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Original Article

Toxoplasma gondii Microneme Protein 3 (TgMIC3): Computational Probing for Improved Vaccine Design

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Received 15 Oct 2025
Accepted 13 Jan 2026

Keywords:
Toxoplasma gondii,
Bioinformatics,
In silico,
Microneme proteins,
Vaccine

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Abstract

Background: Microneme protein 3 (MIC3) is a key adhesion molecule in *Toxoplasma gondii* that is expressed during multiple stages of infection. We aimed to computationally characterize the immunological and structural features of the *T. gondii* MIC3 protein to assess its potential suitability as a vaccine candidate.

Methods: A comprehensive set of bioinformatics tools and web servers was employed to predict the physicochemical properties, allergenicity, antigenicity, solubility, post-translational modification sites, subcellular localization, transmembrane domains, signal peptides, secondary and tertiary structures, potential B- and T-lymphocyte epitopes, and simulated immune responses of the TgMIC3 protein.

Results: A total of 75 post-translational modification sites were predicted in TgMIC3. Furthermore, secondary structure analysis using GOR IV, SOPMA, and NetSurfP-3.0 indicated that random coils and extended strands were the predominant structural elements. In addition, several high-affinity B- and T-cell epitopes were identified across the protein sequence. Subsequent structural validation revealed that 82.91% and 98.60% of residues were located in favored regions in the initial and refined 3D models, respectively. The findings of the allergenicity and antigenicity assessments indicated that the MIC3 antigen seemed to be a non-allergen with an immunogenic nature. Moreover, immune simulation using the C-ImmSim server demonstrated that TgMIC3 could induce robust humoral and cell-mediated immune responses following three simulated antigen administrations.

Conclusion: This study provides foundational computational evidence supporting the potential of TgMIC3 as a vaccine antigen and offers a useful framework for future experimental investigations targeting vaccine development against acute and latent toxoplasmosis.

Introduction

Toxoplasma gondii is an intracellular protozoan, and based on published articles, more than one-third of the global human population is chronically affected by this zoonotic parasite (1). It can infect a wide variety of warm-blooded vertebrate species (2). Throughout its life cycle, the parasite exists in at least three distinct forms, including sporozoites in oocysts, bradyzoites in tissue cysts, and tachyzoites (3). In addition to vertical transmission from mother to fetus, humans acquire the infection through the consumption of undercooked meat containing tissue cysts, ingestion of raw or unwashed vegetables, ingestion of mature oocysts, organ transplantation, and, rarely, blood transfusion (1). Among these forms, bradyzoites are associated with the chronic stage of infection, whereas tachyzoites are involved in the acute phase. *T. gondii* infection is generally asymptomatic in immunocompetent individuals, whereas severe clinical manifestations with a poor prognosis may occur in immunocompromised hosts (4).

At present, control strategies for toxoplasmosis mainly rely on pharmacological treatment during the acute phase of infection, which is often associated with several side effects. Furthermore, these drugs have limited efficacy against bradyzoites within tissue cysts and cannot completely eliminate the parasite from the host (5). Given the widespread seroprevalence of *T. gondii* worldwide and its associated clinical and economic consequences, together with the inability of current therapies to target tissue cysts, the development of an effective vaccine against *T. gondii* is considered essential for preventing infection (6).

In recent years, numerous parasite proteins have been proposed as potential vaccine candidates against *T. gondii* infection; however, most have provided only partial protection. Consequently, the selection of appropriate antigens represents a critical initial step in the development of a safe and effective vaccine. Four well- characterized protein families, namely microneme proteins (MICs), dense granule antigens (GRAs), rhoptry proteins (ROPs), and surface antigens (SAGs), have been extensively investigated as vaccine candidates (6). Among these, MIC3 appears particularly promising because it plays a key role in parasite adhesion, is expressed in tachyzoite, sporozoite, and bradyzoite stages, and induces strong primary immune responses (7, 8).

In recent years, bioinformatics tools have been increasingly applied to identify potential B- and T-cell epitopes for vaccine development. These approaches enable the efficient selection of epitopes with high antigenic potential. Compared with conventional experimental methods, immunoinformatics offers advantages such as reduced time, lower cost, and acceptable predictive accuracy. Accordingly, bioinformatics-based epitope characterization has become valuable for both vaccine design and diagnostic applications (9, 10).

Therefore, the present *in silico* study aimed to predict the structural features, immunogenic epitopes, and key physicochemical properties of the TgMIC3 protein using a range of bioinformatics web servers.

Methods

The complete protein sequence of *T. gondii* MIC3 (accession no. TGME49_319560) was downloaded from the ToxoDB database in FASTA format and saved for further *in silico* analysis. All bioinformatics web servers employed in this study are provided as hyperlinks to facilitate accessibility and reproducibility.

The physicochemical parameters of the TgMIC3 protein, including the total number of amino acids, molecular weight (MW), theoretical isoelectric point (pI), aliphatic index, instability index, and other relevant features, were predicted through the ExPASy ProtParam web-based server (11).

