Vaccinomic Design of an Epitope-Based Fusion Protein Against *Leishmania infantum* Through Whole-Proteome Screening

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Abstract

Introduction: Visceral leishmaniasis is a common disease between animals and humans which is primarily induced by *Leishmania infantum*.

Objective: This research was conducted to engineer a recombinant vaccine against this disease.

Material & methods: To achieve this, the whole proteome of *Leishmania infantum* was analyzed to identify the most antigenic proteins; the screened proteins were then used for predicting different type of epitopes. For the creation of a recombinant vaccine, the predicted epitopes were linked to the RpfE protein using appropriate linkers. To assess the potency of the designed vaccine, various evaluations including physicochemical parameters, various structures and refinement of three-dimensional structure were performed using reliable and trustable tools. Additionally, the protein-protein docking of our recombinant vaccine and the its receptor was investigated using HDOCK server and the docking result was evaluated by molecular dynamics. Finally, to assess expression of the recombinant vaccine in *Escherichia coli* BL21, its nucleotide sequence was optimized and cloned into pET21a (+).

Results: Our results confirmed that the recombinant vaccine was a robust protein with molecular mass of 49.39 kDa which contain 83.51 % random coil. Furthermore, our findings confirmed that the recombinant vaccine could successfully and stably interact with the TLR4 receptor via the RpfE domain with a docking score of 308 and through five hydrogen bonds. Moreover, it was confirmed that the nucleotide sequence of the recombinant vaccine was optimized with CAI =1 and the optimized sequence with 1401 nucleotides in length was successfully cloned into cleavable site of pET21a (+) vector.

Conclusion: Finally, the current study's findings confirmed that the recombinant vaccine could be a suggestable vaccine to prevent *Leishmania infantum*.

Keywords: Bioinformatics, Epitope-based vaccine, Leishmaniasis, Vaccinomic, Whole proteome

1. Introduction

Leishmaniasis is categorized as a parasitic and zoonotic disease caused by intercellular protozoans belonging to different genera of Leishmania. Due to the importance of leishmaniasis, this disease is classified as group A among newly emerged infections by the Tropical Disease Research (TDR) of the World Health Organization (WHO)[1]. These diseases have been reported in 98 countries across various continents, particularly in Europe, Asia, Africa, and America, with more than 90% of cases occurring in poor and developing regions of the world[2]. Leishmaniasis is easily transmitted to its hosts (e.g., humans and animals) through the bite of infected sandflies. When these flies bite the hosts, the Leishmania parasites can enter the bloodstream, infect immune system cells, and trigger various clinical symptoms[2]. It should be noted that dogs are known to be the primary reservoir for leishmaniasis

In general, two morphological forms of Leishmania have been observed: the promastigote form, which develops in the stomach of sandflies as a vector of infection, and the amastigote form, which is found in macrophage cells of the host after infection[3]. Three types of leishmaniasis can infect humans: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL) or Kala-azar, and mucocutaneous leishmaniasis (MCL). It has been reported that *Leishmania major* and *Leishmania tropica* cause CL disease in the Mideast and Central Asia, while *Leishmania infantum*, *Leishmania chagasi*, and *Leishmania donovani* are known as the main causative agents of VL disease. In Iran (the region of the current study), VL disease is primarily caused by *Leishmania infantum* [4].

Among all types of leishmaniasis, VL, as a tropical disease, not only ranks second in mortality rate but also seventh in loss of disability-adjusted life years; therefore, this disease is considered the most dangerous form of leishmaniasis that threatens human health[5]. A person infected by *Leishmania infantum* may exhibit various symptoms such as irregular fever, weight loss, splenomegaly, hepatomegaly, anemia, and hypergammaglobulinemia. It has been reported that 95% of untreated VL cases can lead to death[6, 7].

To treat VL disease, various antiparasitic drugs (e.g., Glucantime and Amphotericin B) are employed. Glucantime is usually used as the first-line drug for treating VL, while Amphotericin B is widely applied as a second-line treatment when cases show resistance to Glucantime. Recent studies have revealed that approximately 50% of VL cases can be cured by Glucantime, whereas Amphotericin B results in a 100% cure rate for VL cases[8]. Despite the success of Amphotericin B in treating VL cases, several challenges including drug resistance, side effects, treatment availability, and early

detection are significant limitations of this method. Given the limitations of conventional methods which are being applied, it seems that applying preventive methods before infection is a logical strategy. Therefore, vaccination as a key preventive strategy may be more effective than traditional therapeutic methods. Currently, multi-epitope-based vaccines that utilize epitopes from antigenic proteins of specific microorganisms are recommended as a new-generation method for controlling infectious diseases[9, 10]. Safety, affordability, effectiveness, and design flexibility are considered the main advantages of these vaccines[10]. Hence, this research was performed to introduce a potent recombinant vaccine (RV) using whole proteome screening.

2. Material and Methods

2.1. Whole Proteome Extraction and Antigen Screening

For this study, the whole proteome of *Leishmania infantum* (8150 proteins), was extracted from the NCBI database (https://www.ncbi.nlm.nih.gov/). To identify antigenic proteins, the proteins were screened for antigenicity and allergenicity via VaxiJen (https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) (cutoff of 1.0), and AllerTOP tools (https://www.ddg-pharmfac.net/allertop/), respectively. Additionally, proteins shorter than 50 amino acids and those with high sequence similarity were excluded from further analysis.

