicted by analyzing the features of the IEDB server. The linear B-cell epitope regions prediction of TgGRA7 showed the maximum estimated length (118 amino acid residues). In addition, antigenicity and hydrophilicity index showed similar tendencies among the three tested proteins, TgGRA7, TgPrx1, and TgPrx3. Thus, the current computational analyses represented TgGRA7, TgPrx1, and TgPrx3 proteins as efficient diagnostic and vaccine candidates suggesting further research and assessments. Additional validation of bioinformatic analysis tools in predicting potent diagnostic and vaccine antigens will greatly contribute to the success of control policies against *T. gondii* and other infectious agents.

Keywords: *Toxoplasma gondii*, Bioinformatics, Antigen, Antibody, Vaccine

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Introduction

Toxoplasmosis is a heteroxenous zoonotic disease infecting approximately the third of the world's human population caused by the protozoan parasite *Toxoplasma gondii* (*T. gondii*). It invades almost all endothermic animals. *T. gondii* infections in humans are generally inapparent in immunocompetent individuals, and they cause a self-limiting disease. However, the effects of infection are much more severe in immunocompromised patients. Additionally, primary *T. gondii* infection can be transmitted vertically from the mother to the fetus and can induce severe diseases like abortion, cerebral lesions, and ocular complications.

Toxoplasmosis in animals, mainly sheep, goats, and pigs, is of great economic impact as it causes abortions, stillbirths, and neonatal loss. The consumption of food contaminated with tissue cysts of *T. gondii*, such as meat from infected livestock, is the main route of the parasite transmission to humans (Jones and Dubey, 2012; Robert-Gangneux and Dardé, 2012). Numerous and diverse parasite-derived molecules were evaluated as vaccine candidates. Some related to the major parasite organelles essential for the establishment and sustainability of infection, such as rhoptries, dense granules, and micronemes, while others were concerned with specified roles as profilin, actin depolarizing factor, and cyclophilin.

T. gondii derived enzyme proteins were widely targeted as vaccine candidates and reported as potent protective factors against the parasite challenge, such as protein disulfide isomerase (Wang et al., 2013), calcium-dependent protein kinase-3 (Zhang et al., 2013), and glutathione reductase (Hassan et al., 2014). Dense granule protein 7 (TgGRA7) is one of the extensively studied molecules of *T. gondii* in various *Toxoplasma*-related research, including vaccine and diagnosis aspects. The TgGRA7 accumulates within

the parasitophorous vacuole in tachyzoite-infected cells and co-localizes with its delimiting membrane. In bradyzoite-infected cells, TgGRA7 is present within the host cell cytoplasm as detected by immunofluorescence staining (Fischer et al., 1998). Additionally, TgGRA7 exhibited a robust efficiency as a diagnostic antigen for detecting *T. gondii* antibodies in various animal species either on experimental or field platforms (Jacobs et al., 1999; Terkawi et al., 2013; Fereig and Nishikawa, 2016).

Despite its robustness as a diagnostic antigen, few studies have investigated and nominated TgGRA7 as a vaccine antigen for *T. gondii*. However, a recent study revealed the success of the recombinant DNA vaccine of TgGRA7 in eliciting humoral and cellular immune responses and conferring protection in immunized mice (Sun et al., 2021). Peroxiredoxin (Prx) is a recently described family of antioxidants that are highly conserved in eukaryotes and prokaryotes (McGonigle et al., 1998), which has been called thioredoxin peroxidase (Tpx) or thiol specific antioxidant (TSA). The Prx acts as an antioxidant enzyme by sweeping hydrogen peroxide and hydroxyl radicals. The catalytic mechanism of the enzyme involves the redox-active cysteine, which is highly conserved in the vicinity of the 47^{th} position of its amino acid sequence (Chae et al., 1994). Three Prxs were identified in *T. gondii* tachyzoites; 2-cys cytosolic Prx1, 1–cys cytosolic Prx 2, and 2-cys mitochondrial Prx 3, protecting them from oxidative stressors (Ding et al., 2004; Akerman and Müller, 2005).

Parasitic peroxiredoxins have shown potency as vaccine antigens as reported in the case of the mouse and non-human primate models against cutaneous leishmaniasis induced by *Leishmania major* (Campos-Neto et al., 2001) and for mice against microfilaria *Brugia malayi* infections (Anand et al., 2008). Furthermore, goats immunized with *Fasciola hepatica* recombinant peroxiredoxin exhibited a 33.04% reduction in fluke burdens and lowered gross and microscopical liver damage compared to the control group (Mendes et al., 2010). Our previous studies on TgPrx1 and TgPrx3 demonstrated the usefulness of both antigens as vaccine candidates against *T. gondii*. Immunization of mice with recombinant protein of TgPrx1 and TgPrx3 enhanced the survival rates and decreased cerebral parasite burdens against the control groups. Simultaneously, both TgPrx1 and TgPrx3 elicited humoral and cellular immunities (Fereig et al., 2016, 2017). Moreover, our recent study revealed that mice with *T. gondii* still experienced a severe infection compared to those infected with mutant *T. gondii* (Fereig and Nishikawa, 2022).

