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Original Article

Design of A Potential Multi-Epitope Vaccine against *Toxoplasma gondii* Based on Microneme Proteins Using A Comprehensive Immunoinformatics Approach

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Abstract

Background: *Toxoplasma gondii* (*T. gondii*) is an intracellular apicomplexan parasite responsible for toxoplasmosis in humans and animals worldwide. Despite its high prevalence, no effective vaccine is currently available for human use. This study aimed to design and evaluate a multi-epitope vaccine candidate based on *T. gondii* microneme proteins (MIC2, MIC3, MIC4, MIC6, MIC8, and MIC13) using immunoinformatics approaches.

***Methods*:** B-cell, cytotoxic T-lymphocyte (CTL), and helper T-lymphocyte (HTL) epitopes were predicted and screened based on their antigenicity, allergenicity, toxicity, and immunogenicity. Selected epitopes were linked using appropriate peptide linkers and fused with the 50S ribosomal protein L7/L12 adjuvant. The construct was further assessed for physicochemical properties, structural features, antigenicity, allergenicity, solubility, immune responses, and codon optimization.

***Results*:** The final vaccine construct consisted of 624 amino acids with a predicted molecular weight of 64.97 kDa. The vaccine was predicted to be antigenic, stable, soluble, and non-allergenic. Structural analyses supported the quality of the refined model. Immune simulation indicated the induction of strong humoral and cellular immune responses, including enhanced antibody production and T-cell activation.

***Conclusion*:** The designed multi-epitope vaccine demonstrated promising immunological and physicochemical characteristics.

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Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite and the causative agent of toxoplasmosis, a globally distributed zoonotic disease affecting both humans and animals. Humans can acquire the infection through the ingestion of tissue cysts present in undercooked meat or by consuming food and water contaminated with sporulated oocysts shed by felids (1). Toxoplasmosis may present as either an asymptomatic latent infection or a severe disease, particularly in immunocompromised individuals and congenitally infected infants (2, 3). Toxoplasmosis in humans, primary infection during pregnancy may lead to congenital toxoplasmosis, resulting in miscarriage, fetal abnormalities, or neurological and ocular complications (4). Current therapeutic options are associated with several limitations, including adverse effects, limited accessibility, high costs, and poor efficacy against tissue cysts (5, 6). These drugs have limited efficacy against tissue cysts and are generally less effective during the early, often asymptomatic stages of infection (5).

Over the past decades, numerous vaccine strategies, including live-attenuated, recombinant protein, DNA-based, and epitope-based vaccines, have been investigated against *T. gondii* (7). Among these approaches, epitope-based vaccines designed through immunoinformatics offer advantages such as high specificity, safety, cost-effectiveness, and reduced development time (8). Secretory proteins are mainly released from three specialized secretory organelles, including microneme proteins (MICs), rhoptries proteins (ROPs), and dense granule antigens (GRAs) (9, 10). Secretory antigens of *T. gondii*, particularly MICs, are considered promising vaccine targets because of their essential roles in host-cell recognition, attachment, invasion, and induction of protective immune responses (11). MICs are specialized secretory organelles and constitute one of the three main secretory organelles of *T. gondii*, that serve critical functions in the invasion process (12). MIC2 is a well-established invasion/adhesion factor, MIC3 and MIC4 are microneme adhesions, MIC6 functions as part of the MIC1-MIC4-MIC6 complex involved in invasion, and MIC8 is an essential invasion factor (13-16).

We aimed to identify immunodominant B-cell and T-cell epitopes from MIC2, MIC3, MIC4, MIC6, MIC8, and MIC13 proteins and to design and evaluate a potential multi-epitope vaccine candidate against *T. gondii* using various immunoinformatics tools.

