Original Article

In Silico Vaccine Design and Expression of the Multi-Component Protein Candidate against the *Toxoplasma gondii* Parasite from MIC13, GRA1, and SAG1 Antigens

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Abstract
Background: We aimed to design a B and T cell recombinant protein vaccine of *Toxoplasma gondii* with *in silico* approach. MIC13 plays an important role in spreading the parasite in the host body. GRA1 causes the persistence of the parasite in the parasitophorous vacuole. SAG1 plays a role in host-cell adhesion and cell invasion.
Methods: Amino acid positions 73-272 from MIC13, 71-190 from GRA1, and 101-300 from SAG1 were selected and joined with linker A(EAAAK)A. The structures, antigenicity, allergenicity, physicochemical properties, as well as codon optimization and mRNA structure of this recombinant protein called MGS1, were predicted using bioinformatics servers. The designed structure was synthesized and then cloned in pET28a (+) plasmid and transformed into *Escherichia coli* BL21.
Results: The number of amino acids in this antigen was 555, and its antigenicity was estimated to be 0.6340. SDS-PAGE and Western blotting confirmed gene expression and successful production of the protein with a molecular weight of 59.56kDa. This protein will be used in our future studies as an anti-*Toxoplasma* vaccine candidate in animal models
Conclusion: *In silico* methods are efficient for understanding information about proteins, selecting immunogenic epitopes, and finally producing recombinant proteins, as well as reducing the time and cost of vaccine design.

Introduction

*T*oxoplasma gondii is an obligate intracellular parasite that has a worldwide spread and infects a variety of mammals and birds (1). The life cycle of the parasite consists of three stages, namely tachyzoite, bradyzoite, and sporozoite (2). This protozoan is one of the most important food-borne parasites that is transmitted to humans through cysts or oocysts (3).

The disease is usually asymptomatic in immunocompetent individuals. The disease may affect behavior and physiological processes (4). Certain diseases, such as rheumatoid arthritis (5) and cancer (3), may be associated with toxoplasmosis. Chronic infection may be activated in immunocompromised individuals, leading to toxoplasmic encephalitis and even death (6). In pregnant women, the infection can cause miscarriage, malformations of the newborn, and chorioretinitis leading to blindness (3).

Available drugs for toxoplasmosis chemotherapy are limited, and these drugs have toxic effects on humans (1). Most importantly, the current drugs cannot kill the parasitic cyst (7). Therefore, it is valuable to develop an effective vaccine against toxoplasmosis that can be used in animals or humans (3).

In recent years, vaccine development strategies have focused on proteins and DNA vaccines. Protein vaccines may cause adverse immune reactions (8), and DNA vaccines may induce the production of antibodies against themselves or cause tolerance (9).

Due to the mentioned problems, the use of recombinant peptides seems to be a smart approach to vaccine production because the use of minimal antigenic epitopes only stimulates the desired immune responses (10).

Since these vaccines contain a variety of B and T cell epitopes, they can stimulate cellular and humoral immune responses long-term (6, 11). The first step in achieving the most effective vaccine is to identify strong, protective, antigenic, and immunogenic compounds of the parasite (12).

Dense granule (GRA), microneme (MIC), rhoptry (ROP), and surface antigens (SAG) are the most effective vaccine candidates against *T. gondii* (13). MICs participate in the complex process of parasite entry into the host cell. MIC13 appears to play an important role in the diffusion of the parasite in the host body by binding to the gut epithelium (14).

One of the protective antigens of the parasite is the GRA (15). GRA1 has been identified in cases of chronic human toxoplasmosis (16). In addition, it is expressed by all three forms of the parasite, which causes the parasite to remain in the parasitophorous vacuole (6). GRA1 is a calcium-binding protein that demonstrates its role in invading host cells (17).

The *Toxoplasma* parasite surface is the main target for the host immune system, and the SAG1 is the main antigen involved in this process (18). Most antibodies produced during infection react with the SAG1 (19). Not only humoral immunity but also host cellular immunity is also stimulated by the SAG1 (20).