Bioinformatics is one of the most recent research fields applied to improve the tackling and monitoring of biological issues (Romano et al., 2011). It is extensively applied to assess gene and protein expression and to predict protein structure, antigenicity, immunogenicity, and general characteristics. Such aspects will greatly improve our understanding of protein molecules and assist the researchers in selecting the correct proteins or specific epitopes for vaccine development or diagnosis research.

Herein, the bioinformatic analysis was conducted on TgPrx1 and TgPrx3; the recently characterized immunogenic *T. gondii* candidates and both antigens were compared with TgGRA7; the highly recognized immunogenic and antigen molecule. The amino acid sequence of TgGRA7, TgPrx1, and TgPrx3 was assessed using numerous bioinformatics tools to analyze its physico-chemical, antigenic, and immunogenic properties. All of the studied antigens might be useful diagnostic and vaccine antigens based on bioinformatic analysis tools.

Materials and methods

Retrieval of complete amino acid sequence

The sequence of TgGRA7, TgPrx1, and TgPrx3 amino acids was obtained from the GenBank database in FASTA format with the accession number (EPT30138.1), (AAG25678.2), and (AAO62417.1), respectively (https://www.ncbi.nlm.nih.gov). Then, the multiple sequence alignment of the three proteins was applied by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Post-translational modification (PTM) sites and transmembrane domains

SignalP online tool (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) was used to determine signal peptides in the sequence. The protein can have a Sec signal peptide (Sec/SPI), a Lipoprotein signal peptide (Sec/SPII), a Tat signal peptide (Tat/SPI), or no signal peptide at all (Other). Also, the potential transmembrane domains of TgGRA7, TgPrx1, and TgPrx3 were predicted using TMHMM 2.0 online server (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) (Petersen et al., 2011). The isoelectric point (pI) of TgGRA7, TgPrx1, and TgPrx3 was measured using an isoelectric point online calculator (http://isoelectric.org/). The molecular weight and diameter of selected amino acids of TgGRA7, TgPrx1, and TgPrx3 were calculated using Calc Tool to predict the protein size (https://www.calctool.org/CALC/prof/bio/protein_size).

Physical, chemical, and morphometric characteristics

Serine, threonine, and tyrosine phosphorylation sites were demonstrated by ensembles of neural networks in the NetPhos 3.1 server (https://services.healthtech.dtu.dk/service.php?NetPhos-3.1). To clarify the secondary structure of TgGRA7, TgPrx1, and TgPrx3, a web server of position-specific iterated prediction (PSIPRED) analysis on outputs from PSI-BLAST (http://bioinf.cs.ucl.ac.uk/psipred) was used (Buchan and Jones, 2019).

Prediction of B-cell epitopes, antigenicity, and hydrophilicity

The Immune Epitope Database (IEDB) (http://tools.immuneepitope.org/bcell/) was used for predicting the B-cell epitopes of TgGRA7, TgPrx1, and TgPrx3 by the IEDB server based on the physicochemical properties of amino acids, including hydrophilicity (Parker

