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In Silico Design and Analysis approach, Multi-Epitope Antigen against Coccidiosis in Broiler Chickens

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Abstract Background: The spread of coccidiosis in poultry has been accompanied by challenges in control and treatment, such as resistance to repeated treatments used in the field, and limited immunity due to antigenic diversity, which prompted researchers to adopt the concept of immunogenic antigens. Objectives: To generate a vaccine using computational methods to create broader immunity against mixed infections and longer-lasting immunity to control them. Methods: Using bioinformatics design techniques to provide the highest and most comprehensive level of protection against coccidiosis, the current project introduced the 19 kDa sporozoite antigen as an immunogenic protein genetically conserved among Eimeria species to design a multi-antigen vaccine. Bioinformatics tools were used to generate a vaccine antigen with a total of 18 antigens (B cells, cytotoxic T cells, and high-density T cells) consisting of 274 amino acids. Outcome: The reconstituted vaccine was physically and chemically evaluated, and binding to TLR15 and TLR4 was achieved. Dynamic immunological simulations were performed, using results from immunological compounds and molecules, including antigen levels of 700,000/ml and 500,000/ml, INFy about 4,75,000 ng/ml, and enhanced T and B cell proliferation to produce IgG (30,000/ml). Conclusion: The results of the vaccine design and evaluation simulations were acceptable expectations, and further in vivo experiments are needed to confirm the vaccine's defensive efficacy
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Keywords: *Eimeria, immunobioinformatic, antigen, vaccine*, *epitope*

Introduction One of the most important diseases affecting the poultry sector is coccidiosis, which is widespread in large areas of the world, causing significant production losses of up to $11 billion (1). The causative agent of the disease is a protozoan parasite called Eimeria that invades the intestines of chickens with host specificity (2). Coccidiosis is often reported to be caused by more than one species include *E. acervulina, E. brunetti, E. maxima, E.imitis, E. mivati, E. necatrix, E. praecox*, and *E. tenella* (3). Rapid multiplication of the parasite within a certain area of the intestine leads to severe effects(4). Oocyst development and formation of infective sporozoites in the fecal environment (5). Diagnosis is based on direct smear and flotation, and there is a need for molecular and immunological methods to identify the species and their importance in proving the virulence of the oocysts (6). Control plans suffer from resistance to anticoccidial drugs, and thus the veterinarian fails to control successive production cycles within the rearing area (7). The most conservative and immunogenic antigens such as 3-1E, Etcab,

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EtROP35 and the 19 kDa sporozoite antigen which are profilin-like protein present in the cytoplasm of most eukaryotic organisms and appear in sporozoites and merozoites in the developmental stages of the parasites, stimulate interferon-gamma, hormones and cellular immunity for experimental studies which functionally interfere with actin polymerization and cause blocking of parasite invasion of epithelial cells and limiting the shedding of oocysts (8). Field studies to control coccidiosis have implemented shuttle treatment programs that recommend switching treatments at successive intervals to avoid resistance and thus increase costs, which makes control attempts fail and return to the preference for the idea of vaccination (9-10). Veterinary research institutions continue to support the idea of preferring vaccination against local strains with the development of broad-spectrum immunity to avoid therapy resistance and reduce economic costs and use it in an attempt to enter the field of biotechnological vaccines.

Methodology

The study was conducted on a multi-genotype vaccine against coccidiosis based on bioinformatics protocols from previous research methods (11-12):

The retrieval of protein sequences: sporozoite antigen is belong to "Profilin liked protein" of many species reported in NCBI https://www.ncbi.nlm.nih. gov/protein and identify many sequence alignment for conserved level among different Eimeria spp. https://www.genome.jp/tools-bin/clustalw.

Antigenicity, toxicity and allergist properties of protein: The antigenic propensity of the protein The antigenic readiness of a protein is determined by https://www.ddgpharmfac. net/vaxijen/VaxiJen/VaxiJen.htmltoxicityhttps://webs.iiitd.edu.in/raghava/toxinpred/ and allergy score https://www.ddg-pharmfac.net/AllerTOP/method.html. Transmembrane domains predicated by TMHMM server https://services.healthtech.dtu.dk/services/TMHMM-2.0/, the solubility value greater than 0.45 is predicted to have a higher solubility https://protein-sol.Manchester.ac.uk/ (13-14).