With the arrival of informatics, vaccine research has entered a new epoch (21). Different tactics, such as *in silico*, are used to reduce the cost and timing of vaccine access (22). We aimed to design a vaccine candidate against *T. gondii* based on recombinant peptides of MIC13, GRA1, and SAG1 of this neglected parasite (23) using immunoinformatics techniques in the field of *in silico*.

Materials and Methods

Retrieval of Sequence

In 2019 at Mazandaran University of Medical Sciences, northern Iran the sequence of the amino acids MIC13, GRA1, and SAG1 of the *T. gondii* RH strain was obtained from the NCBI GenBank and UniProt for further anal-

ysis. Based on the previous study, the best sequence of MIC13 (UniProt: H9BC62), GRA1 (UniProt: B9PHR1), and SAG1 (UniProt: C7E5T3) was selected (24).

Constructional analysis

The TMHMM server was used to transmembrane topology in the selected proteins based on the Hidden Markov Model (HMM) (24). UniProt and SignalP servers were also used to investigate the peptide signal of these proteins (20).

B-cell epitope prediction

This prediction was executed using the IEDB server. This server predicts B cell epitopes in terms of accessibility, hydrophilicity, antigenicity, flexibility, linear epitope, and beta-turns (13).

MHC-I and MHC-II epitope

According to the BALB/c mouse strain alleles, to predict the affinity of epitopes of the antigens to the major histocompatibility complex (MHC) class I (H2-Kd, H2-Ld, H2-Dd) and II (I-Ad, I-Ed), the sequences of these proteins were submitted to IEDB websites. Depending on the desired length of each protein (maximum possible score) and according to the IEDB recommendation using peptides with a length of 9 amino acids and their score (0-10), different epitopes were predicted (22).

Peptides assembly

To achieve the best placement of epitopes derived from proteins MIC13, GRA1, and SAG1 in the structure of chimeric protein antigen, three immunodominant peptide domains with different arrangements were fused by a helix forming linker containing the A(EAAAK)A motif (25).

Predicting structures and validity

To identify the probability of α-helix, β-sheet, and random coil in the secondary structure of the designed antigen, Garnier-Osguthorpe-Robson (GOR) IV and PSIP-ERD servers were used respectively (13).

To evaluate the three-dimensional (3D) structures of the designed sequences, the I-TASSER service was used (20). For estimating the quality of models, I-TASSER provides a confidence score (C-score) (26). To make the 3D models of the protein sequences, Molegro Molecular Viewer software produced by the SWISS-MODEL was employed (27).

The validity of the 3D structure of the MGS1 protein was investigated by the Ramachandran plot using the SWISS-MODEL program (13).

Antigenicity and allergenicity

The VaxiJen v.2.0 site was used for the antigenic analysis of different arrangements of antigen fragments. Threshold scores greater than 0.5 indicate the possible antigenicity of the structure. Server accuracy ranges from 70% to 89%, depending on the target organism (28). To predict the allergenicity of the protein, the Algpred server was used. The server can predict allergenicity with 85% accuracy at a threshold of -0.4 (29).

Physicochemical properties

SOLpro server was used to evaluate the solubility of the structures (20). Moreover, the ProtParam server was employed to predict the different physicochemical properties of the chimeric protein (13).

Codon optimization

Codon optimization is used to maximize protein expression (30). For reverse translation and optimization of the codon, the Jcat tool was used (20).

mRNA structure

To determine the free energy corresponding to the 5' end of mRNA of the recombinant gene, the mfold tool was used. This server determines the thermodynamic properties of the mRNA molecule (20).

Protein production

Biomatik Corporation (Ontario, Canada) synthesized the chimeric gene and cloned it into the corresponding sites of the pET-28a (+) vector to create the pET-MGS1 plasmid.

Calcium chloride was employed to transform the pET-MGS1 into the *Escherichia coli* BL21 cells. Transformed *E. coli* was cultured in LB agar. The bacteria were then passaged to the LB broth, and after reaching the logarithmic phase, gene expression was induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG). The LB broth was centrifuged at 7000 g, and bacterial sediment and supernatant for analyzing the expressed proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20).