The potential allergenicity of the TgMIC3 protein was evaluated using the AllerTOP v. 2.0, AllergenFP v. 1.0, and AlgPred 2.0 online servers. Protein antigenicity was assessed using ANTIGENpro and VaxiJen v2.0. Furthermore, protein solubility was predicted using the SOLpro and Protein-sol online tools (12).

Potential PTM sites of TgMIC3, including N-glycosylation, O-glycosylation, phosphorylation, and acetylation regions, were predicted using the NetNGlyc 1.0, NetOGlyc 4.0, NetPhos 3.1, and GPS-PAIL 2.0 web servers, respectively (12, 13).

The DeepLoc 2.0 online server was used to predict the subcellular localization of the TgMIC3 protein based on its amino acid sequence. Additionally, the DeepTMHMM server (release 1.0.24) was employed to identify potential transmembrane domains. Furthermore, signal peptide prediction was performed using the SignalP-6.0 server (12).

Secondary structure elements of TgMIC3 were predicted using the GOR IV and SOPMA servers (12, 13). In addition, the NetSurfP-3.0 tool was applied to analyze solvent accessibility, structural disorder, and secondary structure features, providing a high-resolution graphical representation. Furthermore, the SWISS-MODEL web server was used to construct the three-dimensional (3D) structure of TgMIC3 through homology modeling (14).

The best model generated by SWISS-MODEL was selected and refined using the GalaxyRefine tool to improve structural quality. Additionally, using the structure assessment tool, a Ramachandran plot was generated to validate the 3D structure of the TgMIC3 protein (13). The overall quality of the refined model was further evaluated using ProSA-web (11).

Several servers were used to predict linear B-cell epitopes. Initially, predictions were performed using the SVMTrip server (13). Subsequently, BepiPred 2.0 was applied with a threshold value of 0.56 to identify potential linear B-cell epitopes. The BcePred server was also used to evaluate linear epitopes based on physicochemical properties, including flexibility, hydrophilicity, polarity, surface accessibility, and beta-turn propensity, with reported prediction accuracies ranging from 52.92% to 57.53% (11). Default parameters were applied in this analysis. Additionally, the ABCpred server was employed (14) to predict linear B-cell epitopes using a window length of 16 amino acids (overlapping filter: ON) and a threshold value of 0.83.

The positional distribution of continuous epitopes has been associated with factors such as hydrophilicity, flexibility, surface accessibility, polarity, and antigenic propensity. Therefore, propensity scale-based methods were applied using the Immune Epitope Database (IEDB) to predict beta-turns, surface accessibility, flexibility, linear epitopes, hydrophilicity, and antigenicity as previously described (13). Discontinuous (conformational) B-cell epitopes were predicted using the ElliPro server based on the 3D structure of TgMIC3, with default settings (11).

The IEDB server was used to predict epitopes capable of binding major histocompatibility complex (MHC) class I and MHC class II molecules by calculating half-maximal inhibitory concentration (IC_{50}) values. According to IEDB recommendations, epitopes with percentile ranks ≤1% were selected to ensure broad immune coverage. Based on the human leukocyte antigen (HLA) reference set, the top 20 cytotoxic T-lymphocyte (CTL) and helper T-lymphocyte (HTL) epitopes associated with MHC-I and MHC-II, respectively, were screened. These epitopes were further evaluated for antigenicity, immunogenicity, and their ability to induce interferon-gamma (IFN-γ) or interleukin-4 (IL-4) using the IFNepitope and IL4pred servers, respectively (11, 13). Additionally, seven MHC-I alleles (H2-Kb, H2-Dd, H2-Kd, H2-Ld, H2-Kk, H2-Db, and H2-Lq) and five MHC-II alleles (H2-IAb, H2-IAd, H2-IAk, H2-IEd, and H2-IEk) were selected

to predict mouse-specific epitopes with lengths of 12-mer and 15-mer, respectively.

The C-ImmSim server was employed to simulate the immune response induced by the TgMIC3 protein *in silico* (15). The following parameters were applied: simulation volume of 10, random seed of 12345, 1050 simulation steps, and three injections administered at four-week intervals (time steps 1, 84, and 168).

Results

This protein contained 359 amino acid residues and had an estimated molecular weight of 37,884.24 Da. The theoretical isoelectric point (pI) was calculated to be 6.01, indicating a weakly acidic pH at which the net charge was zero. In addition, 41 negatively charged residues (Asp + Glu) and 34 positively charged residues (Arg + Lys) were predicted across the protein sequence. The estimated half-life of the protein in mammalian reticulocytes was 30 h. Furthermore, the protein demonstrated good stability (instability index: 39.32), moderate thermotolerance (aliphatic index: 49.97), and an overall hydrophilic character (GRAVY score: -0.435).

The TgMIC3 protein was predicted to be non-allergenic by AllergenFP v1.0 and AllerTOP v2.0, whereas AlgPred 2.0 classified it as an allergen without IgE epitopes. High antigenicity scores were obtained using VaxiJen v2.0 (0.8630) and ANTIGENpro (0.9578). In addition, moderate solubility was predicted by the Protein-Sol (0.507) and SOLpro (0.660) servers (Fig. 1A, B).