Materials and Methods

Retrieval of protein sequences

The amino acid sequences of six specific *T. gondii* MICs, including MIC2 (accession No. KAF4642988.1), MIC3 (accession No. KAF4644940.1), MIC4 (accession No. KAF4645987.1), MIC6 (accession No. KAF4638281.1), MIC8 (accession No. KAF4638404.1), and MIC13 (accession No. KAF4641207.1) were retrieved from the National Center for Biotechnology Information) NCBI database in FASTA format. The antigenicity of the selected proteins was then predicted using the VaxiJen v2.0 server with the default threshold of 0.5. The all characterized web servers and bioinformatics tools used in this study are summarized in Supplementary Table 1 (Not Published).

Prediction of Linear B-cell epitopes (LBC)

The sequences were entered into the SVMTriP server in order to ascertain antigens that have the potential to initiate the production of antibodies and elicit a response from B-cells. The linear epitopes of B-cell were chosen and analyzed for allergenicity and antigenicity using AllergenFP v1.0, VaxiJen v2.0 online servers, respectively (17). We utilized

ToxinPred in order to assess the ability of being toxic (18).

Predicting the epitopes of Cytotoxic T-lymphocytes (CTL)

For prediction of epitopes recognized by cytotoxic T lymphocytes, the obtained sequences of amino acids were assessed through the NetCTL 1.2 server (MHC-I weight = 1, Proteasomal cleavage weight = 0.15, TAP transport weight = 0.05 with a threshold set at 0.75) (19). Forecasted epitopes were also checked by VaxiJen v2.0 (20) and for potential to stimulate major histocompatibility complex (MHC) class I responses (21). ToxinPred was utilized for assessing the toxicity.

Prediction of Helper T-Lymphocyte (HTL) Epitopes

Epitopes of HTL were anticipated utilizing the Immune Epitope Database (IEDB) MHCII authoritative forecast device on the IEDB server. Epitopes were chosen based on the percentile rank. Allergenicity and toxicity were foreseen as noted in other sources. In the next step, the antigenicity of each epitope and its ability to induce IL-4 and interferon-gamma (IFN-γ) were forecasted using VaxiJen v2.0, IL4-pred and IFNepitope web servers (22). Prediction of IL-4 induction was performed using the IL4pred server based on the Support Vector Machine (SVM) algorithm with a default threshold of 0.0.

Designing the vaccine

The final multi-epitope vaccine construct was designed by combining the selected CTL, HTL, and B-cell epitopes with appropriate flexible or rigid linkers. The adjuvant sequence was positioned at the N-terminal end of the construct and was connected to the epitope-containing region using an EAAAK linker. This linker was selected to provide structural separation between the adjuvant and the antigenic domain and to minimize undesirable interactions between these regions. The selected CTL epitopes were joined using AAY linkers, used to separate adjacent CTL epitopes and facilitate their independent processing. The selected HTL epitopes were connected using KK linkers (23).

Solubility, physicochemical effects, antigenicity, and allergenicity prediction of the developed vaccine

We used the ProtParam web server in order to evaluate the physicochemical characteristics of the developed vaccine construct, such as number of amino acids, molecular weight, amino acid count, extinction coefficients, theoretical isoelectric point (pI), grand average of hydropathicity (GRAVY), estimated half-life, aliphatic and instability index (24). Protein stability was evaluated using the instability index calculated by ProtParam. We used the VaxiJen v2.0 server to predict vaccine antigenicity evidence. Besides, SOLpro predicted solubility (25).

Prediction secondary and tertiary structure plus refinement

The secondary structure of the developed vaccine construct was determined using the SOPMA online server. Throughout the analysis, we kept the server settings at as they were originally set. The tertiary structure of the vaccine was developed with the help of SWISS-MODEL (26), and then, the model we were expecting went through refinement process using the GalaxyRefine server (27). The quality of the predicted three-dimensional structure was assessed using the Ramachandran plot available through the SWISS-MODEL assessment tool (https://swissmodel.expasy.org/assess/help). Model quality was further evaluated using the ProSA-web server by examining the local quality plot and the overall Z-score.

The anticipation of discontinuous B-cell epitopes

In order to forecast the tertiary structure of these discontinuous epitopes of B-cell, the ElliPro server was utilized (28). ElliPro utilizes a three of algorithms to anticipate the structure of proteins in the form of an ellipsoid. The analysis is conducted using a default minimum value of 0.5 and 6 Angstrom.