Western blot

Separated proteins with SDS-PAGE, were blotted onto a nitrocellulose membrane affected by the electric field. Anti-His tag was used to specific binding to the polyhistidine sequence of the protein. The protein band appeared with a horseradish peroxidase staining solution containing H2O2 and diaminobenzidine (DAB) (20).

Ethics statement

The protocol for this study was approved by the Ethics Committee of Mazandaran University of Medical Sciences and the code of ethics of this plan is IR.MAZUMS.REC.1398.4851.

Results

Selective sequences

Fragments to the lengths of 200, 120, and 200 amino acids were selected from each of the MIC13 (468:N_{73}-K_{272}), GRA1 (190:E_{71}-E_{190}), and SAG1 (336:I_{101}-E_{300}) antigens, respectively, to be used as chimeric antigen.

Signal peptides and transmembrane topology

TMHMM server analysis determined that only GRA1 has a transmembrane domain. Since one of the main criteria for an efficient epitope is to be exposed at the cell membrane surface, the transmembrane domain was not selected in the design of the chimeric antigen. SignlP 4.1 server predicted the amino acid positions of 1-22, 1-24, and 1-25 in the three MIC13, GRA1, and SAG1 antigens as peptide signals.

B-cell and T-cell epitope

Tables 1 and 2 show the prediction results of three antigens from the associated servers. The IEDB server classifies the 9-mer T-cell epitopes along with their scores. Table 2 lists the epitopes with scores >7.5 that bind to MHC-I and MHC-II.

Segment selection

Based on the data obtained from different servers, MIC13 (73-272), GRA1 (71-190), and SAG1 (101-300) peptide fragments were selected. These fragments have several strong T and B cell immunodominant epitopes.

Combined peptide

Six chimeric proteins (GSM1, GSM2, MGS1, MGS2, MSG1, and MSG2) from the three selected peptides with A(EAAAK)A or A(EAAAK)2A as a linker, which had the best stability and folding based on the second and third structures, were selected. Among these structures, MGS1 with A(EAAAK)A linker based on the degree of antigenicity, second and third structures, and highest C-score was selected as the best candidate for the vaccine.

Antigenicity and allergenicity

Prediction of the allergenicity of the chimeric proteins showed that none of them were allergens. The antigenicity values of these proteins were estimated as follows: GSM1:0.6286, GSM2:0.6290, MGS1:0.6340, MGS2:0.6343, MSG1:0.6332, and MSG2:0.6334.

Table 1: Parameters of B-cell in MIC13, GRA1, and SAG1 by IEDB tool

B-cell Parameters	MIC13	SAG1	GRA1
Bepipred Linear Epitope	**70-95**	45-65	**67-78**
	172-185	**85-112**	**87-98**
	208-222	**120-138**	**122-145**
	240-260	**190-242**	**170-180**
	305-320	**247-315**	
	330-405		
	417-445		
Beta-Turn	**60-90**	45-65	**65-80**
	105-133	**90-115**	**86-98**
	150-165	**123-140**	**135-145**
	200-220	**160-168**	**173-186**
	305-320	**180-265**	
	330-345	**285-293**	
	395-405	304-318	
	420-445		
Accessibility	**175-200**	60-100	**65-75**
	218-235	**120-155**	**105-114**
	270-278	**165-210**	**98-130**
	285-318	**220-292**	**138-147**
	360-381		**167-180**
Flexibility	**70-97**	**60-140**	**70-80**
	175-185	**152-166**	**85-95**
	205-245	**195-295**	**105-115**
	255-278	305-315	**120-128**
	305-318		**137-145**
	360-380		**165-182**
	400-410		
	420-445		
Antigenicity	**100-130**	25-75	**78-86**
	150-170	**80-120**	**90-103**
	235-270	**140-220**	**145-170**
	280-305	**280-300**	
	330-345	315-336	
	440-453		
Hydrophilicity	**70-95**	55-75	**67-75**
	173-185	**80-115**	**85-100**
	207-222	**95-138**	**106-113**
	240-260	**160-242**	**120-145**
	305-320	**250-318**	**155-180**
	330-345		
	395-407		
	415-445		