2.2. Epitope Screening

To identify, various epitopes from the screened proteins, we utilized reliable prediction tools available on IEBD server (https://www.iedb.org/). It must be mentioned, to predict MHCI and MHCII epitopes, we used HLA-A*01:01 and DRB1*01:01 alleles, respectively. To enhance, the efficiency of epitope prediction, each predicted epitope was evaluated for its antigenicity and toxicity using VaxiJen, ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/protein.php), respectively.

2.3. Recombinant Vaccine Assembling

An adjuvant along with the final predicted epitopes (B and T cells types) were used for assembling RV. The epitopes were linked using a rigid linker to create distinct domains with specific function, while these domains were connected using an appropriate flexible linker.

2.4. Evaluation of Primary and Secondary Structures

The physical and chemical parameters of the primary structure of our RV was assessed by the ProtParam server (https://web.expasy.org/protparam/). Furthermore, the antigenicity and allergenicity of the RV were analyzed using VaxiJen and AllerTOP servers, respectively. The secondary structure of the RV was investigated through the SOPMA server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html). Notably, molecular weight, aliphatic index, GRAVY, and stability index were considered as physical and chemical parameters while extended strand, alpha helix and random coil were analyzed as secondary structure features.

2.5. 3D Structure Evaluation

The 3D structure of our RV was modeled by Robetta server(https://robetta.bakerlab.org/). Following the modeling process, the raw model was refined via GalaxyRefine server (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE). The final refined model was selected based on Ramachandran plot generated by VADAR server(http://vadar.wishartlab.com/). Finally, the final refined model was confirmed by Verify3D tool(https://www.doe-mbi.ucla.edu/verify3d/).

2.6. Protein-Protein Docking

To assess the protein-protein docking between the molecular adjuvant of the RV and the TLR4 receptor, the HDOCK server (http://hdock.phys.hust.edu.cn/) was employed. The potential hydrogen bonds formed in the docking process was specified using LigPlot+ software version 2.2.

2.7. Molecular Dynamics (MD)

To assess the durability of the protein-protein complex, MD process was conducted using GROMACS software. A cubic box of with a minimum distance of 1.0 nm between the protein and edge of box was utilized to solve the protein-protein complex. After system optimization, simulations were run for a total 100 ns. Various graphs, including root mean square fluctuation (RMSF), radius of gyration (Rg) and root mean square deviation (RMSD) were generated.

2.8. Cloning into pET21a (+) vector

To clone the nucleotide sequence of the RV, which was 1401 nucleotides in length, the nucleotide sequence was first optimized for production in *Escherichia coli* BL21using JCat server(www.jcat.de/Start.jsp). Then, *BamHI* restriction site was added in 5' while

restriction site of *XhoI* was embedded at 3'of the sequence. Finally, the fragment containing enzymes and sequence was inserted into pET21a (+) vector.

3. Result

3.1. Whole Proteome Extraction and Antigen Screening

The result of whole proteome screening demonstrated that among of 8150 proteins of Leishmania infantum, only six proteins XP_001469754.1, XP_001462717.1, XP_001465795.2, XP_001469872.1, XP_003392823.1.1 and XP_001467863.1 were classified as antigenic proteins suitable for vaccine design (Table 1).

Table 1: The most antigenic proteins of the *Leishmania infantum*

Protein accession number	Length (amino acid)	Antigenicity score*	Allergenicity
XP_001465795.2	247	1.39	without allergenicity
XP_001469754.1	271	1.18	without allergenicity
XP_001467863.1	244	1.16	without allergenicity
XP_001462717.1	169	1.02	without allergenicity
XP_003392823.1	114	1.02	without allergenicity
XP_001469872.1	156	1.01	without allergenicity

*The table has been arranged based on antigenicity score.

3.2. Epitope screening

In the current study, we predicted the final epitopes (B and T cells) from the most screened proteins. As shown in **Table 2**, our results revealed that all predicted epitopes were non-toxic and antigenic with antigenicity scores ranging from 2.11 and 0.61.

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Table 2: the Final epitopes of the screened proteins

Antigen	Epitope	Sequence	Antigenicity score	Toxicity
XP_001469754.1	B cell	GRSGAQGGYGGDRT	2.03	without toxicity
	MHCI	YSGAGDRTCY	0.61	without toxicity
	MHCII	RTCYKCGEAGHISRD	1.19	without toxicity
XP_001462717.1	B cell	AAAAAATATGQAGAGGSASH	1.69	without toxicity
	MHCI	ATDGRVTMT	1.39	without toxicity
	MHCII	TRLMLRLRPYQSRKT	1.28	without toxicity
XP_001465795.2	B cell	GDGPKEDGRTQKNDGDGPKE	1.53	without toxicity
	MHCI	QNDGNAQEK	1.67	without toxicity
	MHCII	DENLQQNDGNAQEKN	0.93	without toxicity
XP_001469872.1	B cell	DVDSKPDKEIEVGA	1.28	without toxicity
	MHCI	HLTYYPDRY	1.22	without toxicity
	MHCII	SWLLVLVTTHPLTDG	1.04	without toxicity
XP_003392823.1.1	B cell	SIYLSVYPQKQKNETQQQRN	1.32	without toxicity
	MHCI	YTSIYIDIIY	1.29	without toxicity

	MHCII	SSIYLSVYPQKQKNE	1.16	without toxicity
	B cell	APGGKHGRGGGAGR	2.11	without toxicity
XP_001467863.1	MHCI	DSHMTALMESY	1.05	without toxicity
	MHCII	AARKFAVQEDSHMTA	0.81	without toxicity

3.3. Recombinant Vaccine Assembling

Our RV contained RpfE protein as an adjuvant, a B cell domain, a MHCI domain and a MCHII domain, respectively. The domains were linked to RpfE domain using EAAAK linkers (**Figure 1**). It must be mentioned that KP linkers were considered to create each epitope-based domain.