Method of stimulating immunity

By employing the CImmSim online server, the immune responses of the vaccine construct were analyzed (https://wwwold.iac.rm.cnr.it/~filippo/projects/c-immsim-online.html). The server replicates how the host immune system's T-cells and B-cells react to the vaccine structure (29). In accordance with prior research, the vaccine construct's design was administered in three doses, with a four-week gap between each dose. The simulation was included different time intervals of 1, 84 and 168 time steps, respectively. Other significant parameters were set at 50 for simulation volume and 1050 for simulation steps (29).

Codon optimization

The nucleotide sequence was obtained by back translating the amino acid sequence utilizing the tool for reverse translation available on the Sequence Manipulation Suite server. To optimize the codons for expression in a specific host, we used the JCat online software. This software provided a codon adaptive index (CAI) with an optimized sequence and a percentage of GC content (GC %).

Results

Prediction antigenicity and retrieving proteins

The amino acid sequences of six *T. gondii* MICs, including MIC2 (769 aa), MIC3 (383 aa), MIC4 (580 aa), MIC6 (349 aa), MIC8 (684 aa), and MIC13 (468 aa), were retrieved from the NCBI database. Antigenicity assessment using the VaxiJen v2.0 server demonstrated that all selected proteins were antigenic, with scores exceeding the threshold value of 0.5. The predicted antigenicity scores were 0.6774, 0.8537, 0.6115, 0.5800, 0.8297, and 0.5616 for MIC2, MIC3, MIC4, MIC8, MIC6, and MIC13, respectively, confirming their suitability for epitope prediction and vaccine design.

LBC prediction

Following screening for antigenicity, allergenicity, and toxicity, seven epitopes satisfied all selection criteria and were considered suitable for inclusion in the vaccine construct. All selected epitopes were predicted to be non-allergenic, non-toxic, and antigenic (Supplementary Table 2) (Not published).

Prediction epitopes of CTL

Overall, 53 CTL epitopes were predicted from the six target proteins using the NetCTL v1.2 server. CTL epitopes were selected using a NetCTL score threshold of 0.75. The study's chosen epitopes have a cumulative score of greater than 0.75 points. Only 25 epitopes were found to be immunogenic after their immunogenicity was assessed; 18 of these met the other selection requirements (Supplementary Table 3) (Not published).

Predicting the epitopes of HTL

Epitopes with percentile ranks below 5 were selected because lower percentile ranks indicate stronger binding affinity to MHC class II molecules. The selected epitopes were further evaluated for antigenicity, allergenicity, toxicity, and their ability to induce IFN-γ and IL-4 cytokines. Following comprehensive screening, seven HTL epitopes met all predefined criteria and were included in the final vaccine construct (Supplementary Table 4) (Not published).

The creation of a vaccine with different epitopes

The vaccine construct had 32 immunogenic epitopes at the end (seven HTL, eighteen

CTL, and seven LBC) that were connected by appropriate linkers. In addition, the N-terminal of the construct was combined with the adjuvant, 50S ribosomal protein L7/L12, to make its immunogenicity better. The epitope sequence and the adjuvant were joined by the EAAAK linker. The final vaccine's design is depicted in the diagram in Fig. 1.