Bold numbers are present in MGS1

Table 2: T-cell epitopes predicted from IEDB server

Protein	Amino acid start position[a]					Number of binding epitopes		Total
	H2-Kd	H2-Ld	H2-Dd	I-Ad	I-Ed	MHC-I	MHC-II	
MIC13	156	218	222	107	137	48	95	143
	104	92	228	200	76			
	229	190	229	165	144			
	195	221	190	182	185			
	197	229	80	222	168			
GRA1	177	109	137	148	79	19	51	70
	146	72	122	94	110			
	83	176	74	114	115			
			71	142	165			
			182	144	155			
SAG1	242	259	259	112	241	47	93	140
	273	227	149	140	110			
	135	203	198	169	142			
	212	190	206	171	221			
		195	139	173	271			

[a] Maximum 5 epitopes with a score higher than 7.5

Secondary and 3D structures and validity

The MGS1 structure, which contains 555 amino acids, consists of 27.39% alpha-helix, 52.43% random coil, and 20.18% extended strand (Fig. 1). The 3D structure of this protein is shown in Fig. 2.

Protein validation using the SWISS-MODEL server indicated 83.54% of residues are located in favored, 12.48% in allowed, and 3.98% in outlier regions (Fig. 3).
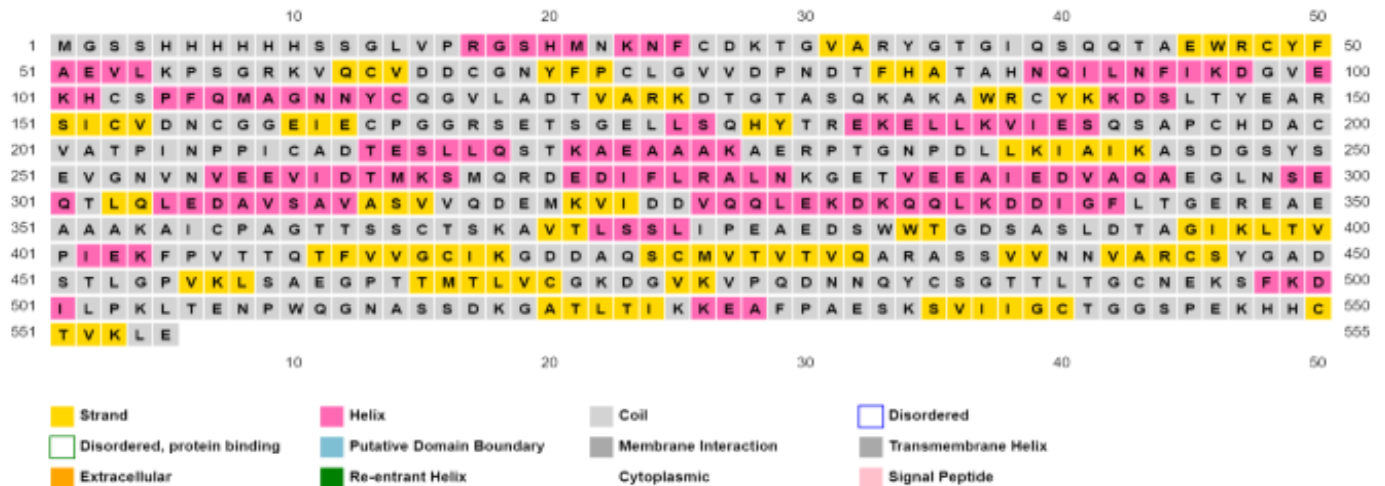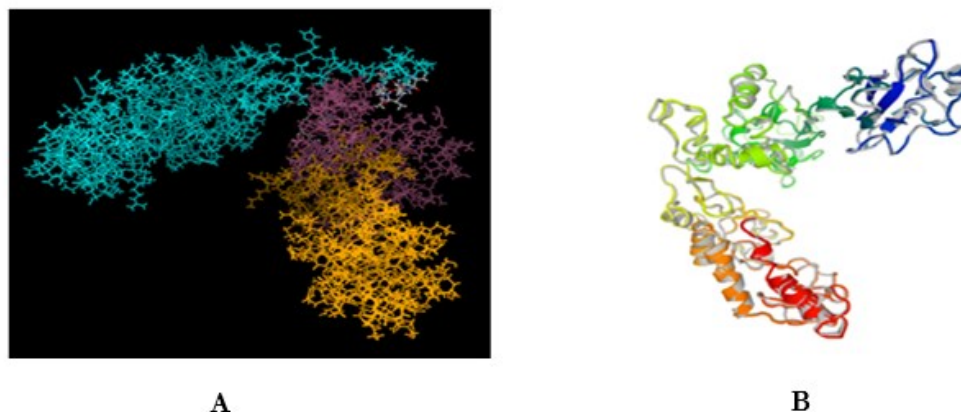