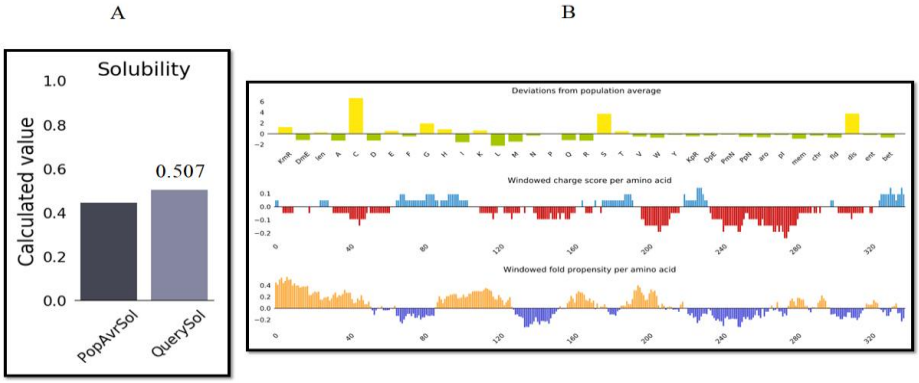

Fig. 1: **(A)** Solubility and **(B)** deviation from the population average, charge score, and fold propensity of TgMIC3 predicted using the Protein-Sol online server

A total of 75 PTM sites were predicted in TgMIC3. Specifically, 46 phosphorylation sites were identified, including 31 serine, 10 tyrosine, and 5 threonine residues (Fig. 2 A, B). In addition, 25 O-glycosylation sites were predicted at positions 49, 51, 55, 59, 61, 63, 64, 67, 69, 73, 79, 92, 101, 186, 260, 268, 272, 273, 309, 311, 313, 315, 316, 323, and 341. Moreover, one N-glycosylation site (position 201) and three lysine acetylation sites (positions 320, 356, and 358) were identified (Fig. 2C).

According to the DeepTMHMM server, no transmembrane helices were detected (Fig. 2D). According to the SignalP-6.0 server, this protein possesses a conventional signal peptide of the Sec/SPI form with a probability score of 0.7271 and a possible cleavage site between residues 26 and 27 (probability score: 0.602349) (Fig. 2E). The DeepLoc 2.0 server output emphasized that the TgMIC3 protein is "extracellular" (probability score: 0.9148).

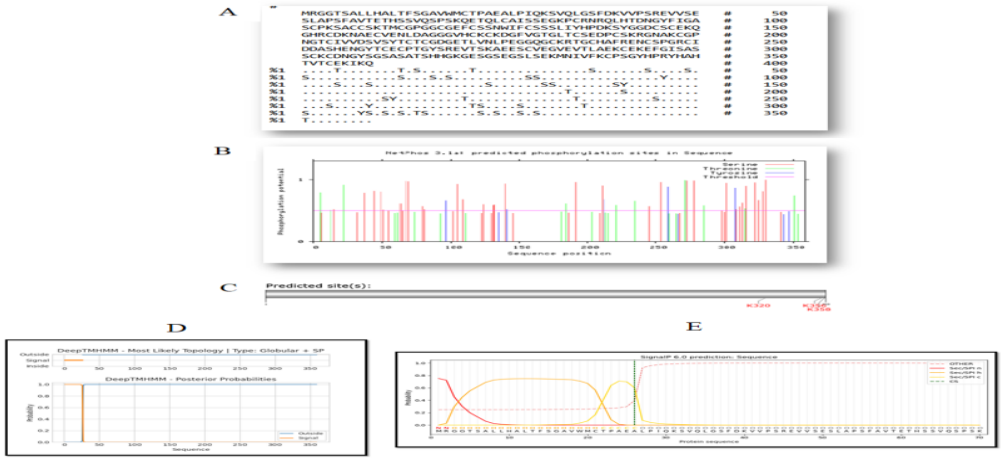

Fig. 2: Bioinformatics analysis of phosphorylation-related features of the TgMIC3 sequence. **(A)** Number of predicted phosphorylation sites based on S (serine), T (threonine), and Y (tyrosine) residues; **(B)** phosphorylation site prediction map; **(C)** predicted acetylation sites; **(D)** predicted transmembrane domains; and **(E)** signal peptide prediction

Three independent servers were used to analyze the secondary structure of TgMIC3. Both GOR IV and SOPMA predicted random coils as the dominant structural element, accounting for 246 residues (68.52%) and 305 residues (84.96%), respectively, followed by extended strands (90 residues, 25.07% and 33 residues, 9.19%). These findings were further supported by NetSurfP-3.0 outputs (Fig. 3).