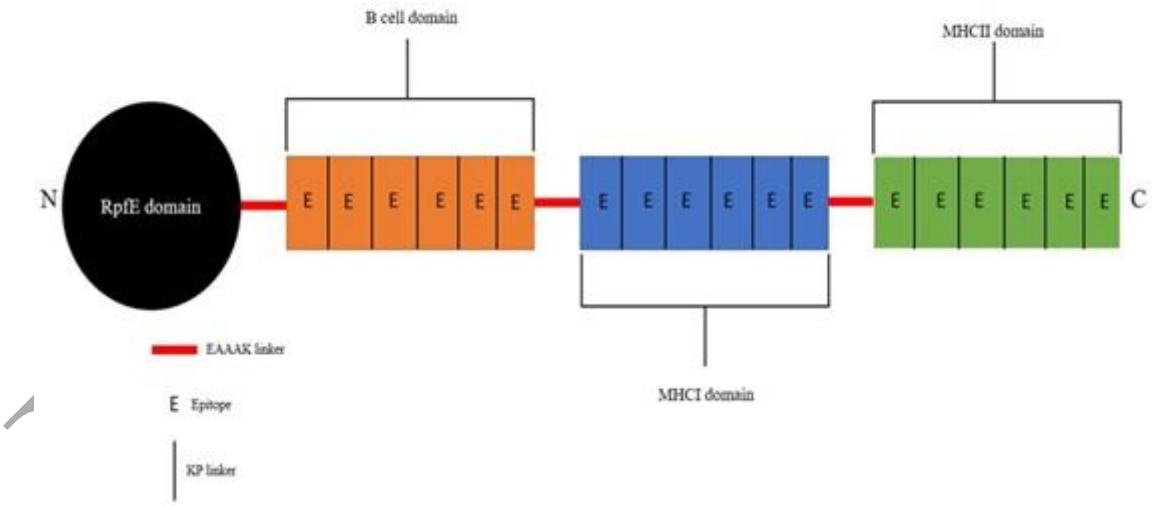


Figure1: The RV contained a RpfE domain and 18 different epitopes which have been embedded in various domains.

3.4. Evaluation of Primary and Secondary structure

The primary structure analysis showed that our RV contained 467 amino acid residues, as well as its weight was 49.39 kDa. Other parameters of our protein including; instability index, theoretical pI, and grand average of hydropathicity (GRAVY) were found to be 37.58, 8.89, and -0.831, respectively. Besides, estimate half-life of the RV exceeded 10 hours in *Escherichia coli* and more than 20 hours in yeast. Our results indicated that antigenicity score of our RV was 0.93 that is able to begin immunogenic reactions. Furthermore, the secondary structure analysis revealed that the RV contained 8.57% alpha helix, 7.92% extended strand and 83.51 % random coil (**Figure2**).

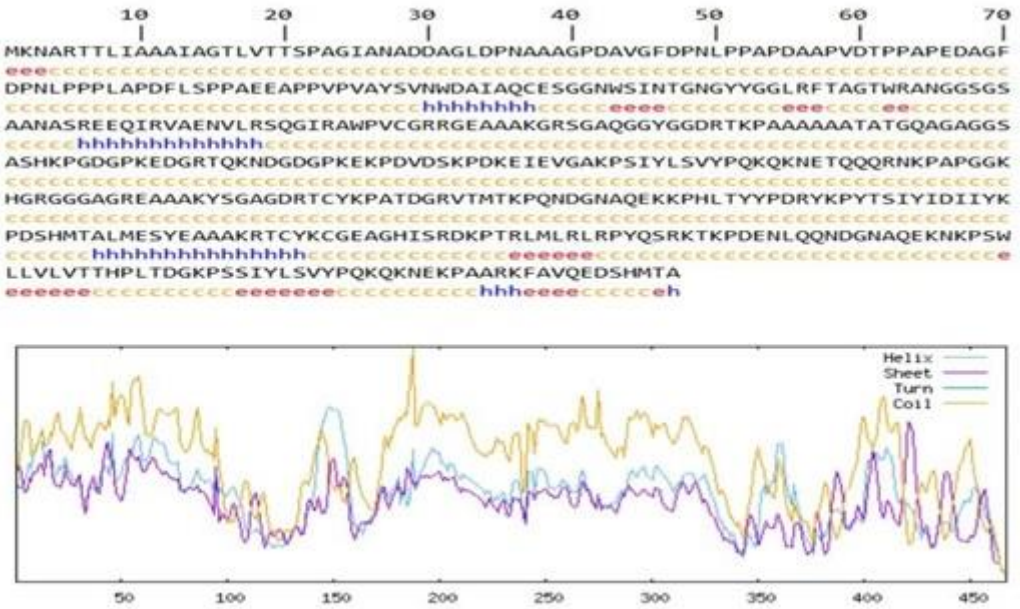


Figure 2: RV secondary structure, the majority of residues (390 amino acids) belonged to random coil states.