B cell ,TCL and HTL epitopes prediction: A sequences prediction of assembly vaccine by use http://tools.iedb.org/mhci, the lack of data currently available in the immunoinformatics tool for chicken alleles that bind to the MHC1 and MHC2 epitopes, it's used a human options that related to the MHC1 and MHC2 epitopes as similar to (15) ,B cell epitopes that stimulate the antibodies responses (16).

Assembly of multi-epitopes vaccine: to build the final structure of the vaccine, (CD4)HTL epitopes were linked by GPGPG,(CD8)CTL epitopes were linked by AAY, andB-cell epitopes linked by KK during perpetrated a vaccine and a design of the chemical structure. The linkers support the flexibility of the peptides making up the vaccine, giving it greater stability, if added an adjuvant may o the vaccine as enhance the immunogenicity of a multi-epitope vaccine(17).

Antigenicity, Allergencity, physicochemical properties of the constructed vaccine: as same the previous steps of the protein analysihttps://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html and https://www.ddg-pharmfac.net/AllerTOP/method .html, protein – sol server to find solubility https://protein-sol.manchester.ac.uk/ , ProtParam 53 web server of the (EXPASY) system and for calculate the number of amino acids of the vaccine, molecular weight(kDa),hydropathicity, half-life, instability, Gravy and aliphatic index https://web.expasy. org/protparam/.

Secondary structure of the constructed protein: The focus directed towards the localized folding patterns that forms inside certain regions of the protein sequence designed by NetSurfP-3.0 server as helical, sheets, and loops important aspect in protein architecture. http://www.proteus2.ca/proteus2/Result.do?ID=0794877 is a server for analysis the proportions of the secondary protein structure, such as prediction of a helix, β-strand, and coils(18).

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3D structure prediction, validation and refinement of the constructed protein: https://swiss_model.expasy.org/ server was designed 3D-structural from the PDB format and refinement of the structural building(Waterhouse *et al*.,2018). The predicted 3D structure was refined quality and of the vaccine protein and lead to made five protein structural models resulting from related on https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE and using the Ramachandran plot of the procheck server https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/ for recognize a residuals of the different regions of favorable, unfavorable, acceptable, or low, whether allowed or not,

The docking of the constructed protein: The Toll-like receptors is a molecular linking in birds involved in the development of the immune response to various pathogens such as Eimeria infection(20-24). Molecular docking predicts the molecular interaction between receptor and ligand to demonstrate the stability and tight affinity of their docked complex using Molecular Operating Environment software (25).

Molecular dynamics simulation of the constructed vaccine: The C-ImmSim online server is immunological computing tool rely on http://kraken.iac.rm.cnr.it/C-IMMSIM, C-ImmSim refers to determine the behavior of the immune system, that documenting in the many plots format(26). For user can be input designed vaccine data and simulation running, then evaluate the findings for considered as a important tool of the immunity prediction.

In silico, cloning and optimization of vaccine sequence: the manufactured vaccine may be add them to pET-28b(+) vector and design a https://en.vectorbuilder.com/design /pRP_Exp.html (27).

Results

The retrieval of protein sequences:

The design was carried out by reviewing a set of proteins reported for the parasite in the previous studies included 19kda sporozoite antigen(AAW31899.1) of Eimeria as an immunogenic antigen available among Eimeria spp. of the stages of development inside a chicken. An evolutionary tree was designed for comparison among samples documented in NCBI web as shown in figure 1.

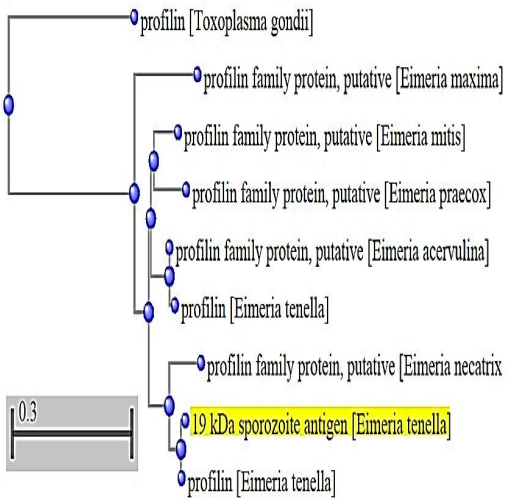

Figure (1)The phylogentic tree of the 19 KDA sporozoite antigen for Eimeria isolates registered on the NCBI (BLAST

Antigenicity, toxicity, solubility and the physicochemical features of protein:

A sequences converted into the FASTA format then inserted in many servers, the vaxijen2.0 server is approved to determine the antigenicity of the protein based on threshold= 0.5 as in the table 1 and other scores. As for determining the presence of the Transmembrane helices in proteins, which will indicate the segments of the protein that lie inside, outside or within the membrane found in figure and the probability reaches much less than 75.0%, the outside site of the cell membrane in the following figure 2.