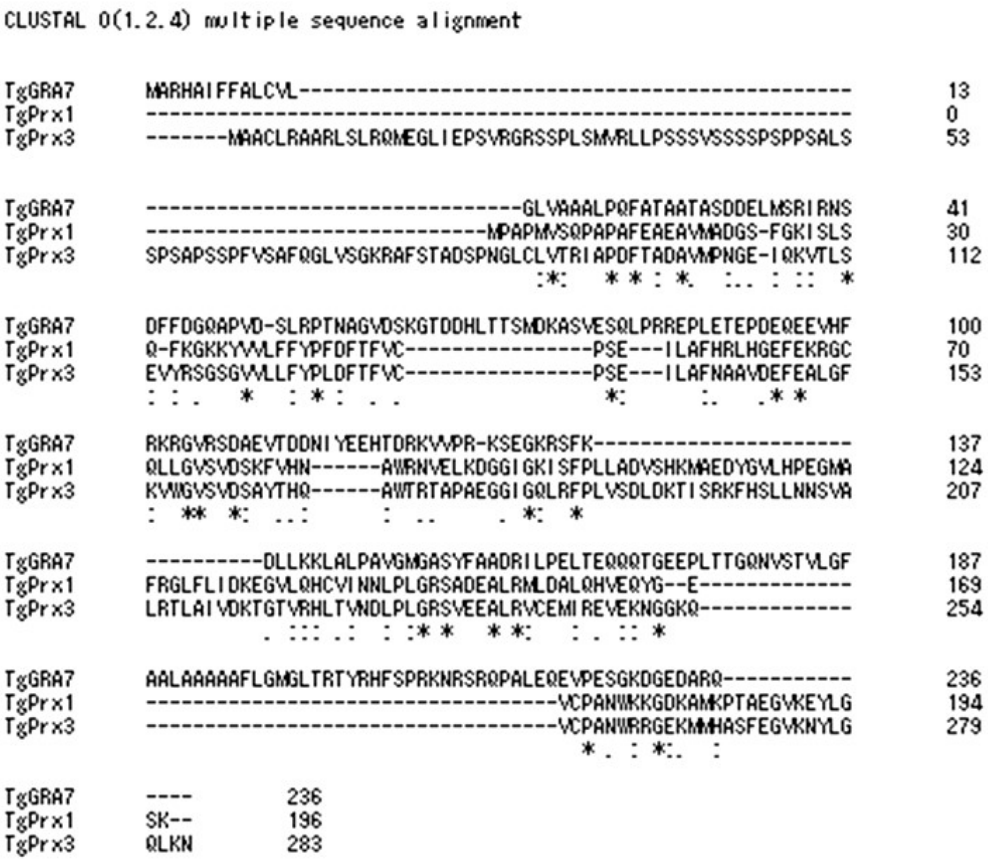

Figure 1: Multiple sequence alignment of TgGRA7, TgPrx1, and TgPrx3 amino acids. Sequence alignment was applied by CLUSTALO against amino acid sequences. Similarity was 45.41%, 18.88% and 14.41% for TgPrx1 vs TgPrx3, TgGRA7 vs TgPrx1, and TgGRA7 vs TgPrx3, respectively (https://www.ebi.ac.uk/Tools/msa/clustalo/).

et al., 1986), and antigenicity (Kolaskar and Tongaonkar, 1990).

Results and discussion

Computer-based assessment of different *T. gondii* proteins involved in various life cycle stages can open new insights towards discovering potent diagnostic and vaccine antigen candidates by identifying highly immunogenic, nonallergenic, and nontoxic B- and T-cell epitopes (Flower, 2013). The present study was performed to evaluate the crucial biochemical features and immunogenic epitopes of the TgGRA7, TgPrx1, and TgPrx3 proteins using different bioinformatics servers. The amino acid sequences of TgGRA7, TgPrx1 and TgPrx3 proteins were obtained from GenBank.

The amino acid sequences of TgGRA7 protein include 236 amino acid residues with an estimated molecular weight of 28.32 kDa, with a diameter equal to 4.26403 nanometers (nm). Consistently, TgPrx1 and TgPrx3 include 196 and 283 amino acids, 23.52 and 33.96 kDa, and 4.00806 and 4.53014 nm, respectively. Antigens with MW of <5–10 KDa are considered weak immunogens (Berzofsky and Berkower, 1993). Then, CLUSTALO-based multiple sequence alignment of TgGRA7, TgPrx1, and TgPrx3 was applied. Homology was 45.41%, 18.88% and 14.41% for TgPrx1 vs TgPrx3, TgGRA7 vs TgPrx1, and TgGRA7 vs TgPrx3, respectively (Figure 1). These results demonstrated the high similarity of TgPrx1 and TgPrx3, which may be attributable to both antigens being members of the same protein family (Ding et al., 2004; Akerman and Müller, 2005). Such protein also showed a similar tendency for immunological performances (Fereig and Nishikawa, 2016; Fereig et al., 2017).

Based on the SignalP-5.0 and TMHMM output, no signal peptides or transmembrane domains were found for TgPrx1 or TgPrx3 (Figure 2 and Figure 3). On the contrary, TgGRA7 amino acid residues include a signal peptide at the cleavage site between 26-27 amino acids ATA-AT. In addition, two transmembrane domains were detected in TgGRA7 amino acid residues (Figure 2 and Figure 3). Signal peptides and transmembrane domains are short peptides that play a significant role in recombinant protein production (Petersen et al., 2011; Peng et al., 2019). However, a previous study succeeded in producing a recombinant antigen of TgGRA7 lacking the first 27 amino acid sequences with high diagnostic potentials (Terkawi et al., 2013).

Whereas the theoretical average of the isoelectric point (PI) of TgGRA7, TgPrx1, and TgPrx3 was 5.13, 6.1, and 8.08, respectively (Figure 4). The accuracy of PI estimation through the amino acid sequence is