Fig. 1: Secondary structure of MGS1

A   **B**

Fig. 2: (A) 3D structure of MGS1 using I-TASSER service. MIC13:blue, GRA1:purple, SAG1:yellow.
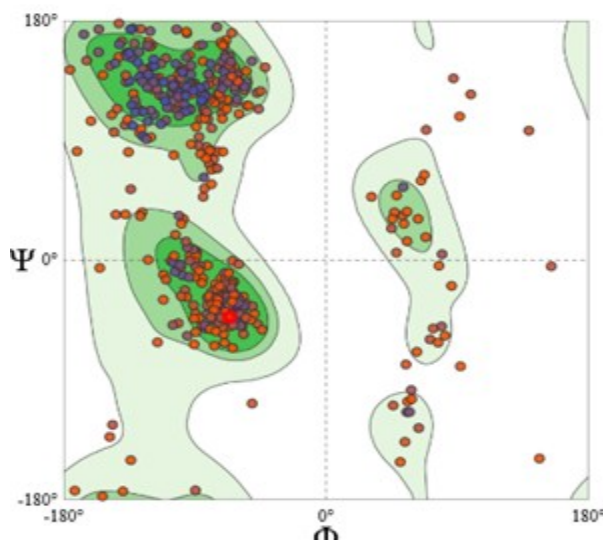**(B)** Computed 3D model by the SWISS-MODEL server

Fig. 3: Validation of the 3D structure of MGS1

Physicochemical properties

The MGS1 has a molecular weight of 59.56kDa and an isoelectric point (IP) of 5.22. The number of negatively (Asp+Glu) and positively charged residues (Arg+Lys) were 75 and 55, respectively. The half-life was estimated to be 30 h (mammalian reticulocytes, *in vitro*), more than 20 h (yeast, *in vivo*), and more than 10 h (*E. coli*, *in vivo*). The instability index for this protein was computed to be 35.42 classified as a stable protein. Furthermore, the grand average of hydropathicity (GRAVY) of MGS1 was -0.404. The SOLpro server estimated the solubility of MGS1 was at 0.744677.

Codon optimization

By reverse translation in JCAT and EBI servers, codon optimization was performed for maximum protein expression in *E. coli*. This means that for a particular amino acid, its codon in the host can have the highest expression efficiency.

mRNA secondary structure

According to prediction in the mfold server, the best second structure of the mRNA molecule had a ΔG=-451.90kcal/mol. The minimum free energy (MFE) for the first 10 amino acids at the 5' end was estimated to be -2.8, based on which the structure of the stable pseudoknot and hairpin will not be formed (Fig. 4).
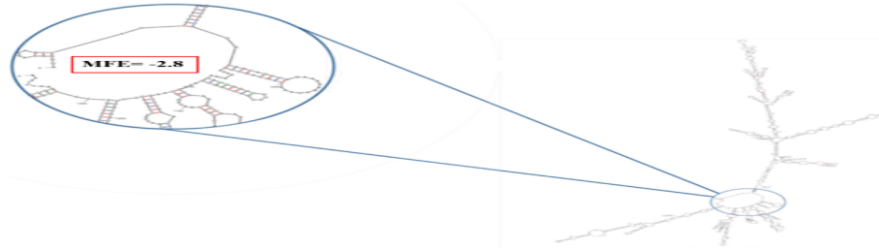

Fig. 4: Secondary structure of mRNA by mFold (The enlarged fragment shows the 5' end of the mRNA)

Cloning, transformation, and expression

The recombinant gene of chimeric protein (1617bp) was cloned into the pET-28a(+) vector between two NdeI and XhoI restriction enzyme sites and production of MGS1 in *E. coli* was evaluated using the SDS-PAGE technique (Fig. 5, Left).