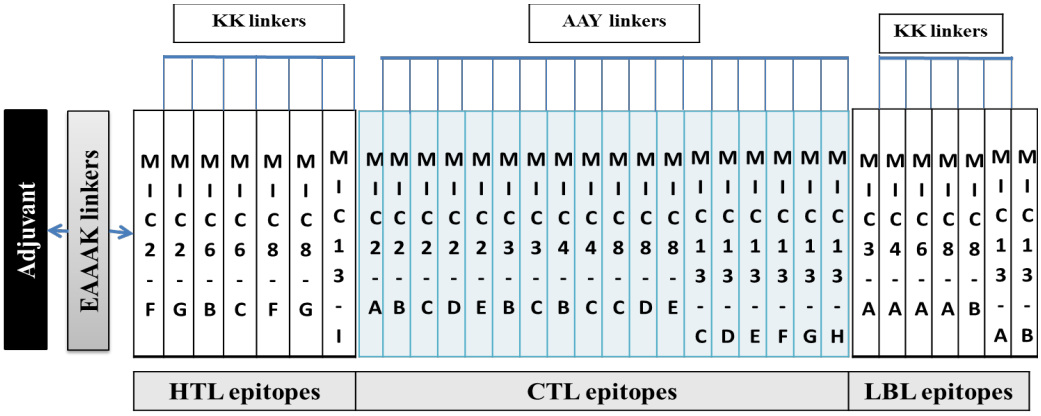


Fig. 1: Schematic representation of the designed multi-epitope vaccine construct against *T. gondii*. The construct consists of the 50S ribosomal protein L7/L12 adjuvant at the N-terminus linked via an EAAAK linker, followed by HTL, CTL, and LBC epitopes joined by KK and AAY linkers

The prediction of solubility, allergenicity, antigenicity, and physicochemical effects of the designed vaccine

Table 1 displays the physicochemical features of the vaccine. The molecular weight of the vaccine was 64.97 kDa (theoretical pI: 6.98). The vaccine instability index would be 30.13, indicating stability. Additionally, the aliphatic index was 87.93 and the GRAVY score was 0.126, respectively, suggesting a slightly hydrophobic overall character of the construct. Additionally, the antigenicity score (0.7333) was used to gauge the vaccine's immunological potency. Solubility score of the construct was 0.8615, and it was soluble and non-allergic (Table 1).

Table 1: Prediction of the physicochemical properties, antigenicity, allergenicity, and solubility.

Characteristics	Finding	Remarks
Number of amino acids	624	Suitable
Molecular weight	64970.20	High
Theoretical PI	6.98	
Chemical formula	$C_{2894}H_{4560}N_{756}O_{885}S_{27}$	-
Extinction coefficient (at 280 nm in H20)	70960	-
Estimated half-life (mammalian reticulocytes, in vitro)	30 h	-
Estimated half-life (yeast, in vivo)	>20 h	-
Estimated half-life (*E. coli*, in vivo)	>10 h	-
Instability index of vaccine	30.13	stable
Aliphatic index of vaccine	87.93	Thermostable
Grand average of hydropathicity (GRAVY)	0.126	Hydrophobic
Antigenicity	0.7333	Antigenic
Allergenicity	No	Non-Allergen
Solubility	Soluble	0.8615

Predicting the structure and refinement of secondary and tertiary structures

SOPMA was able to predict 117 (18.75 %) random coils, 137 (21.96 %) beta strands, and 299 (47.92 %) α-helices. For the protein that was designed, the SWISS-MODEL results revealed five templates that matched the target sequence. We selected the model with the highest coverage and similarity (Fig. 2A and 2B). There were numerous residues in the preferred region, and the z-score reflecting the model's overall quality was -4.1. After refinement, the quality of the 3D structure was enhanced (z-score of -4.2) (Fig. 3A, B). Prior to refinement, protein validation revealed that, in the main model, 85.16% of residues were in favored areas, 13.31% were in allowed areas, and 0.56% were in outlier areas. After the 3D model was refined, 96.9% of residues were in the Ramachandran plot's favored, 0.78% in allowed, and 0.0% in outlier areas (Fig. 3C, D)