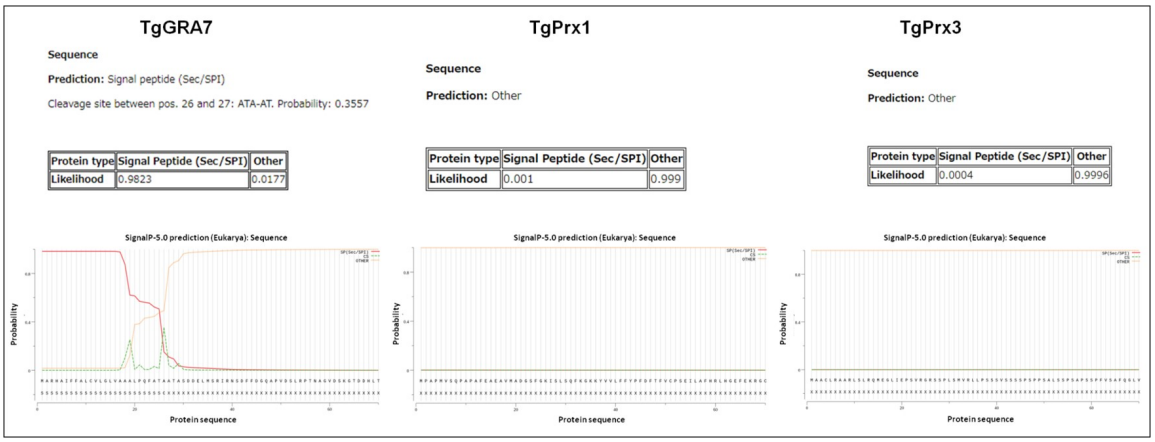

Figure 2: Signal peptide prediction of the TgGRA7, TgPrx1, and TgPrx3 proteins using SignalP-5.0 online tool. SP (Sec/SPI): type of signal peptide predicted; CS: the cleavage site; Other: the probability that the sequence does not have any signal peptide (https://services.healthtech.dtu.dk/service.php?SignalP-5.0).

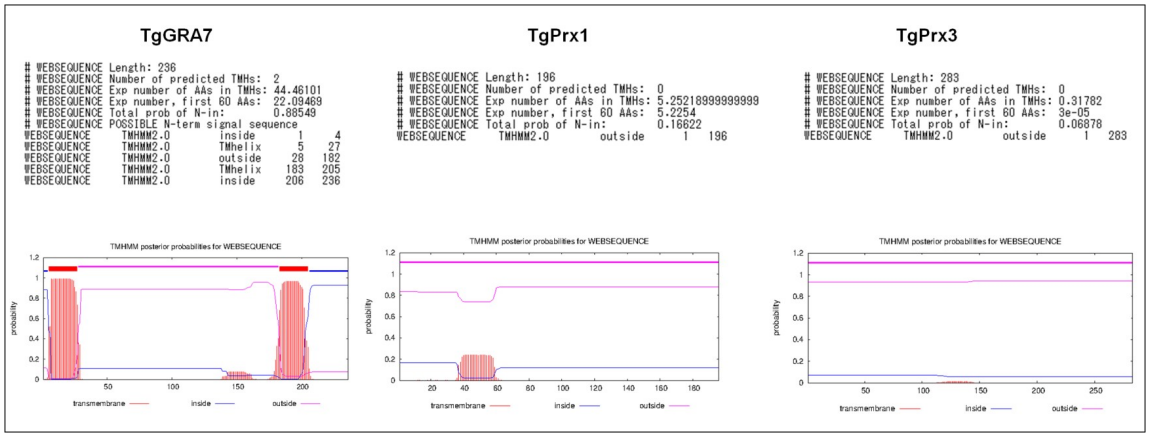

Figure 3: Transmembrane domains expected in TgGRA7, TgPrx1, and TgPrx3 proteins. Some statistics and a list of the location of the predicted transmembrane helices and the predicted location of the intervening loop regions. Length: the length of the protein sequence; the number of predicted TMHs: the number of predicted transmembrane helices; Exp number of AAs in TMHs: the expected number of amino acids in transmembrane helices. If this number is larger than 18, it is very likely to be a transmembrane protein (or have a signal peptide); Exp number, first 60 AAs: the expected number of amino acids in transmembrane helices in the first 60 amino acids of the protein. If this number is more than a few, the predicted transmembrane helix in the N-term could be a signal peptide; total prob of N-in: the total probability that the N-term is on the cytoplasmic side of the membrane (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0).

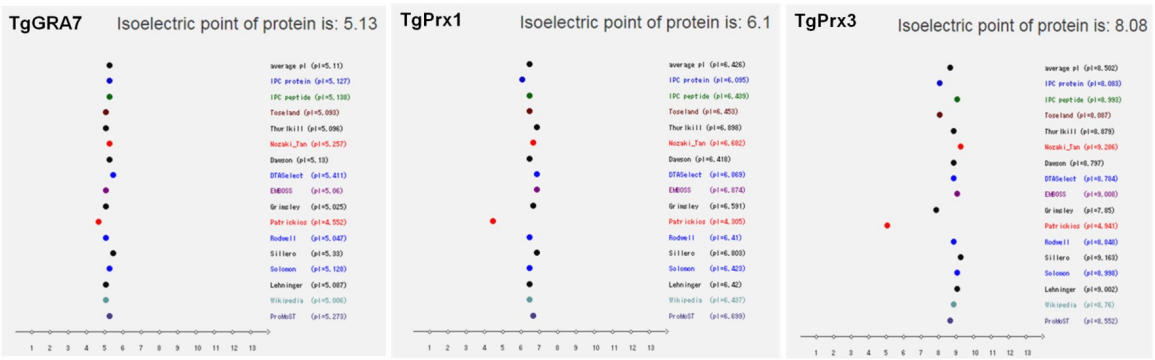

Figure 4: The theoretical isoelectric point pI of TgGRA7, TgPrx1, and TgPrx3 proteins showing average and different predictors values (http://isoelectric.org/).