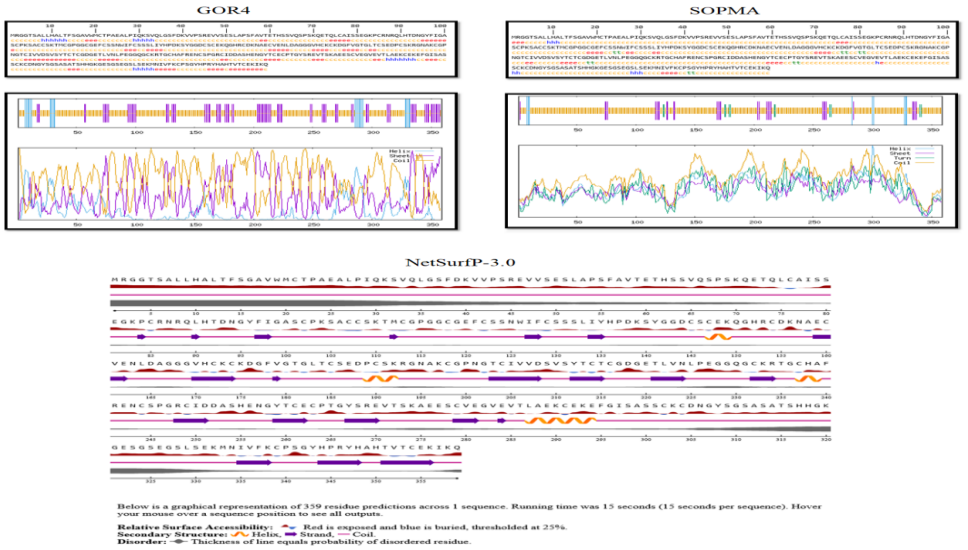

Fig. 3: Secondary structure prediction of the TgMIC3 sequence using the GOR IV, SOPMA, and NetSurfP-3.0 servers

Homology modeling using SWISS-MODEL identified template 1 with 100% sequence identity and full coverage (Fig. 4A, B).

The GalaxyRefine server was employed to refine the predicted 3D structure of TgMIC3. The initial model showed values of GDT-HA (1.0000), RMSD (0.0000), MolProbity (2.374),

clash score (3.0), poor rotamers (6.1), and Rama favored (82.9%). Following refinement, model 1 was selected, with improved values for GDT-HA (0.8823), RMSD (0.677), MolProbity (1.551), clash score, (10.8), poor rotamers (0.7), and Rama favored (98.6%). Ramachandran plot analysis showed that 82.91% of residues in the initial model and 98.60% in the refined model were located in favored regions (Fig. 4C, D).

ProSA-web analysis further supported this improvement, with Z-scores of −6.3 and −6.35 for the initial and refined models, respectively (Fig. 4E, F).

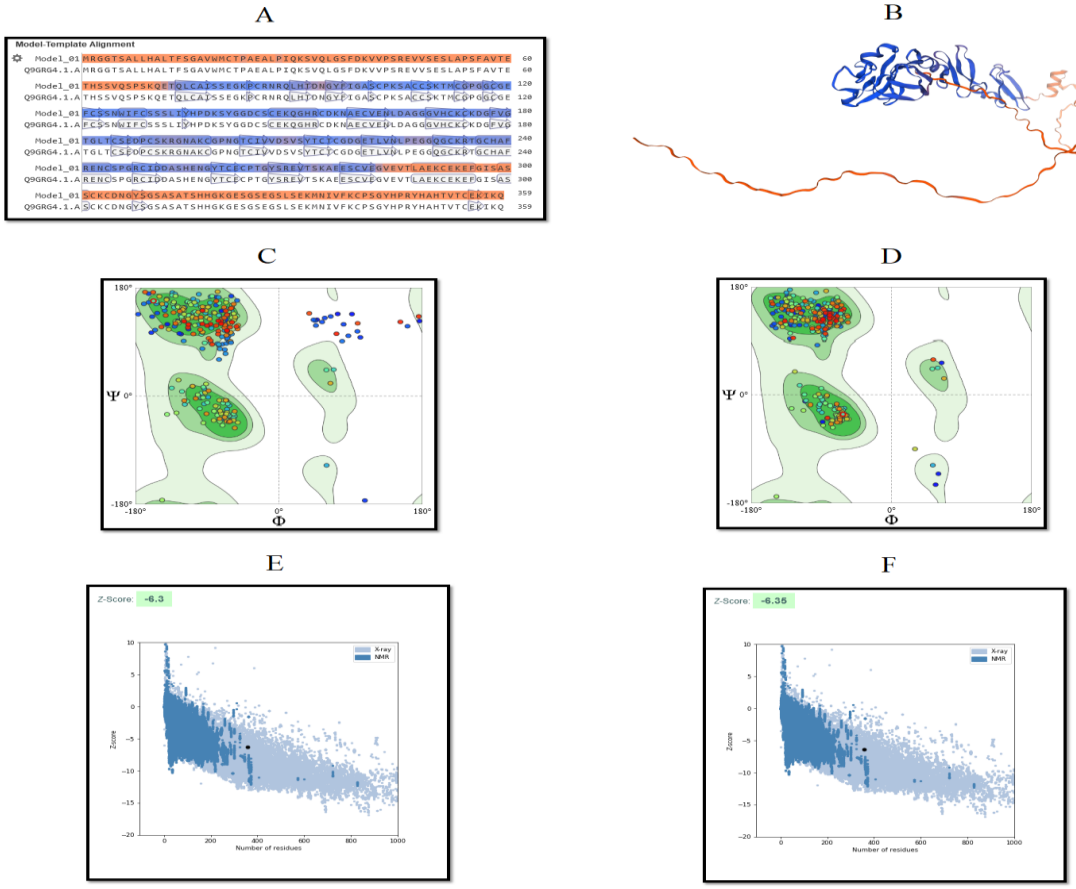

Fig. 4: Output of the SWISS-MODEL server. **(A)** Model-template alignment; **(B)** 3D structure of the TgMIC3 protein. Validation of the 3D model using the structure assessment tool and ProSA-web for the initial (**C and E**) and refined models (**D and F**)