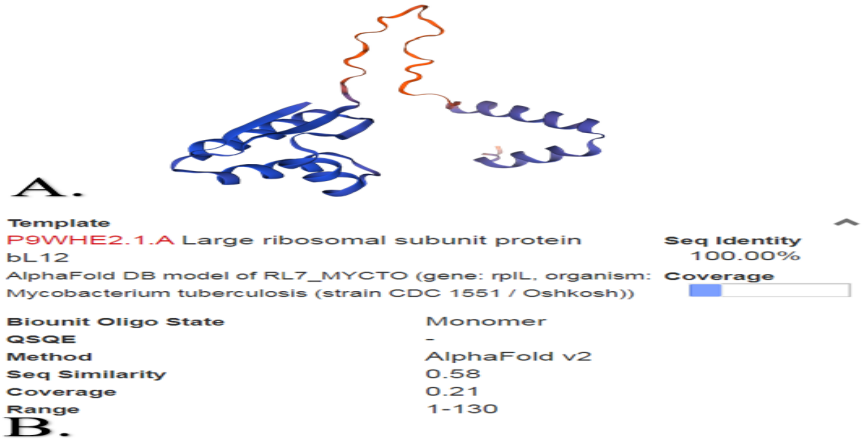


Fig. 2: Three-dimensional (3D) structural modeling and refinement of the multi-epitope vaccine construct. (A) Initial 3D model generated using the SWISS-MODEL server; (B) Refined 3D model obtained after processing with the GalaxyRefine server

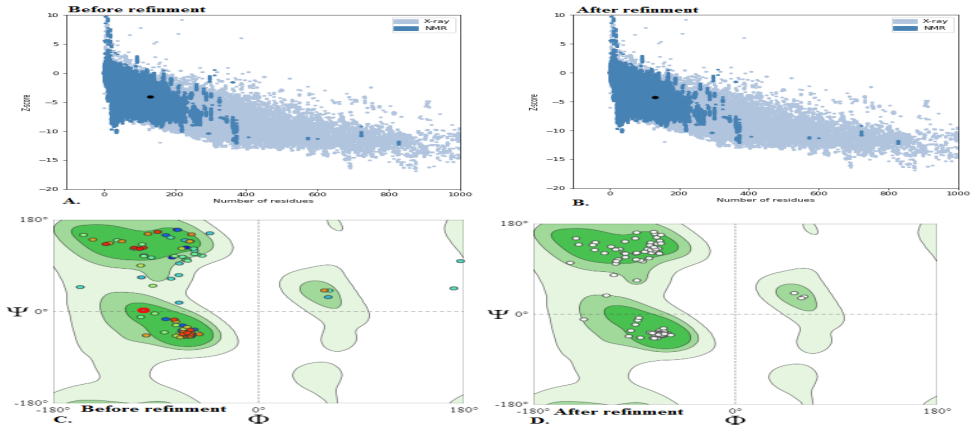


Fig. 3: Structural validation and quality assessment of the multi-epitope vaccine construct before and after refinement. (A, B) ProSA-web overall quality plots showing Z-scores of -4.1 (initial model) and -4.2 (refined model); (C, D) Ramachandran plot analysis of the initial (85.16% in favored regions) and refined (96.9% in favored regions) models

The recognition of structural B-cell epitopes

The ElliPro web server successfully predicted 5 non-contiguous B-cell epitopes. These epitopes were selected based on their score values, which ranged from 0.551 to 0.804 (Fig. 4A-E and Table 2).

Table 2: Predicted discontinuous B-cell epitope residues of the designed vaccine construct.

No.	Residues	Number of residues	Score
1	A:M1, A:A2, A:K3, A:L4, A:S5, A:T6, A:D7, A:E8, A:L9, A:L10, A:D11, A:A12, A:F13, A:K14, A:E15, A:M16, A:T17, A:L18, A:L19, A:E20, A:L21, A:S22, A:D23	23	0.804
2	A:V40, A:A41, A:V42, A:A43, A:A44, A:A45, A:G46, A:A47, A:A48, A:P49, A:A50, A:G51, A:A52, A:A53, A:V54, A:E55, A:A56, A:A57, A:E58, A:E59	20	0.752
3	A:A70, A:G71, A:D72, A:K73, A:K74, A:I75, A:G76, A:K79, A:A122, A:A123, A:G124, A:A125	12	0.619
4	A:F24, A:K27, A:E30, A:T31, A:F32	5	0.602
5	A:K91, A:K94, A:D95, A:D98, A:G99, A:A100, A:P101, A:K102	8	0.551