3.5. 3D Evaluation

The 3D structure of the RV was created by Robetta server and visualized with PyMol software (**Figure 3**). The evaluation of the raw model showed that core region contained 83% amino acid residues (**Figure 4A**), after refinement, the model exhibited an improvement, so that 89% of amino acid residues were located in the core region (**Figure 4B**). The overall quality factor of the refined model was determined to be 79.11 (Figure

4C), and majority of the amino acid residues (88.01%) had averaged 3D-2D >=0.1(**Figure 4D**).

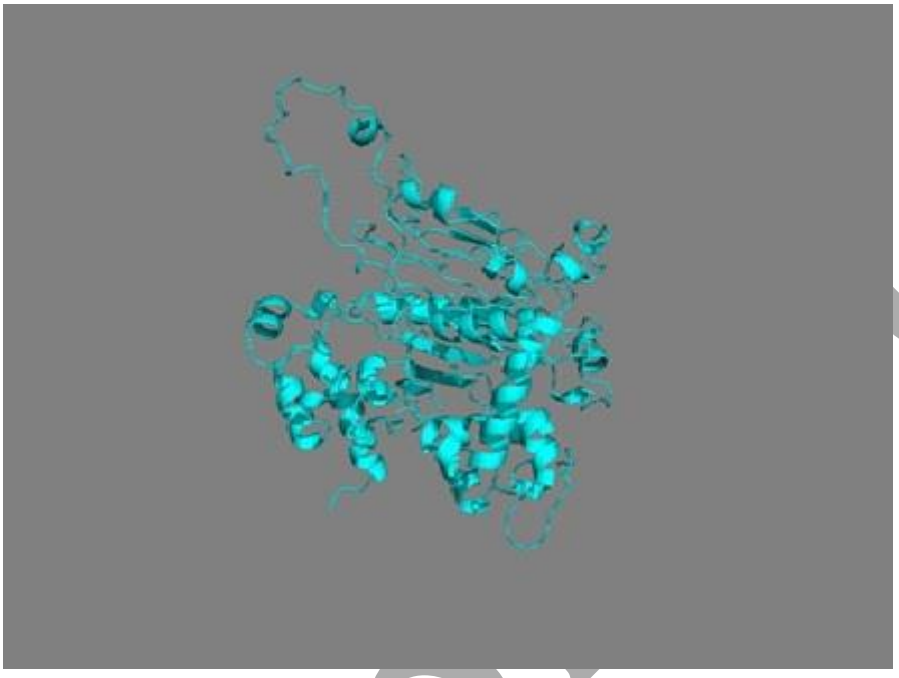


Figure 3: 3D structure of the RV modeled by Robetta server and visualized by PyMol software.

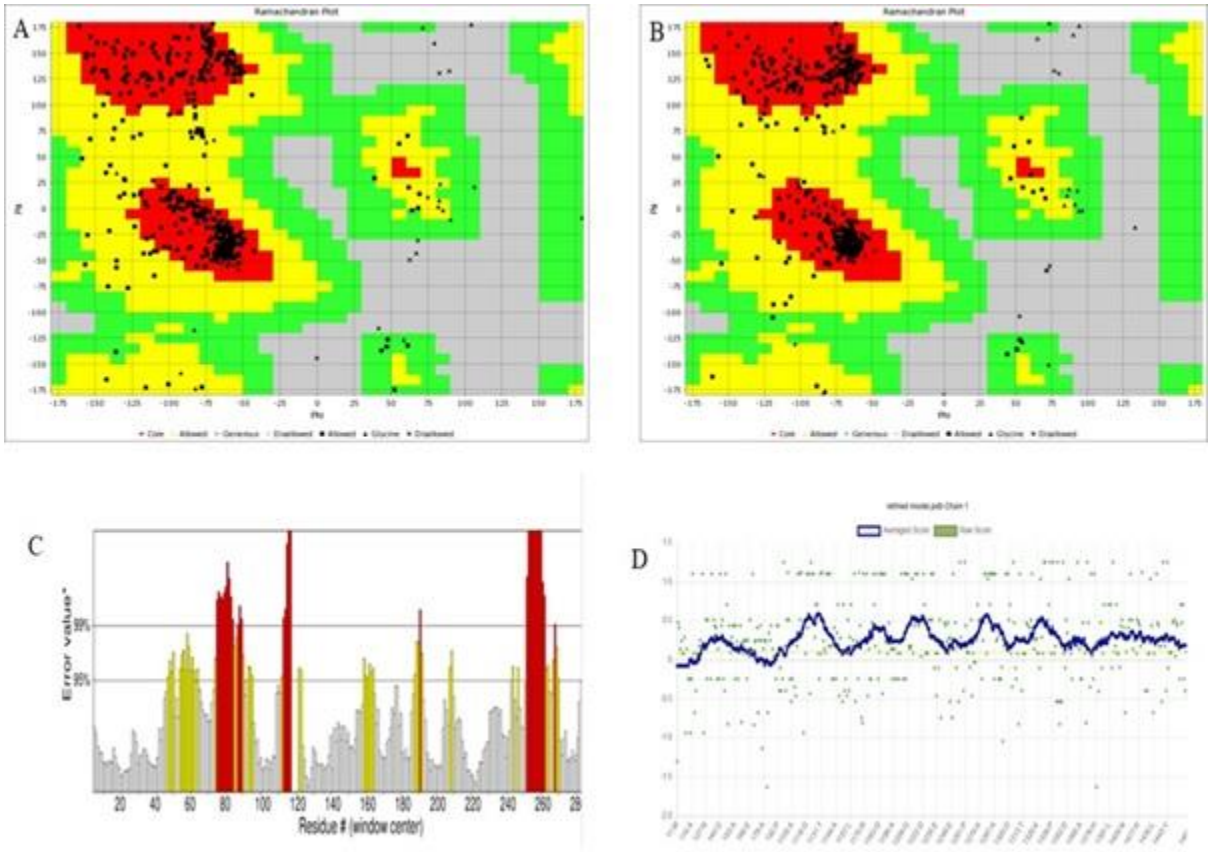
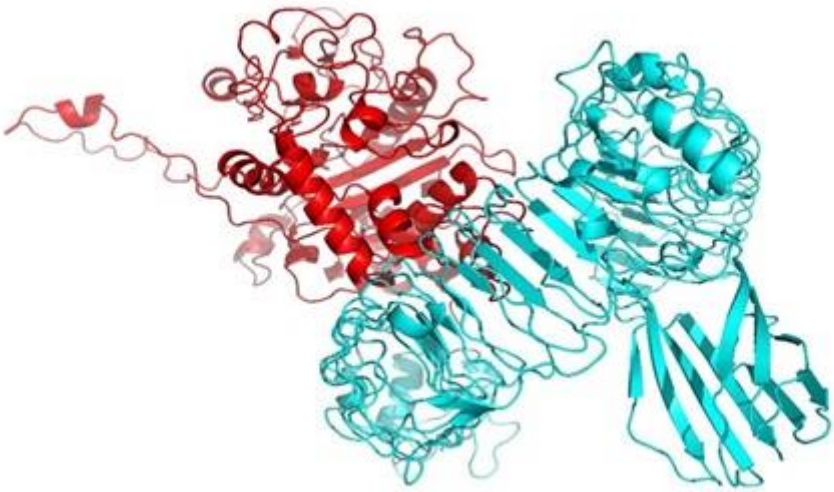