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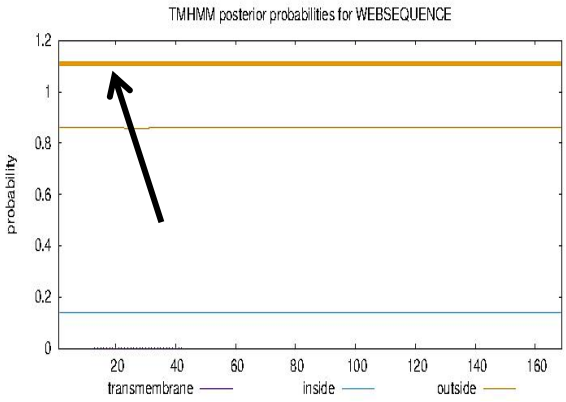
Table (1)the physicochemical, toxicological, and antigenic

physicochemical features	value
Number of amino acids	169
Molecular weight:	18420.31
Theoretical pI	4.37
classifies the protein	stable
Aliphatic index	77.93
average of hydropathicity	-0.386
Antigenicity	0.7387
solubility	0.869



Figure(2)The Transmembrane helices of 19 sporozoite antigen

Table(2) Properties Each epitope is a genetic predictor for protein structure design

Epitopes	Sequences	Antigenicity	Allergic	Toxicity	Gravy
HTL	MGEADTQAWDTSVRE	0.502	non	non	-1.053
	ADGCRLFGAAVEGEG	1.4931			0.179
	NAWEELVKTNYQIEV	0.7238			-0.665
	ETLRQAVVDGRAPNG	1.2343			-0.687
	GRAPNGVYIGGTKYK	1.2198			-0.564
BCL	MEGEGNAWEELVKTNYQIEVPQ EDGTSISVDCD	1.0478	N on	non	-0.958
	VDGRAPNGVYI	1.8207			-0.045
	TFNDQNY	1.2304			-1.886
	DEEKEQNKAD	1.2642			-3.05
CTL	NGVYIGGTK	1.0516	non	non	-0.211
	GVYIGGTKYK	0.9265			-0.36
	VYIGGTKYK	0.5873			-0.356
	NYDVAILGK	1.7413			0.189
	KGGGFLIKTP	0.7013			-0.02
	NENVVIALY	1.3361			0.794
	DEEKEQNKAD	1.2642			-3.05
	ADALTTALN	0.7291			0.511
	AEYLHQSGF	0.5859			-0.478

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Antigenicity, allergencity and toxicity ofselected epitopes:

The lack of data currently available in the immunoinformatics tool for chicken alleles ,either used a IEBD server in predicted B cell linear epitopes explain that stimulate the antibiotic responses within the following figure 3. A number of immunogenic epitopes (5 HTL,4 BcL ,9 CTL) are evaluated a features of each epitope which are detailed in table 2.each epitope.

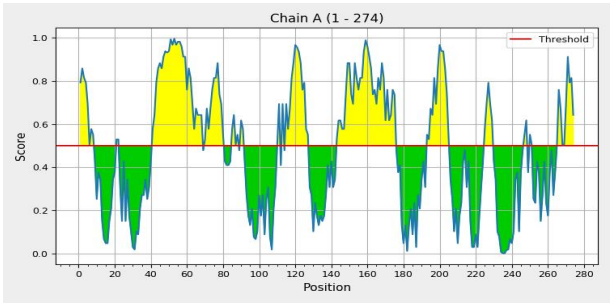

Figure (3) a IEBD server in predicted B cell linear epitopes, Green area= less immunogenic, yellow area= potentially immunogenic

Assembly of multi-epitopes vaccine: the number of amino acids combined to compose the manufactured vaccine were 274 amino acids and linkers approved in the previous studies, as shown in the figure4 and sequence of constructed protein include:

Antigenicity, Allergencity, physicochemical properties of the constructed vaccine:

Entering the sequence of the manufactured antigen into the many servers mentioned above to evaluate immune-antigenic features of complete protein, which are detailed in table (3).