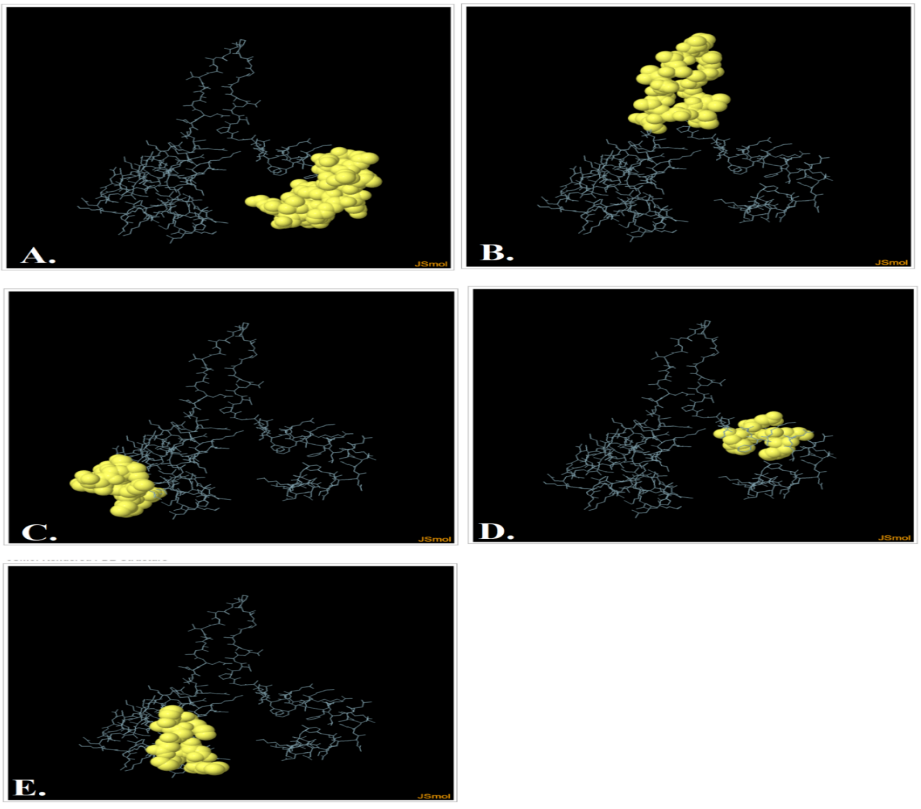


Fig. 4: Conformational (discontinuous) B-cell epitopes predicted on the 3D structure of the vaccine construct using ElliPro (A–E). The core polypeptide backbone is represented by grey sticks, while the predicted discontinuous B-cell epitope residues are highlighted as yellow molecular surfaces

Immune simulation

The dynamic in silico immune simulation via C-ImmSim demonstrated the induction of both robust humoral and cellular immune responses (Fig. 5). Immunologically, the kinetics of antibody titers displayed classical characteristics of adaptive immune maturation. A sharp initial rise in IgM was observed following the first immunization, signifying a rapid and potent primary immune response triggered by naive B-cell activation. With subsequent booster administrations, a pronounced isotype switching occurred, evidenced by high and sustained titers of secondary immunoglobulins, specifically IgG1 and IgG2 (and total IgG + IgM), alongside a substantial generation of B-cell memory pools. Concurrently, the cellular arm exhibited marked proliferation of active CTLs and HTLs, accompanied by a lasting reservoir of memory T-cells. The cytokine profiling revealed a pronounced peak in interferon-gamma and IL-2, maintaining prolonged elevated levels throughout the post-immunization period.