A multi-server strategy was applied to predict linear B-cell epitopes using BepiPred 2.0, SVMTriP, and ABCpred (Table S1, S2, and S3). Shared epitopes predicted by at least two servers were selected for further analysis. In total, 11 common epitopes were identified, among which four peptides ("HSSVQSPSKQET", "SSEGKPCRNR", "YGGDCSCEKQGHR", and "TSHHGKGESGSEG") were predicted to be water-soluble, antigenic, and non-allergenic (Table 1). Next, the BcePred tool was used to predict the hydrophilicity, turn, polarity, and exposed surface of several linear B-cell

epitopes (Table S4). The IEDB epitope prediction tool also illustrated different features, such as B-cell epitopes, antigenicity, beta turns, surface accessibility, flexibility, and hydrophilicity, with average prediction scores of 0.527, 1.042, 1.077, 1.000, 1.010, and 2.828, respectively (Fig. 5A-F). Moreover, 12 conformational B-cell epitopes with scores ranging from 0.504 to 0.905 were predicted using the ElliPro server (Fig. 6).

Table 1: The final screening of shared linear B-cell epitopes of TgMIC

B-cell epitopes	Antigenicity	Allergenicity	Solubility
HSSVQSPSKQET*	0.8565	No	Good
SSEGKPCRNR*	1.6503	No	Good
YGGDCSCEKQGHR*	1.3413	No	Good
TSHHGKGESGSEG*	2.4878	No	Good
SCVEGVEVTL	0.0317	Yes	Good
IQKSVQLGS	1.3244	No	Poor
GEFCSSNWIFCS	1.0919	No	Poor
GRCIDDASHENG	1.1787	Yes	Good
CEKQGHRCDK	1.5106	Yes	Good
GGCGEFCSS	1.8946	Yes	Good
AGGGVHCKCK	2.2525	Yes	Good

* Indicates potent epitopes

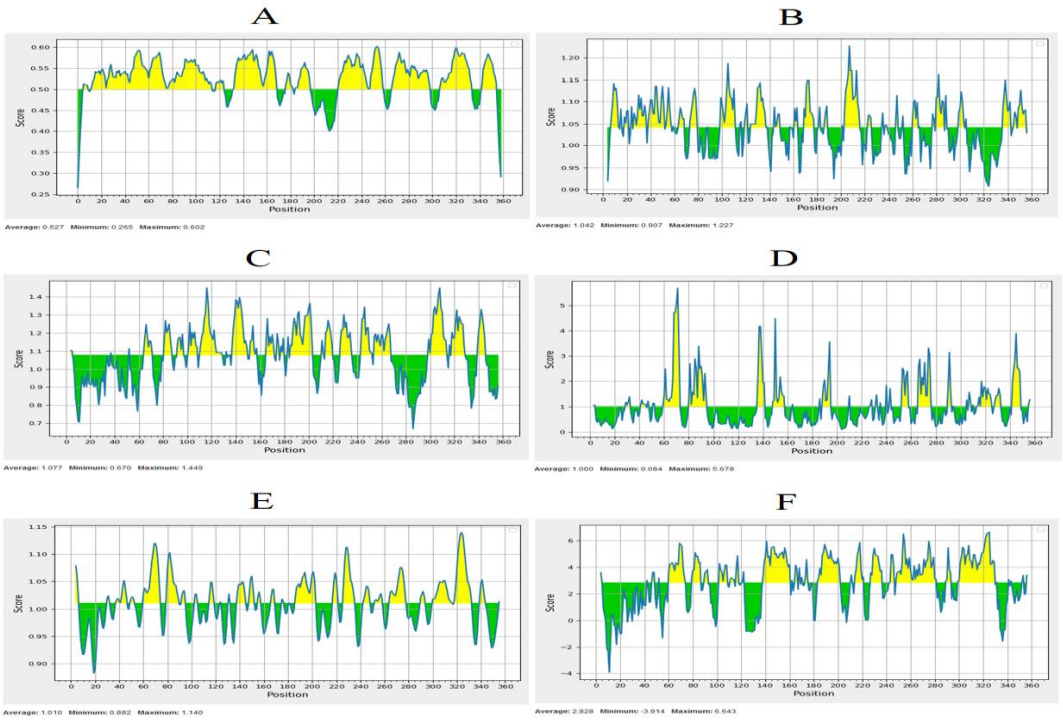

Fig. 5: Output of the IEDB analysis. **(A)** B-cell epitopes; **(B)** antigenicity; **(C)** beta turns; **(D)** surface accessibility; **(E)** flexibility; **(F)** hydrophilicity