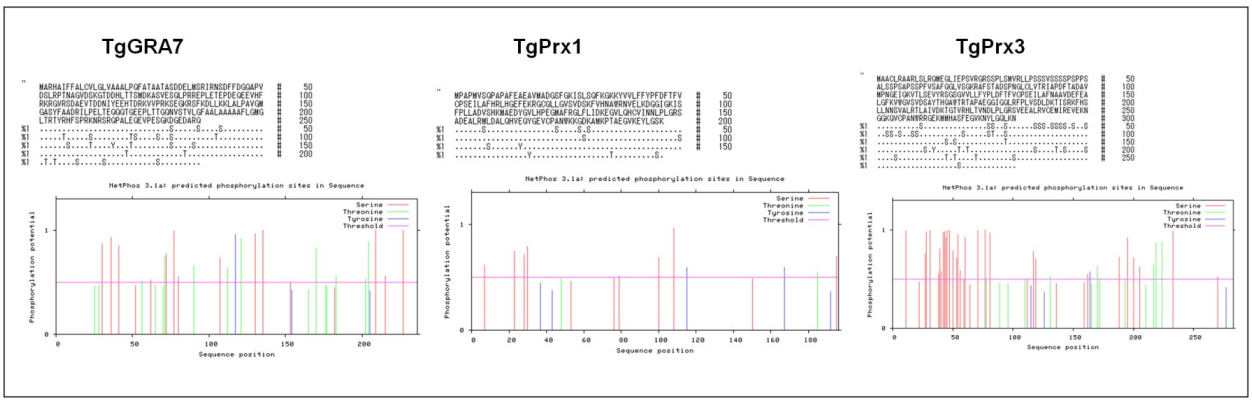

Figure 5: NetPhos server output for TgGRA7, TgPrx1, and TgPrx3 protein phosphorylation sites. The number of predicted sites, based on S (serine), T (threonine) and Y (tyrosine); and the prediction diagram of TgGRA7, TgPrx1, and TgPrx3 proteins phosphorylation sites (https://services.healthtech.dtu.dk/service.php?NetPhos-3.1).

useful for predicting many analytical biochemistry and proteomics processes (Kozlowski, 2016).

Phosphorylation sites in TgGRA7, TgPrx1, and TgPrx3 proteins were detected by the NetPhos server based on serine, threonine, and tyrosine amino acids. Analysis results revealed phosphorylation sites at 23, 11, and 39 positions of the TgGRA7, TgPrx1, and TgPrx3 protein sequences, respectively. In the case of TgGRA7, 13 serine (S), nine threonines (T), and one tyrosine (Y) were reported. TgPrx1 includes eight serine (S), one threonine (T), and two tyrosine (Y). While TgPrx3 includes 30 serine (S), eight threonines (T), and one tyrosine (Y) (Figure 5). The presence of phosphorylation sites reveals the high possibility of post-translational modifications (PTMs) processes in protein. PTMs in eukaryotic cells such as parasites are important in selecting the correct expression system for producing recombinant proteins and thus might affect the biological function of the protein (Hansson et al., 2000; Walsh, 2005).

The secondary structures of TgGRA7, TgPrx1 and TgPrx3 proteins were analyzed by PSIPRED servers. Three major secondary structure constituents in the TgGRA7 protein consisted of 53.4% random coil, 45.76% alpha helix, and 0.85% extended strand. TgPrx1 comprises 45.41% random coil, 32.14% alpha helix, and 22.45% extended strand. While, TgPrx3 protein contains 54.77% random coil, 31.1% alpha helix, and 14.13% extended strand. The high portion of alpha-helix that exist in the internal portions of the protein will maintain the protein's structure resulting in better interaction with antibodies (Figure 6) (Shaddel et al., 2018). In addition, the random coil plays an essential role in the flexible nature of the protein (Zhou et al., 2016). The secondary structure is determined by the hydrogen bonds pattern between carboxyl oxygen and amino hydrogen atoms in a polypeptide chain, with α-helices and β-structures being the most prevalent forms (Yada et al., 1988).

Using the IEDB server, high values calculated for epitopic regions with good hydrophilicity and strong antigenicity were identified. The linear B-cell epitope regions are predicted by Bepipred Linear Epitope Prediction 2.0. TgGRA7 showed 3 expected peptides and a threshold of 0.500 with an estimated maximum length ranging from 24 to 140 amino acids. TgPrx1 revealed nine predicted peptides and a threshold of 0.500 with an estimated maximum length ranging from 5 to 30 amino acids. TgPrx3 showed eight expected peptides and a threshold of 0.500, with an estimated maximum length ranging from 10 to 117 amino acids (Figure 7).