Table(3)Immunologicaland physicochemica

physicochemical features	value
Number of amino acids	274
Molecular weight	29108.11
Theoretical pI	5.02
classifies the protein	stable
Aliphatic index	62.45
average of hydropathicity	-0.642
Antigenicity	0.8954
solubility	0.726

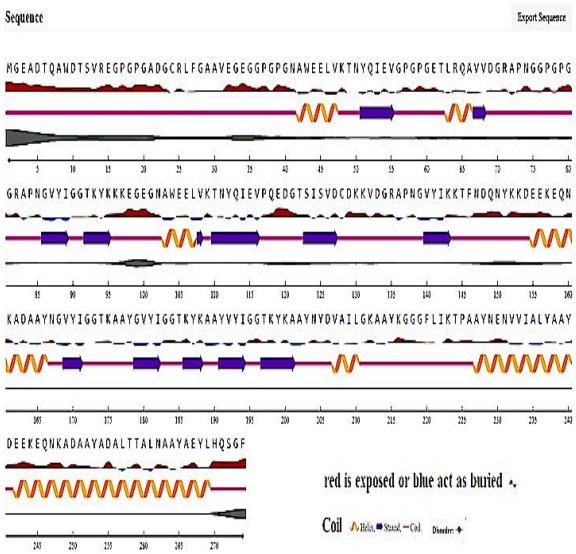

Figure (4) The composition of the recombinant protein with adding adjuvants and linkages.

Secondary structure of the constructed protein:

A hydrogen bond formed between the oxygen atom in the basic carbonyl group of one amino acid and the hydrogen atom in the basic amino group of another amino acid. The secondary structure of recombinant protein analyzed by Proteus 2.0 server explain confidence value =72% as moderately reliable in the secondary structure prediction, helixes= 19%, Beta sheets =24% and Coils = 57% as showed in figure(5).

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Identify surface or buried regions. a graphical image of 274 residue predictions, Surface regions refers to red is exposed or blue act as buried, while secondary Structure present include Strand, Coil, Helix, Strand. Disorder Disorder: thickness of line equals probability of disturbed residue

3D structure and refinement of the constructed protein:

Swiss-model server is a platform for creating 3D models (PDB format) of the recombinant protein explain in figure 6A, using a comparative scores of the best quality by alpha fold method with seq. identical =86.75(score >30%) related to the origi**A** template and QMGE=0.64% and Ramachandran plot of Swiss-model server =77.57% high favored regions as explain in figure 7.

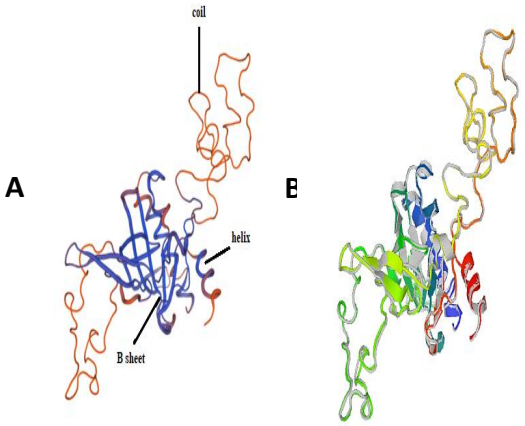

Figure (6) A-3d structure predicted by Swiss Model server, B- refinement of recombinant protein using GalaxyRefine

Using Galaxy Server led to significant improvements in infrastructure quality and verification metrics showed in figure 6B. the five models of the 3D protein based on the results listed in the table 4. MODEL 5 exhibits GDT-HA, Clash Score was lower thus near to initial model. After that in figure 9, the Ramachandran plot analysis evaluated by the procheck server 180residues within favored regions representing 82.6% of the protein tertiary structure.