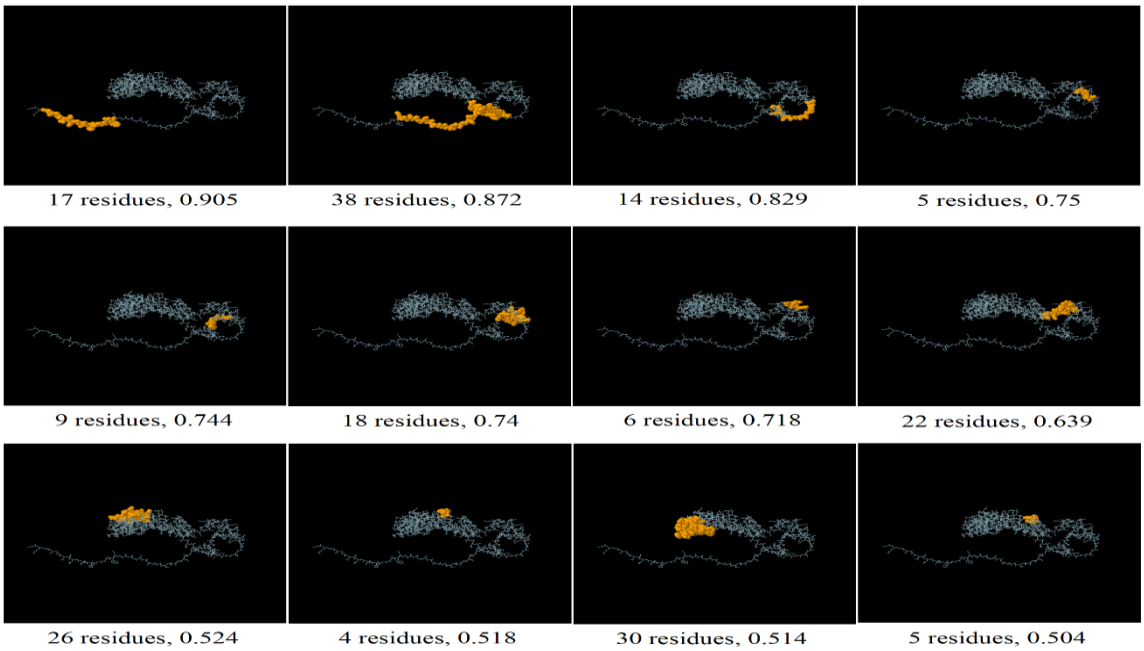

Fig. 6: Discontinuous 3D structure of B-cell epitopes predicted by ElliPro

Although most of the human CTL epitopes predicted in the present study for MIC3 were of low immunogenicity, as substantiated by the class I immunogenicity tool of the IEDB server, one of them (CPSGYHPRY$_{59-67}$) exhibited a good immunogenicity score (0.02813) and positive IFN-γ induction (Table S5). Among the top five mouse CTL epitopes predicted in terms of MHC-I alleles (H2-Db, H2-Dd, H2-Kb, H2-Kd, H2-Kk, H2-Ld. H2-Lq), only a single epitope (ASHENGYTCECP$_{43-54}$) was found to have good immunogenicity (0.1744) and IFN-γ induction capability (Table S6). In contrast, multiple IFN-γ-inducing (n = 4) and/or IL-4-inducing (n = 12) human HTL epitopes were found to have good antigenicity. Among these, two epitopes could simultaneously stimulate both cytokines (SSSLIYHPDKSYGGD$_{130-144}$ and TGYSREVTSKAEESC$_{265-279}$); hence, these two epitopes can be considered potent candidates for eliciting both humoral and cellular responses (Table S7). With respect to the mouse HTL epitopes, four and fifteen fragments were capable of eliciting IFN-γ and IL-4, respectively. Of these, three epitopes (SKQETQLCAISSEGK$_{69-83}$, SSSLIYHPDKSYGGD$_{130-144}$, and TGYSREVTSKAEESC$_{265-279}$) could simultaneously induce both cytokines (Table S8).

The highest IgG+IgM responses reached approximately 160,000 at 65 days post inoculation, among which the highest IgG1 and IgM responses were 68,000 and 90,000, respectively, during this period (Fig. 7A). The observed increase in IgM and IgG+IgM titers reflects the induction of a strong humoral immune response, a key mechanism involved in controlling parasite dissemination and supporting protective immunity.

At 60 days post inoculation, a large increase in B lymphocytes was observed (over 700 cells/mm^3), which subsequently remained relatively constant for more than one year (approximately 480 cells/mm^3). Most of these lymphocyte subpopulations were memory B cells, representing a slight downward trend throughout the year. Additionally, the IgM isotype remained constant, while the IgG1

isotype decreased after the third upsurge (Fig. 7 B, C). The latter trend was also evident for the CD4$^+$ memory Th cell population (Fig. 7D, E). The active CD8$^+$ population increased approximately 50 days post inoculation (~1,000 cells/mm^3) and then decreased, and the CD8$^+$ population remained active without any anergic features for approximately 150 days (Fig. 7F).