Figure 4: 3D structure confirmation. 1-Ramachandran analysis: before structure refinement (**A**), and after structure refinement (**B**). 2- Validation analysis of the refined structure: ERRAT graph (**C**) and Verifyed3D graph (**D**).

3.6. Protein-Protein Docking

For molecular docking analysis, the HDOCK server was employed. The results indicated that our designed vaccine, acting as a ligand was successfully docked to the TLR4 receptor with a docking score of -308.14 and a confidence score of 0.9594 (**Figure 5**). Moreover, LigPlot+ software analysis showed that formation of five hydrogen bonds in protein-protein interaction (**Figure 6**).



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213 **Figure 5**: Interaction between our RV (red moiety) and TLR4 receptor (blue moiety).

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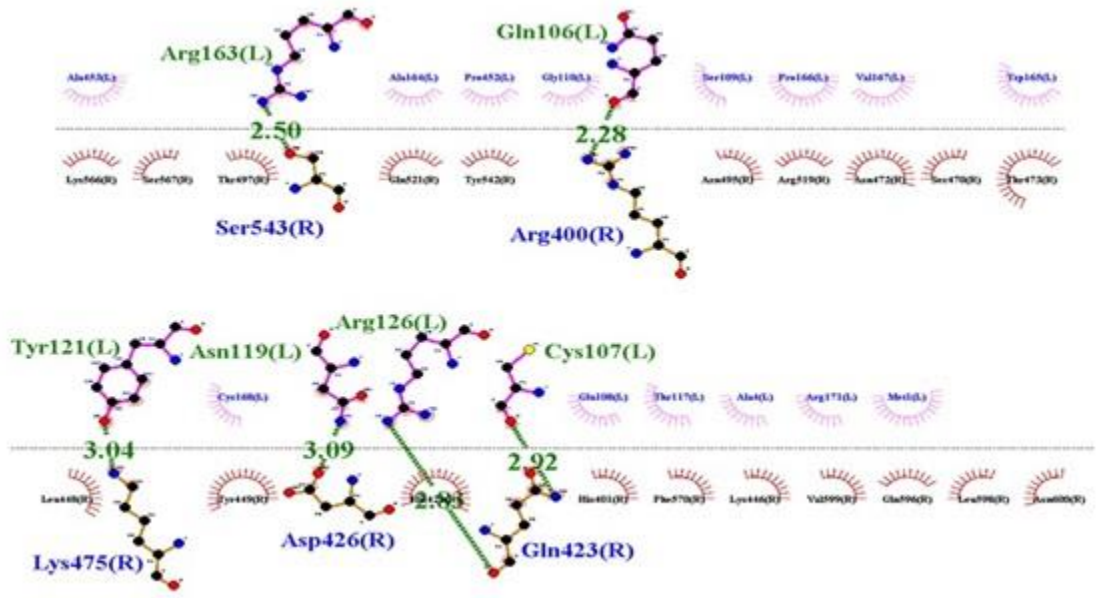

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216 **Figure 6**: Hydrogen bond formation, between the RV and TLR4, the green bars represent
217 hydrogen bonds.