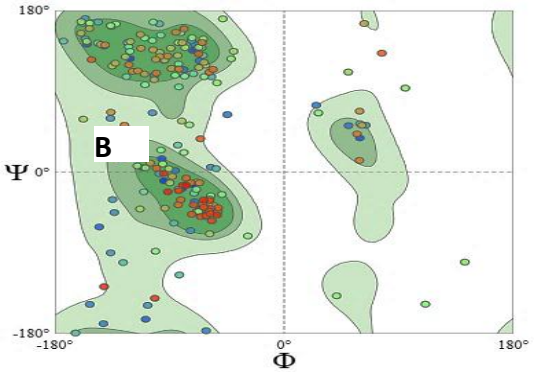

Figure (7) Investigation of recombinant protein model using Ramachandran plot Red dots: high favored regions, Blue and green dots: less common angles, Green regions: allowed regions Dense regions represent the most likely angles based on the

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Table(4) The five improved models of the Galaxy Server

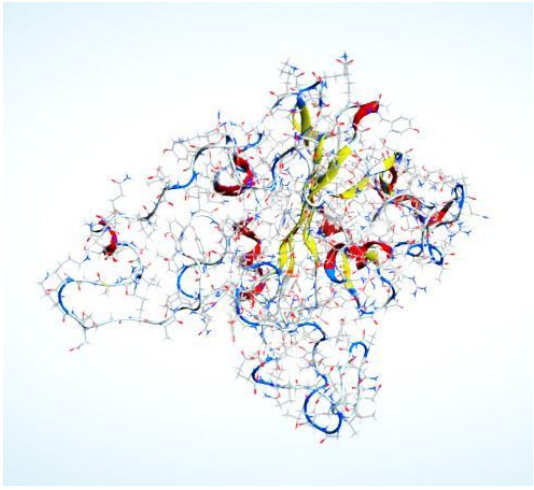
Model	GDT-HA (↑ Better)	RMSD (↓ Better)	MolProbity (↓ Better)	Clash Score (↓ Better)	Poor Rotamers (↓ Better)	Rama Favored (↑ Better)
Initial	1.0000	0.000	2.380	9.0	1.5%	77.9%
MODEL 1	0.9516	0.414	2.138	12.0	0.5%	90.1%
MODEL 2	0.9599	0.380	2.208	13.9	0.0%	89.7%
MODEL 3	0.9498	0.411	2.181	12.7	0.5%	89.3%
MODEL 4	0.9571	0.406	2.147	12.9	0.5%	90.8%
MODEL 5	0.9507	0.408	2.171	13.9	1.0%	91.2%

The docking of the constructed protein:

Design and preparation of the crystal structure for simulation with receptors as seem in the figure 10. A comparison was made with TLR4, TLR15 receptors, as shown in the values in the table may be the best confidence score showed in figure11. The more negative of docking score between ligand and receptor as seem in the table5.

Table (5) Comparison after docking scores of several ligand 19kda and chicken receptors (TLRs)

RANK	Recombinant E19Kda antigen	
	TLR4	TLR15
Binding strength	-17.5313,-7.59155	-15.3464,-8.037



Figure(10) preparation of crystal structure vaccine

thantigenic decline and continue for periods of 35 days after vaccination. Finally, the immune cytokines, interferon gamma and interleukin 2, it's find that they increase significantly and reach the fifteenth day after vaccination and then

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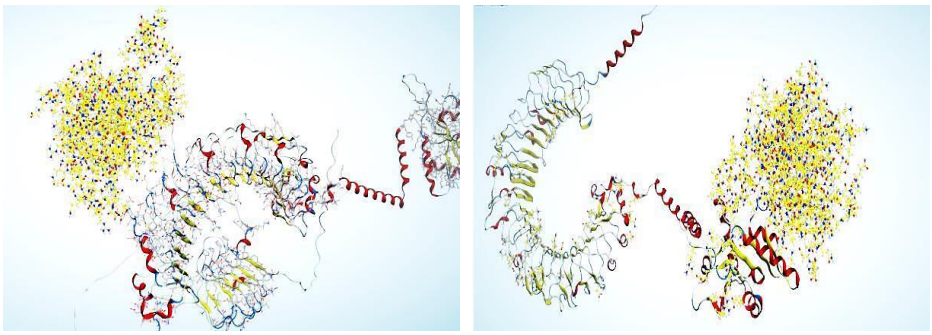
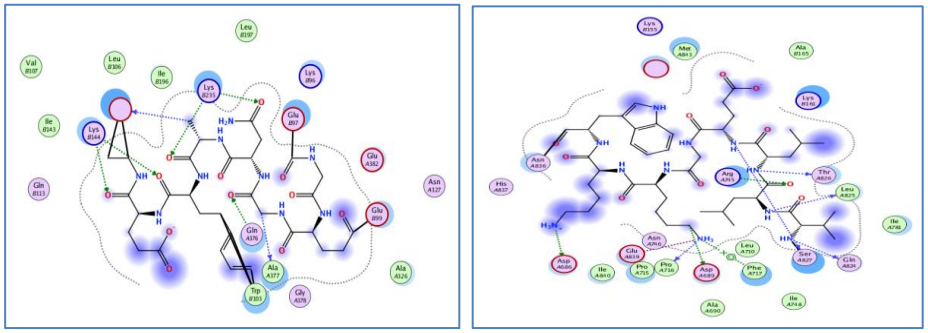