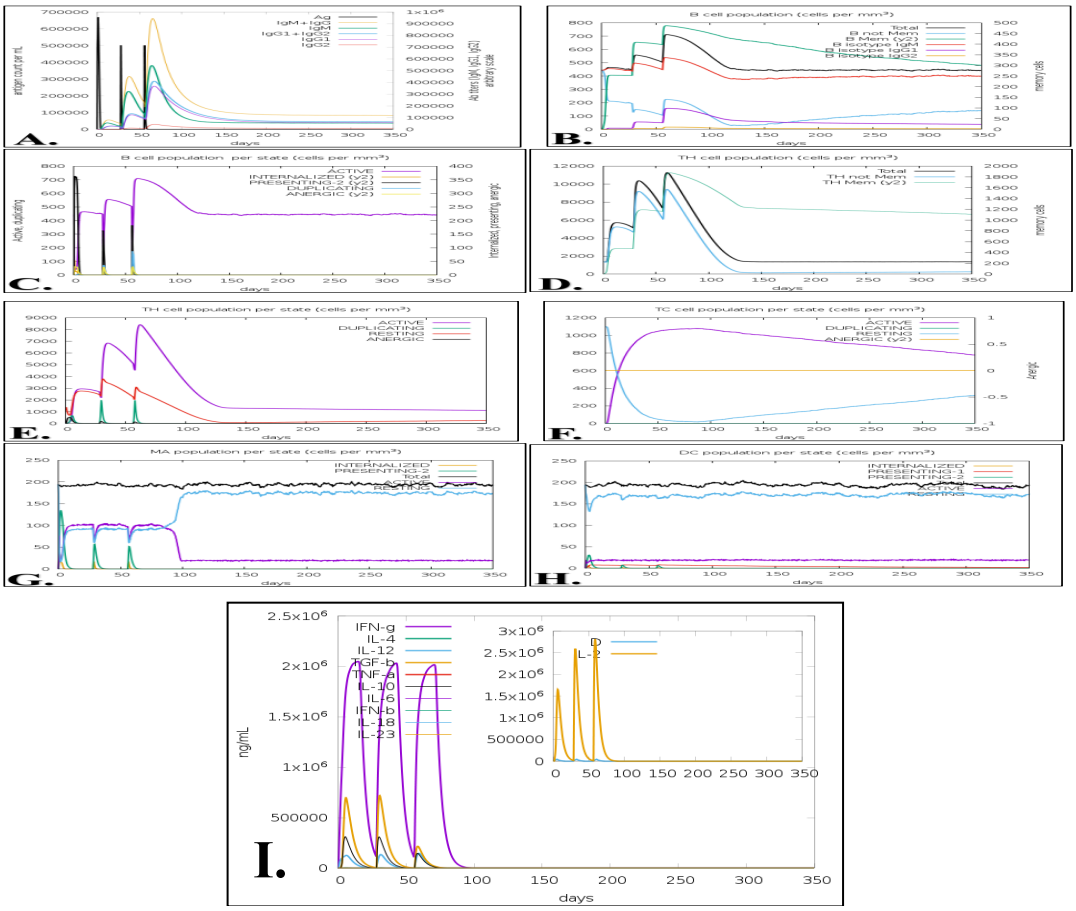


Fig. 5: In silico immune simulation of the proposed multi-epitope vaccine construct using the C-ImmSim server. (A) Antigen count and immunoglobulin titers following three doses administered at 4-week intervals; (B) B-cell population per state; Total B-cell count; (D) Helper T-lymphocyte (HTL) population per state; (E) Total HTL count; (F) Cytotoxic T-lymphocyte (CTL) population per state; (G) Macrophage population per state; (H) Dendritic cell population per state; (I) Production of induced cytokines and interleukins (including IFN-γ and IL-2) with Simpson index

Reverse translation and modification of the genetic codon

Following the protein sequence's reverse translation, codon adaptation was carried out in accordance with *Escherichia coli* (*E. coli*) K12 strain. The codon-adapted sequence had an improved CAI-value of 1.0 and a GC percent of 49.83, respectively, compared to the initial

sequence's estimated values of 0.53 and 58.44 percent.