Regarding antigenicity, TgGRA7 consists of seven expected peptides with high antigenicity index were predicted by Kolaskar & Tongaonkar Antigenicity with a threshold of 1.013 with an estimated maximum length ranging from 4 to 26 amino acids. In the case of TgPrx1, nine expected peptides with antigenicity threshold 1.042 and estimated maximum length ranging from 47 to 60 amino acids were reported. Also, TgPrx3 revealed 11 expected peptides with an antigenicity threshold of 1.043, and an estimated maximum length ranging from 119 to 138 amino acids (Figure 8). Kolaskar & Tongaonkar Antigenicity index is based on the analysis of finding the hydrophobic residues Cys, Leu, and Val on the surface of a protein, the more likely to be a part of antigenic sites. Application of this method to a large number of proteins has shown 75% accuracy was reported, which is better than most of the known methods (Kolaskar and Tongaonkar, 1990).

Additionally, the Parker Hydrophilicity Prediction scale detected the hydrophilicity of TgGRA7, TgPrx1, and TgPrx3 proteins and showed high water solubility with thresholds of 2.296, 0.962, and 1.293, respectively (Figure 9). Based on the score of residues, the larger score represented a higher probability of being part of the epitope (yellow graphs). Parker hydrophilicity prediction implies surface amino acids that are potentially antigenic regions (Parker et al., 1986). These results revealed the high efficacy of the three antigens (TgGRA7, TgPrx1, and TgPrx3) for interacting with immune cells and generating specific antibodies. Such effect was also reported in numerous previously published studies.

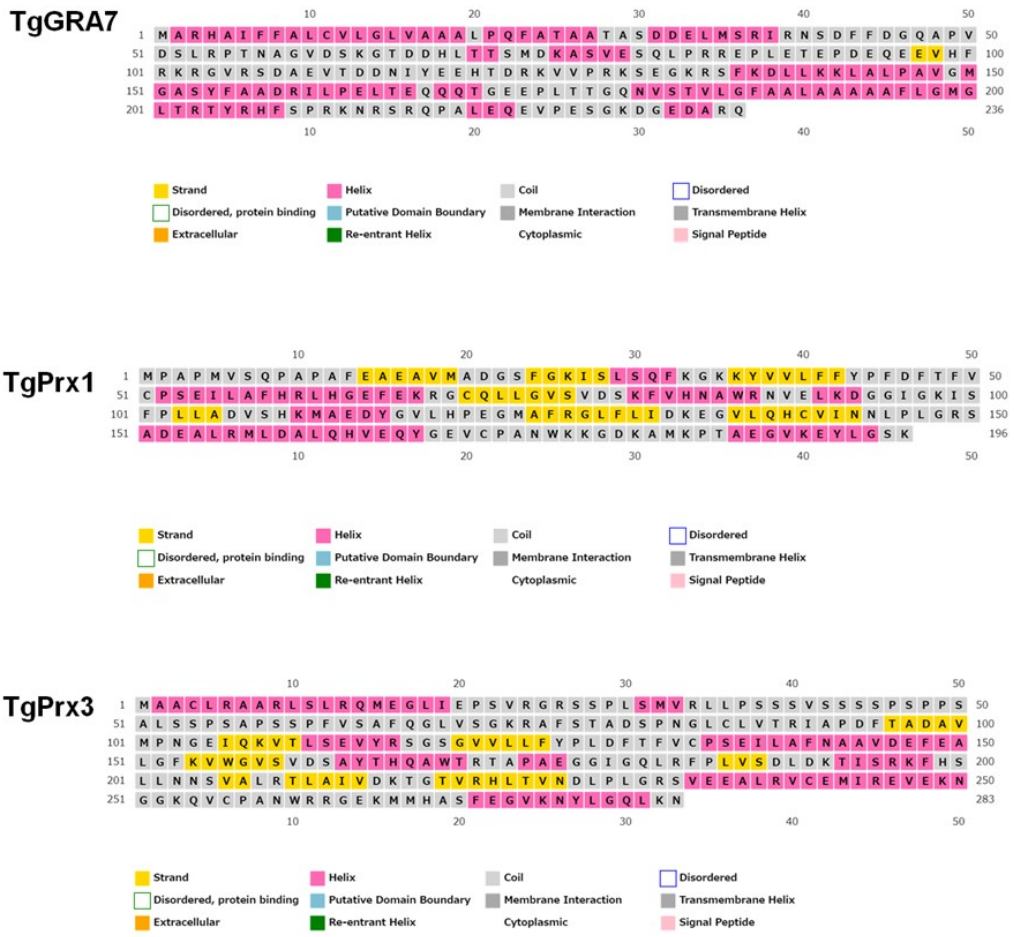

Figure 6: Graphical output of secondary structure of TgGRA7, TgPrx1, and TgPrx3 proteins. Illustration of the secondary structure prediction of TgGRA7, TgPrx1, and TgPrx3 protein using PSI-BLAST (http://bioinf.cs.ucl.ac.uk/psipred)showingPSIPRED).