The highest peak of IFN-γ was approximately 400,000 ng/ml, indicating strong stimulation of the Th1 immune responses, which are required for the clearance of invasive free or intracellular tachyzoites (Fig. 7I).

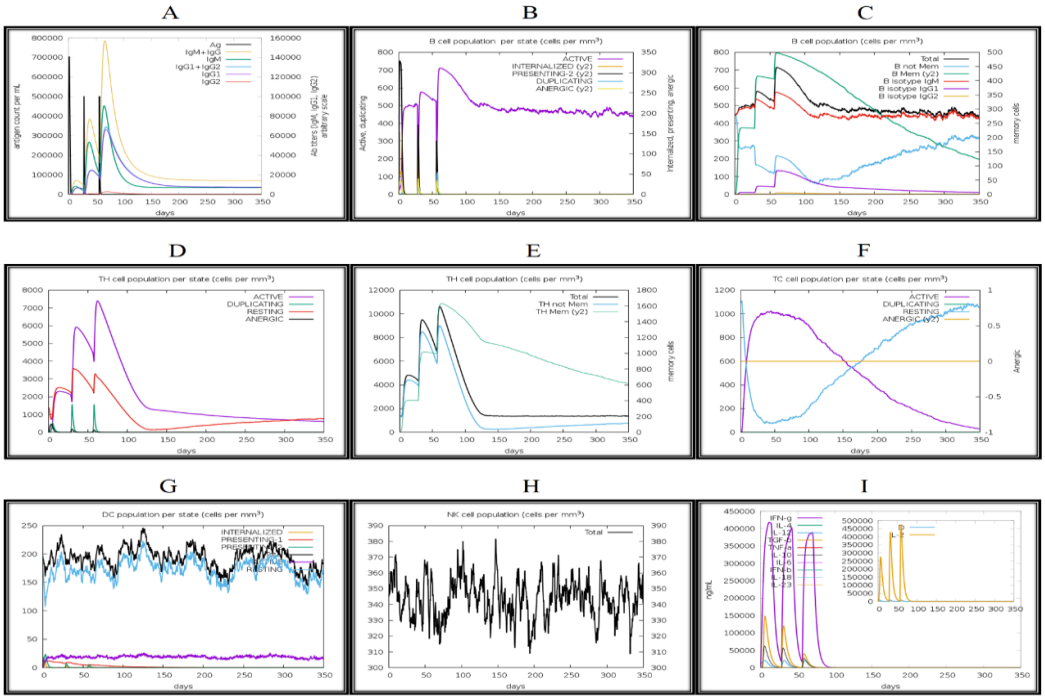

Fig. 7: Immune simulation results generated by the C-ImmSim server. **(A)** Immunoglobulin production; **(B, C)** B-cell population (cells/mm^3); **(D, E)** T-CD4$^+$ cell population (cells/mm^3); **(F)** T-CD8$^+$ cell population (cells/mm^3); **(G)** dendritic cell (DC) populations per state (cells/mm^3); **(H)** natural killer (NK) cell population (cells/mm^3); and **(I)** cytokine production levels (ng/ml)

Discussion

This study aimed to identify a potential vaccine candidate against *T. gondii*, a widespread zoonotic parasite of global importance, using an integrated *in silico* approach. Given the growing significance of computational tools in epitope-driven vaccine design, the MIC3 protein was analyzed with respect to its immunogenic potential and physicochemical properties.

The molecular weight of MIC3 was estimated to be 37,884.24 Da, which suggests that the protein may possess immunogenic potential (16). Moreover, the combination of a favorable aliphatic index, hydrophilic properties, and structural stability indicates that the protein is likely to exhibit adequate thermal stability and solubility, supporting its potential applicability in biological systems. Collectively, the physicochemical features support the suitability of TgMIC3 for downstream expression and purification experiments.

Previous *in silico* studies on other microneme-associated antigens of *T. gondii* have reported comparable antigenicity scores using established prediction tools. For instance, *T. gondii* apical membrane antigen 1 (TgAMA1) exhibited a VaxiJen v2.0 score of 0.6685, while MIC4 showed high antigenicity with VaxiJen and ANTIGENpro scores of 0.6182 and 0.9596, respectively. Similarly, MIC13 demonstrated strong antigenic potential with VaxiJen v2.0 and ANTIGENpro scores of 0.5624 and 0.9663, respectively (11, 13, 14). In agreement with these findings, the high antigenicity score for TgMIC3 in the present study (VaxiJen = 0.8630 and ANTIGENpro = 0.9578) further support the relevance of microneme proteins as promising vaccine antigen candidates.

In this study, we identified a total of 75 PTM sites in TgMIC3, including 46 phosphorylation sites, 25 O-glycosylation sites, one N-glycosylation site, and three lysine acetylation sites. PTM regions can critically regulate protein structure, subcellular localization, and interaction networks, thereby influencing functional behavior and immunogenic potential. In particular, modifications such as glycosylation, phosphorylation, and acetylation represent important regulatory mechanisms governing protein activity (17).