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3.7. MD

This process was conducted to investigate the stability of the protein-protein complex over time. The results confirmed that the complex maintained its interactions as observed during the docking studies. The RMSD analysis indicated that the protein complex preserved overall structural stability during the 100 ns simulation. The observed fluctuations indicate a dynamic yet stable binding interaction, supporting the robustness of the complex (**Figure 7a**). our results provide valuable insights into the conformational behavior of the protein complex and its interaction with TLR4, contributing to the understanding of its functional dynamics. Besides, the RMSD analysis confirmed that the TLR4 complex kept overall structural stability all over the simulation. The observed fluctuations indicate a dynamic yet stable binding interaction, supporting the robustness of the complex (**Figure 7b**). The RMSF analysis highlighted the stable nature of the TLR4 complex, with localized flexibility in functionally relevant regions. These fluctuations suggest adaptive movements that may facilitate interactions with its binding partner, supporting its biological function (**Figure 7c**). The RMSF analysis showed that the vaccine-receptor complex was structurally stable during the 100 ns simulation, with flexibility concentrated in loop and solvent-exposed regions. The interaction site with TLR4 exhibits minimal fluctuations, suggesting strong and stable binding **(Figure 7d**). The Rg analysis demonstrated that the protein complex with TLR4 maintained a stable and compact structure over the 100 ns MD simulation. The minor fluctuations indicate localized flexibility while preserving the global structural integrity of the complex. These findings provide valuable insights into the conformational stability of the TLR4-protein interaction, supporting further studies on its functional and therapeutic implications (**Figure 7e**). Moreover, the Rg analysis revealed that the TLR4 complex kept a stable and compact structure over the 100 ns MD simulation. The low fluctuations suggest that the interaction with its binding partner does not induce significant conformational changes, reinforcing the stability of the complex (**Figure 7f**).

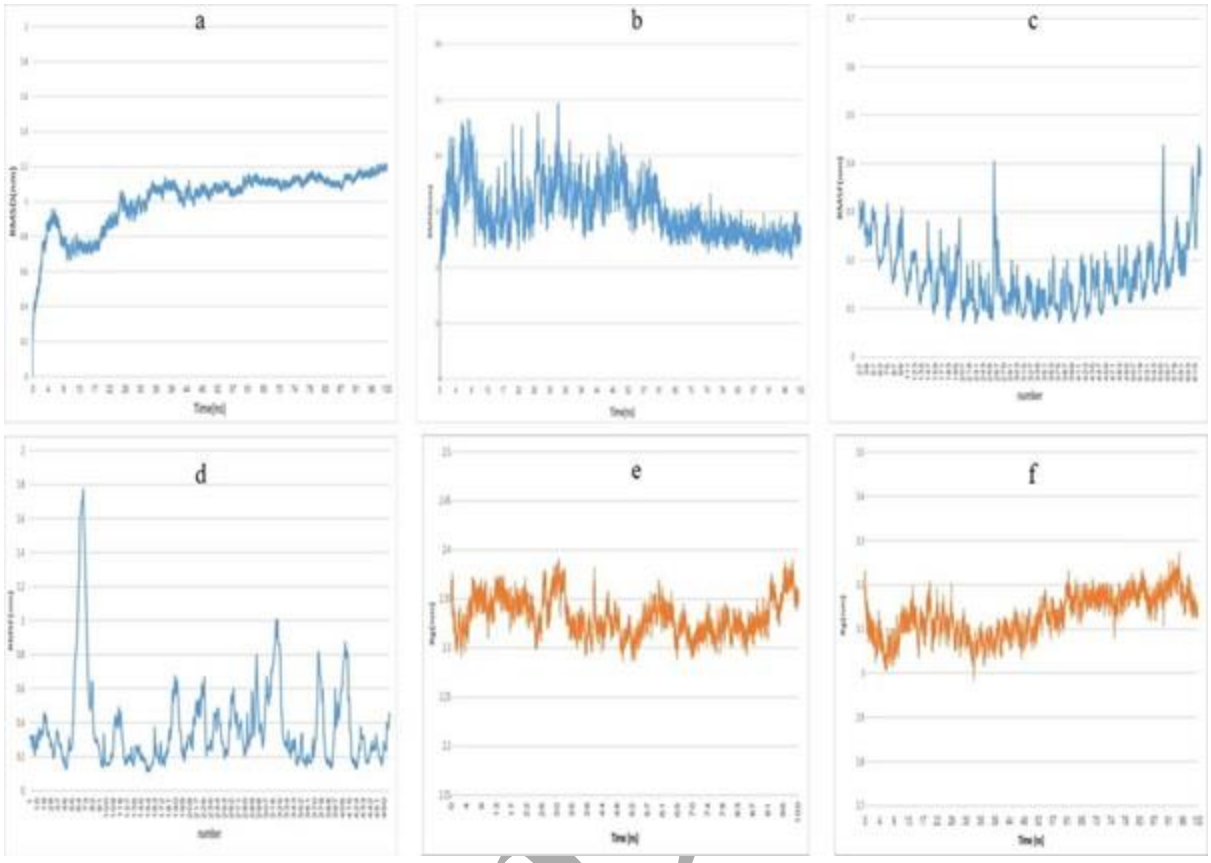

Figure7: Different graphs of MD simulation. **(a)**: The RMSD graph of the protein complex with its binding partner TLR4 during 100 ns MD simulation. **(b):** The RMSD graph of the TLR4 complex with its binding partner protein throughout MD simulation. **(c):** The RMSF graph of the individual amino acid residues in the TLR4 complex with its binding partner protein over a 100 ns MD simulation. **(d):** The RMSF graph of the individual amino acid residues in the protein when in complex with TLR4 during 100 ns MD simulation. **(e):** The Rg graph of the protein in complex with TLR4 throughout MD simulation. **(f):** The Rg plot of the TLR4 protein over a 100 ns MD simulation.