Western blot

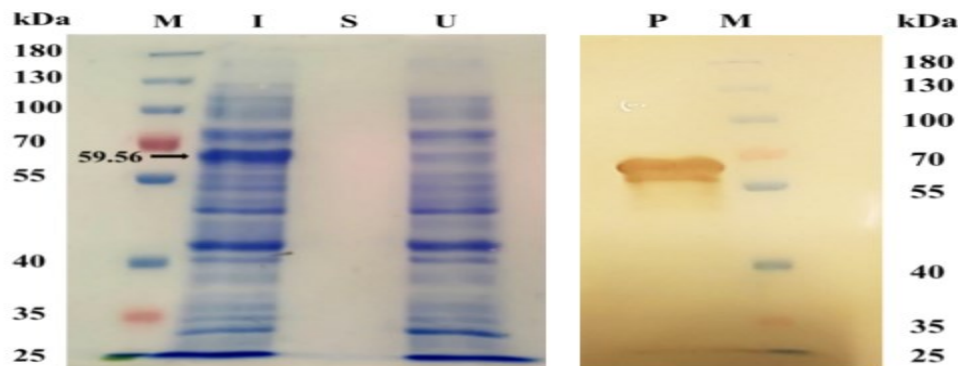The band of chimeric protein was observed using diaminobenzidine in western blot (Fig. 5, Right).

Fig. 5: Left: MGS1 SDS-PAGE. Right: MGS1 Western blot. **M:** Protein MW marker. **I:** E. coli Pellet including pET-MGS1 induced with IPTG. **S:** Induced E. coli Supernatant including pET-MGS1. **U:** Not induced E. coli pellet, including pET-MGS1. **P:** MGS1 band.

Discussion

Considering the deadly complications of toxoplasmosis due to the activation of the chronic form and the problems of congenital toxoplasmosis, research on vaccine production seems necessary (1).

Vaccine for the whole body of the pathogen contains hundreds of proteins, while most proteins are not essential for inducing protective immunity (31). Synthetic peptide vaccines with multiple antigens that have different epitopes can be a successful strategy in developing anti-*Toxoplasma* vaccines (8).

Today, using vaccine databases and *in silico* tools, vaccine design is more possible than in the past (21). These alternative strategies develop effective new-generation vaccines based on immunoinformatics and reverse vaccinology. Reverse vaccinology can identify different structures of parasites that elicit immune responses (22). These methods are cost-effective and can significantly reduction in time. In addition, designed vaccines are more efficient and safer than traditional vaccines (32). With all these advantages, bioinformatics predictions may not always be fully consistent with laboratory and experimental results (33).

Since the spread of bioinformatics, several studies have been conducted with an *in silico* approach on different *Toxoplasma* antigens for vaccine design. In 2022, Li et al. used T and B cell epitopes of *T. gondii* membrane proteins to design a vaccine (34).

The focus of this study is on three *Toxoplasma* antigens that have been identified in previous studies as antigens that are effective in stimulating the humoral and cellular immune systems. These antigens include MIC13 (14), GRA1 (11), and SAG1 (11).

B-cell antibody responses to prevent parasite replication in tissues are essential in the chronic stage of the disease, and humoral and cellular immunity work closely together in host resistance to *T. gondii*. It appears that the role of each immune response is different depending on the stage of infection and the anatomical location of the parasite (35). Therefore, in selecting epitopes from these three antigens, it is attempted to choose fragments that are immunodominant in both B and T cells.