A- 3D interactions diagram of induced fit docking of vaccine and RLR15

B- interactions diagram of induced fit docking of vaccine and RLR4



C- 2D interactions diagram of induced fit docking of vaccine and RLR15

D- 2D interactions diagram of induced fit docking of vaccine and RLR4

Figure(11) docking of the vaccine and toll liked receptors A,B 3D structure and C,D 2D structure of protein-protein binding

Molecular dynamics simulation of the constructed vaccine:

The curve of B cells and their activity in general, the most important of which are memory cells that provide immunity for a longer period accompanied by an increase in helper T cells of the first class as shown in Figure 12. IgM and IgG from the fifth day directly after

In-silico cloning and optimization of constructed vaccine:

Use the gene design tool (vector builder) inserted to the cloning vector (pET28b(+)) and forming a gene length 838 bp, they are cleaved by the enzymes BamH, Xhol. as showed in the figure 13. Gel electrophoresis of the clone product was performed and the plasmid was digested at 37°C for 30-60 minutes according to GenScript company. The M=LADDER represents,1=plasmid and 2= digested plasmid.

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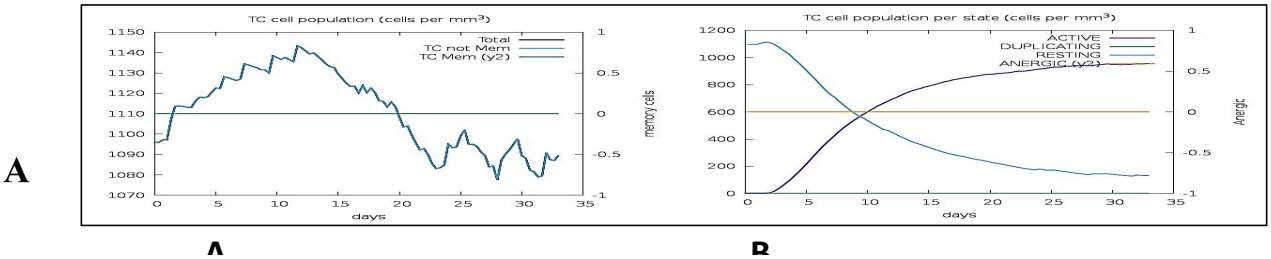
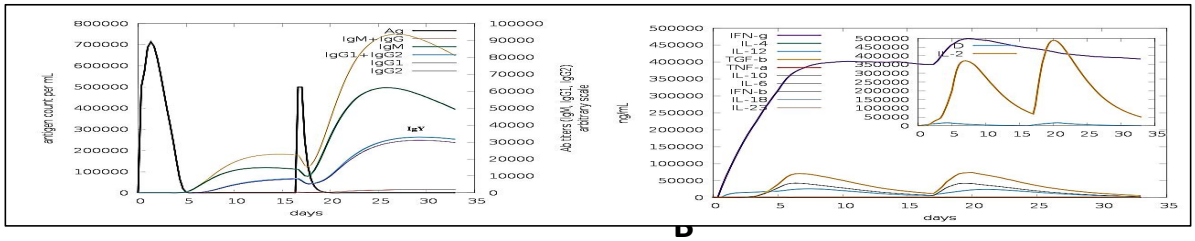


Figure (12) immune simulation of the response to the designed vaccine .A- Increased differentiation of cytotoxic T cells important in immunity against coccidian ,B- Elevated activity of non-memory cytotoxic T cells in immunity against coccidian



Figure(13) immunoglobulin and cytokines simulation for the designed vaccine . A-Antigenicity increases during the first five days after vaccination, followed by a gradual rise of antibodies, B-increase in interferon-y, TGF-b and interleukin2,10,12 in the first days and continuing for 35 days) during injected by two doses of recombinant vaccine.