3.8. Cloning into pET21a (+) vector

In this step, first nucleotide sequence of the RV was optimized. Our results showed that GC content percent of the sequence was changed from 54 to 55 % and also, CAI parameter was improved from 0.21 to 1. Also, the results of cloning confirmed that our fragment of interest with 1401 nucleotides in length was successfully inserted between T7 promotor and His-tag of pET21a (+) vector. Final size of our recombinant vector was 6810 base pair (**Figure 8**).

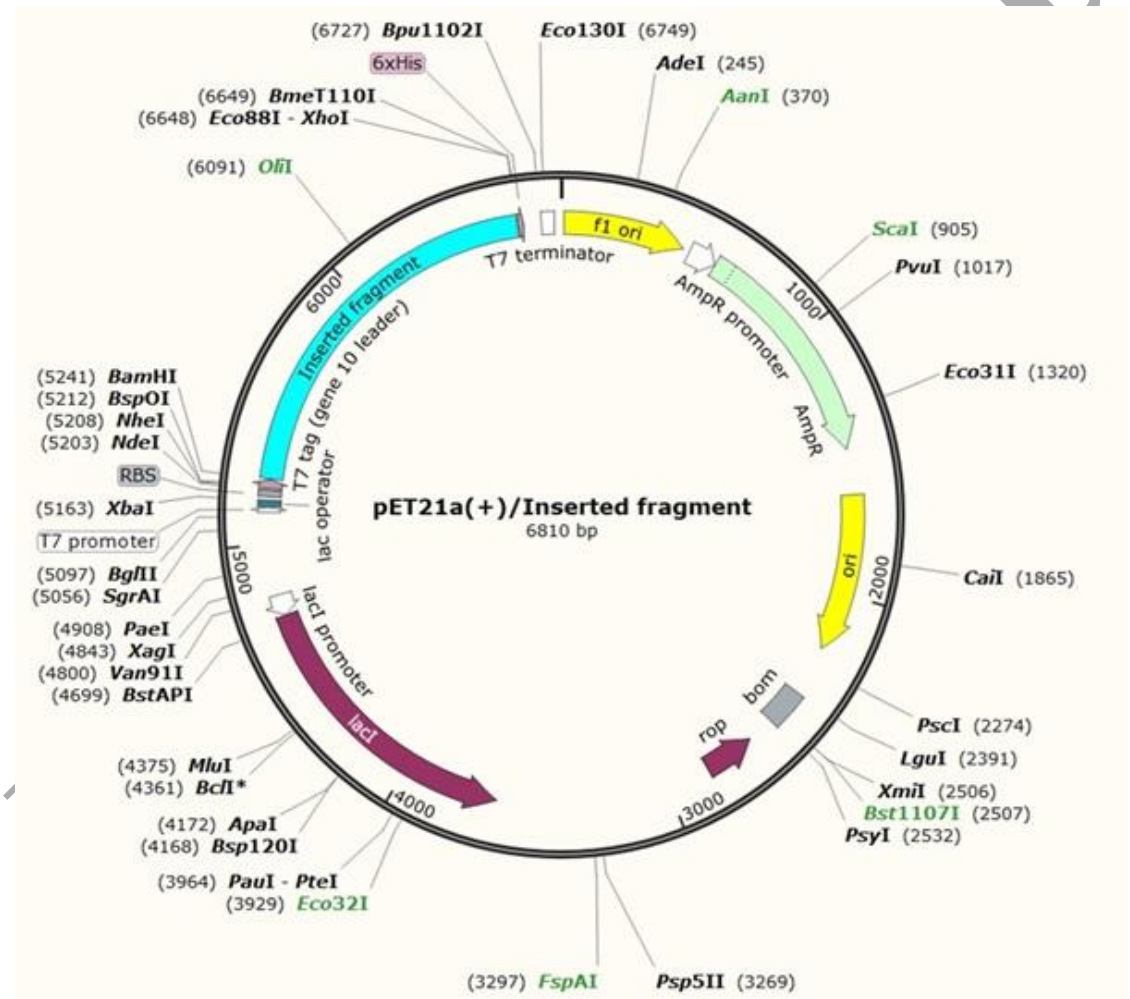

Figure 8: *In silico* cloning of the RV nucleotide sequence (blue region) into pET21a (+) vector.

4. Discussion

Currently, bioinformatics as a robust tool is widely being applied for design and development of recombinant or multi-epitope vaccines[11]. Utilizing various algorithms, bioinformatics can analyze vast biological datasets, including genomic and proteomic information, to identify antigenic proteins and predict B cell and T cell epitopes from pathogens. Consequently, it can be concluded that utilizing bioinformatics can affordably lead to rapid and precise design of efficient vaccines against emerging infectious diseases and even various cancers[12]. More recently, various studies have been done which utilized bioinformatics tools for design vaccine against viral and bacterial infections. Hao Wu et al. (2025) employed a bioinformatics approach to screen over 30,000 protein sequences for the design of a robust vaccine against the influenza B virus. Their findings indicated that the developed vaccine provided broad protection against this infection[13]. Similarly, Kashiri et al. (2025) conducted a bioinformatics study for design a vaccine against Mycobacterium avium complex species. They successfully predicted the epitopes based on conserved immunodominant proteins and presented a wide-spectrum multiple-epitope vaccine[14]. Forouharmehr (2024) applied bioinformatics approach to screen whole proteome of *Coxiella burnetii* for the identification of the most immunogenic proteins. The findings facilitated the design of an epitope-based vaccine that elicited immune responses against this bacterium[9]. In recent years, several studies have focused on vaccine designing against *Leishmania infantum*. For instance, Shams et al. (2022) designed a multi-epitope vaccine against *Leishmania infantum* using the best epitopes from four proteins including histone H1, KMP11, LACK and LeIF. They suggested that their vaccine could be a promising candidate to used[15]. Similarly, Vakili et al. (2020) designed an epitope-based vaccine based on four antigenic proteins (histone H1, sterol SMT, LiHy, and LSAP) to prevent infection by *Leishmania infantum*. The immune responses results of this project revealed that their designed vaccine could trigger a robust Th1-type immune response against the parasite[16].