Discussion

The development of an effective vaccine against *T. gondii* remains a major challenge due to the parasite's complex life cycle, multiple transmission routes, and ability to establish chronic infections (30, 31). The amino acid sequences of MIC2, MIC3, MIC4, MIC6, MIC8, and MIC13 proteins were acquired from the NCBI database through our research. The antigenicity of the selected proteins was assessed using the VaxiJen v2.0 server. The AAY, KK, and KK linkers were utilized as crucial components in vaccine development (32). The conjugation of CTL epitopes with the AAY linker was employed to modulate stability of the protein by determining the site of proteasomal degradation. This strategy aimed to reduce immunogenicity and enhance the presentation of epitopes (33). The inclusion of an adjuvant in the multi-epitope vaccine formulation has been shown to enhance vaccine stability, promote an enhanced immune defense towards the antigens, and prolong the duration of immune protection (34). Incorporation of the 50S ribosomal protein L7/L12 adjuvant was predicted to enhance the immunogenic potential of the construct (18). The sequence of protein is linked to the vaccine through the rigid EAAAK linker, which has minimal impact on the vaccine's efficacy (35). The ultimate vaccine consisted of 624 amino acids that were readily manufacturable and synthesized in large quantities.

Based on the findings related to allergenicity and antigenicity, the vaccine construct was found to display nonallergenic and antigenic properties, making it suitable for use. The predicted molecular weight of the vaccine construct was 64.97 kDa, indicating that it falls within the optimal range of molecular weight for vaccines, which is typically greater than approximately 40-50 kDa. The potential of the vaccine construct to be soluble in the *E. coli* host was determined, and the results were confirmed to be 0.8615. Additionally, the high aliphatic index of 87.93, which exceeds the recommended threshold of 70, suggests that the vaccine construct exhibits thermostability across a broad range of temperatures (30). The integrity of protein structure conformation heavily relies on the presence of protein β-turns and α-helices. These interactions are essential for ensuring the stability and functionality of proteins in various biological processes (30, 31).

The presence of a high percentage of β-strands and α-helices in this study suggests the formation of strong hydrogen bonds, crucial for maintaining the stability of the protein structure and facilitating interactions with antibodies. The 3D structure prediction plays a vital role in understanding the intricate details of the vaccine's structure, with a focus on achieving a close resemblance to the actual protein structure. Structural validation of the refined model demonstrated that 96.9% of residues were located within the favored regions of the Ramachandran plot, indicating a high-quality and reliable three-dimensional structure. Immune simulation analyses showcased the vaccine's capability to elicit a potent and broad immune response, underscoring its potential efficacy. vitro

However, a notable limitation of the research pertains to the absence of in and in vivo assessments, highlighting the need for additional empirical investigations to validate the protective efficacy and safety profile of the vaccine construct against *T. gondii* infection. Several vaccine-related studies have contributed to the development of anti-*T. gondii* strategies in Iran. For example, Selseleh et al. reported the production and evaluation of recombinant GRA7, underscoring the value of *T. gondii* antigen-based approaches for serological and immunological applications (36). Moreover, Abasi E, et al. (2026) have continued to explore multicomponent vaccine strat-

egies, reflecting a shift toward rational vaccine design based on antigen selection, epitope prediction, and immune-response modeling (37). Recent vaccine development efforts in Iran have placed substantial emphasis on multi-antigen and cocktail DNA vaccine platforms to overcome the limited efficacy associated with single-antigen formulations. Notably, studies evaluating dual-promoter expression vectors simultaneously encoding SAG1 and GRA7 demonstrated superior humoral titers, enhanced splenocyte proliferation, and prolonged survival rates in challenge models using the virulent RH strain of *T. gondii* in BALB/c mice compared to single-gene controls (38).

Conclusion

The vaccine exhibited favorable predicted antigenicity, solubility, physicochemical properties, and lack of predicted allergenicity and toxicity. Immune simulation suggested the potential induction of both humoral and cellular immune responses. Nevertheless, these findings are computational predictions and do not establish protective efficacy or safety. Experimental studies, including in vitro assays and in vivo evaluation in appropriate animal models, are required to validate the immunogenicity, safety, and protective efficacy of the proposed construct.

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Supplementary material

Links to all bioinformatics web servers used in this study are provided in Supplementary Table 1. Respected readers may contact the corresponding author for supplementary materials.

Ethics Statement

No human participants, animals, biological samples, or personal data were involved in this research.

Conflict of interest

The authors declare that there is no conflict of interests.

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