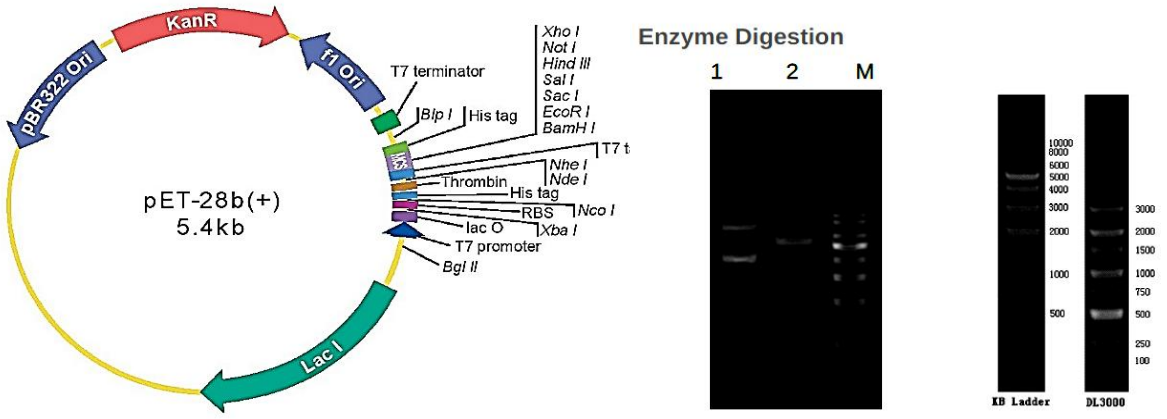


Figure (13) shows the plasmid carrying the E19 Eimeria_pET-28b gene and electrophoresis of product

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Discussion

Preventive programs rely on therapeutic and management plans that may have contributed to reducing the clinical coccidiosis incidence, but problems soon occur such as resistance and the recurrence of the disease within chicken populations. Immunological protection requires extensive study to confront challenges such as antigenic diversity during a complex stages of the life cycle of this parasite(28-29). Profilin liked protein achieves an increase in the response of Toll receptors and increases the secretion of lymphocytes that release interferon gamma (30-31). The recombinant protein properties showed a higher antigenicity and the solubility level more than 0.45, and no allergy or toxicity, thus similar to the previous study on the IMP-1,Gam56,3-1E and AMA antigens(32-33). The researchers explained about this quality of vaccine protein with criteria stating that "vaccine antigens must be a molecular weight <110 kDa are considered a best vaccine candidates as in the current study MW=29108.11 (34-35). The tertiary structure of the constructed vaccine using Swiss model server with sequence identity and GMQE scores which analyzes based on the properties an alignment of the target template and the structure, the higher value for better GMQE observed in the current study reported model1= 0.64, the seq. identity=86.75% which considered ideal at a rate greater than 30% and Ramachandran plot of favored region= 77.57 % (36-37). Galaxy Refine server uses for To improve a structure GDT-HA score= 0.9516 and Rama plot =91.2 indicate that the 3 model maintains a more similar structure to the initial model (38-39). Evaluation of the model according to the Ramachandran plot of the Procheck server indicated the percentage of residues83%, a number of the model's residues =181 in the most favorable regions (a good quality model expected more than 90% of the most favorable regions). In the current model, a value of less than 90% appeared, but it is relatively acceptable (40-42).

It is not necessary to pay attention to the three-dimensional design of the assembled antigens in stimulating T-cell immunity (30-31,43-45). The current study of the TLR group registered against Eimeria spp. infection in poultry showed that TLR15 was the most strongly bound to the ligand (vaccine) Consistent with a TLRs expression study (30). In MOE program, the docking comparison between TLR4 and TLR15 are (-17.5313, -7.59155) and (-15.3464, -8.037) respectively that expected values are considered appropriate for establishing correlation. The vaccine can be subjected to evaluation and simulation of the immune behavior by the C-immsim server, and immunologically it showed results similar to previous studies (33,41). The significant increase in Th1 and Tc cells, which play a fundamental role especially in inflammation and the secretion of interferon gamma (IFN-γ) and interleukin 2,10,12 and their importance in stimulating the response and developing protection against Eimeria (24,46). The suggested vaccine shows high antigenic ability during the first days, then decreases to its lowest levels, so that the humoral response of IgG + IgM immunoglobulin complexes begins and continues for a period exceeding 35 days. Laboratory experiments are needed to achieve the necessary confidence in the results of the current study (47-48).

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Conflict of interest
all authors declare no conflict of interests

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