In general, the current project focuses on designing a highly efficient epitope-based fusion protein against *Leishmania infantum*. Initially we screened whole proteome of *Leishmania infantum* to identify and characterize antigenic proteins. Antigenic proteins as foreign components play key role in vaccine development. These proteins can strongly trigger antibodies production and activation of various immune cell via stimulation of the immune system[17]. In our project, we aimed to identify robust antigenic proteins by setting a cut-off for antigenicity evaluation at ≥1. As detailed in Table 1, the antigenicity scores of our selected antigenic proteins ranged from 1.01 to1.39. In addition to assessing antigenicity, we also considered allergenicity of these proteins. Allergenicity refers to the abnormal reaction of the immune system for foreign substances, such as proteins[18]. Evaluating

whether a protein can trigger allergenic reaction is critical step for its therapeutic applications. As shown in Table1, all screened proteins were classified as non-allergen. Following the identification of antigenic proteins, we proceeded with epitope prediction. An epitope is specific region of an antigenic protein that serves as a communication bridge between antigenic proteins and immune system, activating both B cells and T cells. As listed in Table 2, we predicted 18 epitopes, including those B cell, MHCI and MHCII, based on antigenicity and toxicity profiles. These epitopes along with the RpfE protein and proper linker, were utilized for assembling the vaccine construct. It has been demonstrated that RpfE as a molecular adjuvant leads to activation of dendritic cells, this activation not only facilitates CD4 differentiation, but also enhances IL-6, and TNF-α expressions[19]. The linkers were incorporated for maintain the folding and functionality of the design vaccine. As illustrated in Figure 1, we employed the EAAAK peptide as a rigid to ensure appropriate distance among various domains and to optimize vaccine bioactivity [20]. Additionally, a KP linker was applied to separate the epitopes within each, domain of the designed vaccine. We assessed various functional and structural characteristics of the vaccine, which yielded promising results. The analysis indicated that our designed vaccine with an antigenicity score of 0.93, has the potential to provide protection against Leishmiansis without eliciting allergenic reactions. Our findings also confirmed that the designed vaccine is stable, exhibiting an instability index of less than 40 (37.58), and is hydrophilic with a negative GRAVY of 0.831[21]. Furthermore, the secondary structure analysis revealed that the random coil conformation constituted 83.51 % of the overall structure. It has been reported that there is a direct correlation between percentage of random coil and antibody binding ability of an epitope-based vaccine [22]. Consequently, our designed vaccine with 83.53 % random coil content, is likely to efficiently trigger immune responses. Th accuracy of the vaccine's tertiary structure was further validated using Verify3D tool, which revealed that 88% of the residues achieved averaged 3D-1D score of ≥ 0.1. it must be mention that a correct model should have at least 80% of its residues meeting this criterion. In the current study, to confirm the interaction between RpfE as molecular adjuvant of the designed vaccine and TLR4 receptor, molecular docking process was conducted. Our results demonstrated that RpfE adjuvant could be docked to its receptor via five hydrogen bonds with a confidence score of 0.9594. Moreover, stability of vaccine-receptor complex was confirmed during 100ns MD simulation. In the subsequent phase, we evaluated the capability of the designed vaccine for expression in prokaryotic system. Codon optimization results showed that the optimized sequence with a GC content of 55 % and CAI of 1, can be efficiently expressed in a prokaryotic system (e.g., *E coli*). The results of cloning indicated that the optimized sequence of the RV was successfully inserted into pTE21a (+), which serves as prokaryotic expression vector. Finally, it can be concluded that in this research we successfully assemble a promising RV through whole proteome

screening and a vaccinomic approach. Although the findings of this study are strongly confirming our vaccine, it is crucial to consider the vast complexity of *in vivo* environment compared to *in silico* condition. Physiological factors may affect affinity, protein stability and even immunogenicity. Consequently, the current vaccine requires to be experimentally validated in subsequent phases.

Conclusion

Conventional methods used to treat VL disease have significant limitations, making it essential to explore alternative approaches. Consequently, the development of new strategies, such as epitope-based vaccine is strongly recommended for prevention of this disease. Our study successfully designed a promising epitope-based vaccine through whole proteome screening and a vaccinomic approach. Although our findings are encouraging, the current vaccine requires to be experimentally validated in subsequent phases.

Authors' contribution

The manuscript was completely performed by A.F.

Ethics

Not applicable

Conflict of interest

The author declares there is no conflict of interest. The Z ai online tool version 4.5 was applied to edit only grammatical problems of the text.

Acknowledgement

The author would like to thank research deputy stuffs of Lorestan University due to their supports.

Funding

Not applicable

Data Availability:

The data that support the finding of this study are available on request from the corresponding author.

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