A chimeric protein was made in this study, and unstable elements and restriction sites were removed in the epitope selection process. For this purpose, several bioinformatics tools were used to analyze various aspects of the protein. On their structure, linkers are divided into three categories, namely flexible, rigid, and *in vivo* cleavable. Linkers maintain an appropriate distance between the peptides so

that each has its function (36). Low protein production, impaired biological activity, and proteins misfolding are the consequences of using an inappropriate linker or not using a linker (37). In this study, a rigid linker was used to connect the domains, and it was selected among different MGS1 arrangements with high antigenicity.

The antigenicity of proteins makes it possible for the immune system to detect them (20). On the other hand, care should be taken in the design of recombinant peptide vaccines, as chimeric proteins can be allergenic (22). In this study, among the six investigated structures, MGS1, in addition to not being allergenic, also had a high antigenicity score.

The physicochemical properties of proteins are another important issue that should be considered in the vaccine design process. One of these properties is the aliphatic index, which indicates the presence of aliphatic amino acids (alanine, valine, leucine, and isoleucine) in the side chains of the protein and the higher index indicates the stability of the protein over a wider range of temperatures. GRAVY is the other characteristic, and the negative value of GRAVY indicates the hydrophilicity of the protein, which makes the protein interact better with water molecules in its surroundings (38).

The IP is also one of the important physicochemical parameters that can be used to estimate the solubility of a protein at a certain pH, meaning that the protein precipitates in a solution close to the IP (24). According to the IP, the MGS1 is an acidic structure. The IP is useful to achieve a suitable buffering system for protein purification based on pH-focused methods (38), and based on this; MGS1 dissolves well in basic buffers.

Another very important physicochemical indicator is the MW of the protein, which is directly, related to the stimulation of the immune system, and given that the MW>5-10kDa are known as good immunogens (13),

MGS1 is a good antigen considering its weight of 59.56kDa.

One of the most important factors influencing the function of epitopes is their secondary structure (39). The secondary structure of proteins is the basis of 3D structures and sequences formed by hydrogen bonds between polypeptide chains (40). The results of investigating the secondary structure of MGS1 pointed out that this protein contains 52.43% random coil, 27.39% alpha-helix, and 20.18% extended strand (38).

Discernment of the 3D structure details of a protein can be a great help in determining its molecular function (27). From six structures, the MGS1 model with the highest C-score was selected for further analysis. The quality of the models made can be compared using the C-score (29).

Another parameter in protein structure prediction is the validation of the structure and the Ramachandran plot is useful for evaluating the quality of experimental structures and predicting a protein's biological function (20). The MGS1 antigen was considered a suitable model based on the residues percentage in favored and allowed areas and a relatively low percentage in the outlier area.

The main factor used in codon optimization is the Codon Adaptation Index (CAI), which ranges from zero to one, and the number one means that a gene for each amino acid used synonymous codons the most frequently (41). CAI in our gene reached one after codon optimization.

The secondary structure of mRNA is an important factor in the expression of proteins, and this molecule must be sufficiently stable (33). The mRNA structure is optimized based on the ΔG, and the energy required to open the hairpin loop structure of the start codons. This feature helps connect the ribosome to start the translation (38). The mfold server predicted 37 secondary structures for the mRNA of the MGS1 gene, the ΔG of which from ranged -451.90 to -438.70Kcal/mol. The best structure had the ΔG=-451.90. Based on mfold data, mRNA is stable and suitable for efficient translation and production of chimeric protein in the host vector.

In the future, the protective effect of the designed recombinant MGS1 vaccine will be evaluated on BALB/c mice against *T. gondii*.

Conclusion

Bioinformatics is efficient for understanding information about proteins and selecting immunogenic epitopes and finally producing recombinant proteins. However, although today bioinformatics studies are necessary for faster and more efficient access to vaccine candidates, due to the limitations of *in silico* methods for predicting the physicochemical properties, structures, and immunogenicity of chimeric peptides, the efficacy of designed recombinant vaccine must be confirmed by experimental methods and animal studies.

Acknowledgements

Conflict of Interest

The authors declare that there is no conflict of interest.

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