Secondary structure prediction is a critical step toward understanding tertiary protein organization and functional stability (12, 18). In the present study, three independent servers, including GOR IV, SOPMA, and NetSurfP-3.0, were used to predict the secondary structure of TgMIC3, and the outputs revealed that random coils were the dominant structural element, followed by extended strands. The high proportion of random coil structures may influence protein flexibility and antigenic exposure. Such flexible regions are typically more accessible to immune recognition, which may enhance the overall antigenic potential of the protein (19). Additionally, the presence of alpha-helices and beta-turns within internal protein regions, stabilized by hydrogen bonding, may contribute to structural integrity and enhanced interaction with antibodies (18).

Structural refinement of the predicted 3D models using the GalaxyRefine server resulted in an improvement in overall model quality, as confirmed by Ramachandran plot assessment, ProSA-web analysis, and Z scores. For instance, based on the results of this study, Ramachandran analysis demonstrated that 82.91% and 98.60% of residues in the initial and refined models were positioned within favored regions, respectively, indicating substantial structural improvement. The increased proportion of residues located in the favored regions of the Ramachandran plot indicates that the refined model is more stable and energetically favorable conformational state (20).

Epitope prediction provides valuable insights into immune-recognizable regions of antigens that are capable of eliciting effective humoral responses. In particular, the identification of linear B-cell epitopes is essential for inducing antigen-specific antibodies that can limit parasite attachment and enhance immune-mediated clearance (10, 21). Moreover, IL-4 production and Th2 immune polarization play pivotal roles in B-cell activation and antibody class switching, thereby contributing to the generation of protective IgG responses during *Toxoplasma* infection (21, 22). In addition, innate immune recognition of *T. gondii* antigens involves Toll-like receptors, followed by activation of dendritic cells and macrophages, leading to CD4+ and CD8+ T-cell responses mediated by IL-12 and IFN-γ (21, 23). In the present study, a multi-server approach was employed to identify potential B- and T-cell epitopes using the BepiPred 2.0, SVMTriP, ABCpred, ElliPro, and IEDB online tools. The findings indicated that the *T. gondii* MIC3 protein harbors promising CTL, HTL, and B-cell epitopes, thereby providing a strong foundation for the rational design of a novel vaccine candidate against toxoplasmosis.

Virtual simulation of immune responses is a key step in determining the extent to which a protein can elicit the desired immune response

(15). For this purpose, the C-ImmSim web server was used, and the immune responses elicited upon triple antigen injections were evaluated. Based on predicted results, the activation of humoral and cellular immune compartments, accompanied by the formation of memory B and T lymphocytes, demonstrates the capacity of TgMIC3 to elicit an integrated immune response that is critical for sustained and long-term protective immunity (21-23). Additionally, the significant elevation in IFN-γ production—an essential cytokine in the control of *T. gondii* infection—provides further evidence that TgMIC3 is capable of eliciting protective immune responses (21, 23).

Although immunoinformatics approaches provide a rapid and cost-effective framework for prioritizing vaccine antigens, their predictions are inherently constrained by algorithmic assumptions, reference database coverage, and the absence of biological context (9, 10). Variability among computational tools can result in inconsistencies in predicted antigenicity, allergenicity, solubility, and immunogenicity profiles (10, 12). Moreover, immune simulation platforms are unable to fully recapitulate the complexity of host–parasite interactions observed *in vivo*, particularly with respect to antigen processing, immune regulation, and strain-dependent variability (10, 15). Consequently, the present study should be regarded as hypothesis-generating, and its findings require experimental validation through *in vitro* and *in vivo* assays to confirm the protective relevance of the predicted epitopes.

Future directions

Future studies should focus on experimental validation of the predicted B- and T-cell epitopes to confirm their immunogenicity and protective efficacy. The integration of TgMIC3 epitopes into a rationally designed multi-epitope vaccine construct, potentially combined with other stage-specific antigens, represents a logical next step. Such efforts may include advanced computational optimization of epitope arrangement, immunogenic enhancement strategies, and structural evaluation to improve vaccine stability and immune recognition. Ultimately, *in vivo* challenge studies using appropriate animal models will be required to assess protective efficacy, including cytokine responses, antibody production, parasite burden in tissues, and survival outcomes.

Conclusion

The most immunodominant B- and T-cell epitopes of *T. gondii* MIC3 were identified. Furthermore, the physicochemical properties, antigenicity, allergenicity, structural features, and immune-stimulatory potential of this protein were comprehensively evaluated using bioinformatics approaches. Based on these analyses, MIC3 exhibited favorable antigenicity scores and harbored multiple immunogenic epitopes, supporting its candidacy as a vaccine antigen. The present study represents the first step of a larger vaccine development pipeline, in which TgMIC3 was evaluated as a potential antigenic component. Nevertheless, experimental validation is essential to confirm these computational predictions and to establish the correlation between the identified epitopes and protective immune responses.

Acknowledgments

This study was financially supported by Abadan University of Medical Sciences, Abadan, Iran (Grant No: 1618). Ethics approval Number is IR.ABADANUMS.REC.1401.169.

Conflict of interest

All authors declare no conflicts of interest.

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