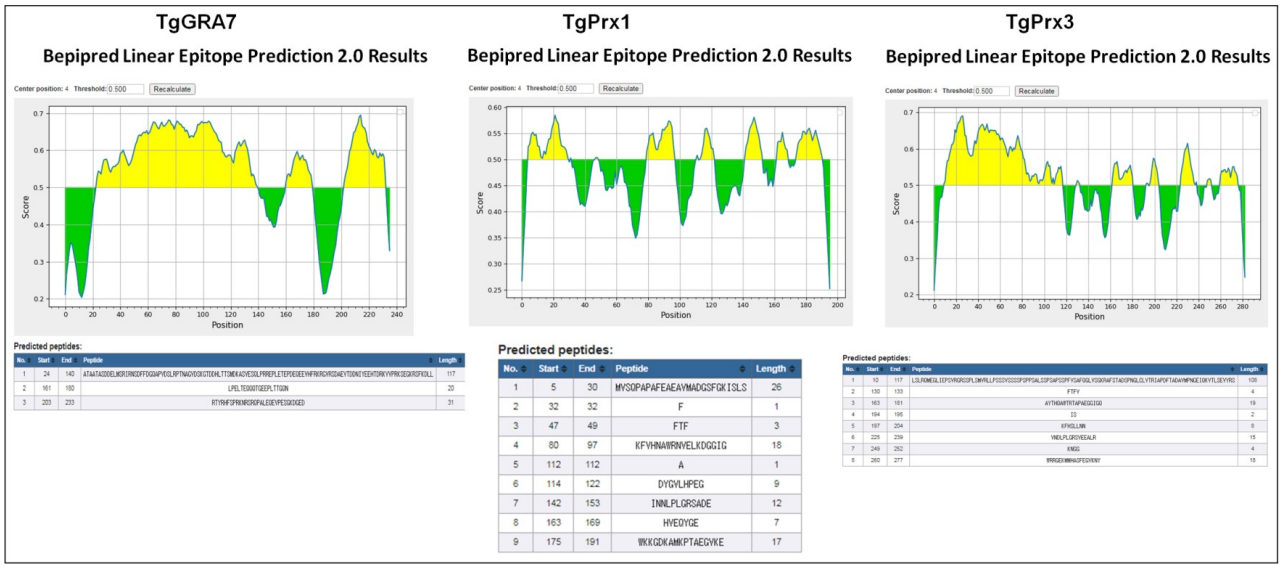

Figure 7: Bepipred linear epitopes prediction of immunogenicity of TgGRA7, TgPrx1, and TgPrx3 proteins. The X-axis and y-axis represent position and score, respectively. The horizontal line indicates the threshold or the average score. Yellow colors (above the threshold) indicate favorable regions related to the properties of interest. The green color (below the threshold) indicates the unfavorable regions related to the properties of interest. The results were analyzed by the Immune Epitope Database (IEDB) (http://tools.immuneepitope.org/bcell/).

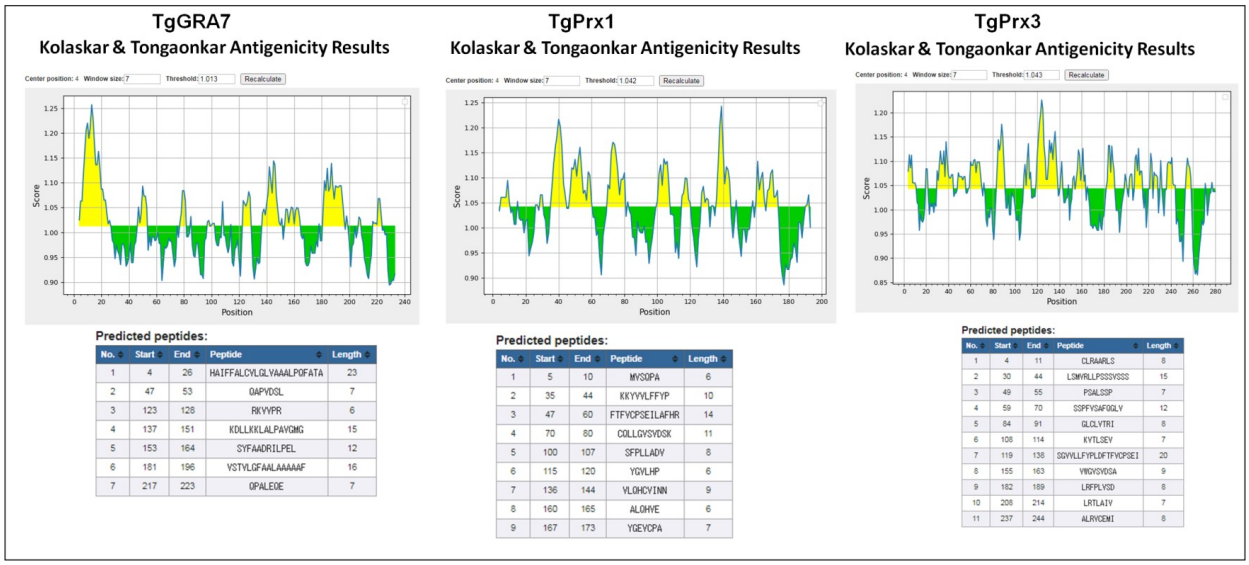

Figure 8: Antigenicity of TgGRA7, TgPrx1, and TgPrx3 proteins protein. The X-axis and y-axis represent position and score, respectively. The horizontal line indicates the threshold or the average score. Yellow colors (above the threshold) indicate favorable regions related to the properties of interest. The green color (below the threshold) indicates the unfavorable regions related to the properties of interest. The results were analyzed by the Immune Epitope Database (IEDB) (http://tools.immuneepitope.org/bcell/).

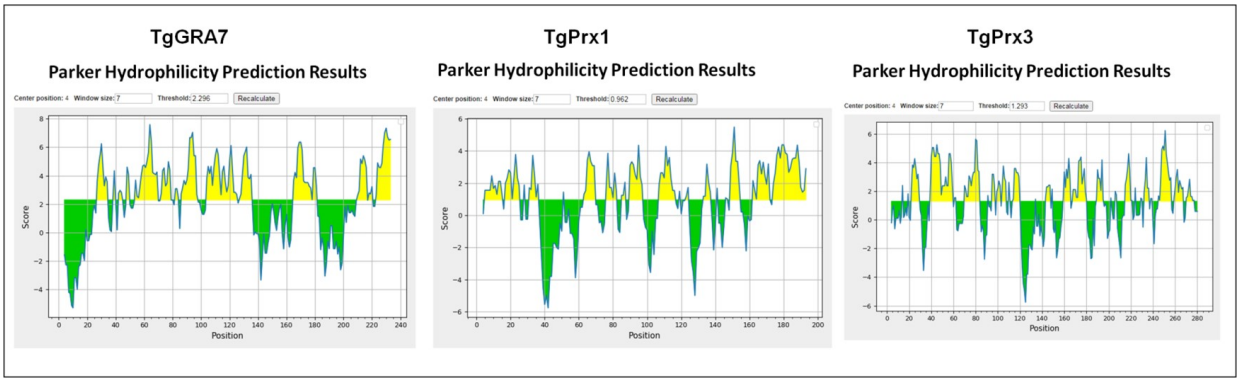

Figure 9: Hydrophilicity of TgGRA7, TgPrx1, and TgPrx3 proteins. The X-axis and y-axis represent position and score, respectively. The horizontal line indicates the threshold or the average score. Yellow colors (above the threshold) indicate favorable regions related to the properties of interest. The green color (below the threshold) indicates the unfavorable regions related to the properties of interest. The results were analyzed by the Immune Epitope Database (IEDB) (http://tools.immuneepitope.org/bcell/).

Vaccination of mice with recombinant DNA vaccine of TgGRA7 alone or those formulated in calcium phosphate nanoparticles induced a strong antibody response, cellular immunity, and significant protection compared to non-immunized mice (Sun et al., 2021). Consistently, TgGRA7-based ELISA exhibited excellent performance in serosurvey of specific antibodies of *T. gondii* in different animal species (Fereig et al., 2016). In the case of TgPrx1 and TgPrx3, our previous studies revealed the role of both antigens as immunomodulatory and vaccine antigens (Fereig and Nishikawa, 2016; Fereig et al., 2017; Fereig and Nishikawa, 2022). Regarding the diagnostic potentials of TgPrx1 and TgPrx3, the data on such aspects are scarce. However, a previous study revealed that the recombinant TgPrx1 prepared in dot-immunogold-silver staining (Dot-IGSS) could detect IgG antibodies in serum from mice and pregnant women (Liu et al., 2020). In the same context, our previous study showed that rTgPrx1-based indirect ELISA detected specific antibodies in mice infected with *T. gondii* (strain PLK), and purified anti-TgPrx1 IgG detected secreted antigen in ascites fluid of *T. gondii* (strains RH or PLK)-infected mice (Fereig et al., 2017).

Conclusion

The current study provides a detailed explanation of the bioinformatic analysis aspects of TgGRA7, TgPrx1, and TgPrx3 proteins, including their physicochemical characteristics, transmembrane domains, secondary structures, and B-epitopes. Based on the findings, such proteins revealed an acceptable antigenicity score. Also, these proteins contain many potential epitopes of B-cells, confirming that TgGRA7, TgPrx1, and TgPrx3 can be considered an

appropriate vaccine candidate against *T. gondii*. This research presented important fundamental and theoretical evidence for further in-vivo investigations on such proteins to establish an effective vaccine against acute and chronic *T. gondii* infection.

Article Information

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Conflict of Interest. The authors declare no conflict of interest.

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