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Original Article

Designing a Multiple-Epitope Vaccine Candidate against *Leishmania major* and *Leishmania infantum* for Monocyte-Derived Exosome Preparation

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Abstract

Background: *Leishmania* is a vector-borne protozoon, which causes visceral, cutaneous and mucocutaneous leishmaniosis in human and animals. Monocyte-derived exosome vaccines can be used as prophylaxis and immunotherapy strategies. The aim of this study was to design a multiple-epitope candidate vaccine using leishmaniolysin (*GP63*) and *rK39* proteins against *Leishmania major* and *L. infantum* for monocyte-derived exosome preparation.

Methods: This study was carried out in Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran, 2023–2024. Effective immunodominant epitopes were selected from two antigenic proteins of *GP63* and *rK39* using various immunoinformatics and bioinformatics approaches. *Vibrio cholerae* β-subunit was used as an adjuvant to stimulate immune responses. Then, appropriate linkers were selected for the fusion of epitopes. The 3D model of candidate vaccine was predicted and validated.

Results: This designed candidate vaccine could effectively be used as a prophylaxis strategy against leishmaniosis.

Conclusion: A candidate vaccine was designed using bioinformatic and immunoinformatic studies with virtual acceptable quality; however, effectiveness of this vaccine should be verified through further *in-vitro* and *in-vivo* studies.

Introduction

Leishmaniosis is a vector-borne parasitic infection in humans as well as animals such as dogs and rodents. *Leishmania* species cause visceral (Vl), cutaneous (CL) and mucocutaneous (ML) diseases. Naturally, *GP63* is a metalloprotease, playing critical roles in parasite migration and escape from the host immune system. Moreover, this metalloprotease plays important roles in enhancing parasite survival via modulating macrophage-killing mechanisms (1). The rK39 is used as a biomarker for visceral leishmaniosis diagnostic assays. Technically, *rK39* is a gene, encoding for a kinesin-linked protein of *L. infantum* with multiple 39-amino acid (AA) repeats. The multiple repeats can induce antibodies with strong affinity against the antigens. High concentrations of antibodies against *rK39* can be detected in patients' sera with VL but not in patients with ML or CL (2). Exosomes are small extracellular vehicles (EVs), secreted by various cells. Naturally, EVs include important cell-derived bioactive molecules such as proteins and RNAs, which regulate immune responses (3). A previous study has shown release of exosomes from cell infected with intracellular pathogens such as *Toxoplasma gondii* and *Mycobacterium tuberculosis*. Furthermore, vaccination of mice with exosomes containing pathogen antigens has resulted in activation of $CD4^+$ and $CD8^+$ T-cells (4). *Leishmania* spp. included important virulence factors such as *GP63* (5).

Studies have used dendritic cell-derived exosomes as vaccines to induce specific immune responses (6, 7). Various pathogen-associated molecules such as peptides, recombinant proteins, whole cells and lysates can be used as sources of antigens. Loading of antigen presenting cells (APCS) with crude antigens leads to stronger stimulation of antigen-specific T-cell responses, compared with peptide-loaded exosomes (8). However, advantage of subunit vaccines include that the host immune responses can be focused on recognition of a few special target antigens, but subunit vaccines do not always induce strong immune responses, compared to that the whole antigen vaccines do (9). In general, EVs derived from stimulated monocytes with multiple-epitope proteins can induce CD_8 and CD_4 responses because of MHC-I and MHC-II molecules as well as high levels of costimulatory molecules (6).

In the current study, a multiple-epitope vaccine was designed using *GP63* and *rk39* virulence factors against leishmaniosis for the preparation of recombinant proteins.

Materials and Methods

Retrieving sequences of rk39 and Gp63 from L. infantum and L. major and the adjuvant

The current study was carried out in Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran, 2023–2024. Furthermore, the study was carried out based on the ethical principles for medical research from Declaration of Helsinki. Complete AA sequences of *rk39* and *GP63* were retrieved from NCBI database (https://www.uniprot.org). These antigens included *L. infantum rk39* (accession no. CAC9469802.1), *L. major rk39* (accession no. XP_001687710.1), *L. infantum Gp63* (accession no. XP_003392249.1) and *L. major Gp63* (accession no. ACL01096.2). Putative adjuvant sequences of *Vibrio cholerae* toxin β-subunits (accession no. AAV67882.1) were downloaded in FASTA format.

Prediction of MHC-I and MHC-II epitopes

Cytotoxic T-lymphocyte (CTL) epitopes play key roles in induction of MHC-I dependent cellular immune responses. In this study, epitopes were predicted using netMHCnpan-4.1 (https://services.healthtech.dtu.dk/service.php?NetMHCpan-4.1). Thresholds for strong and weak binders were reported as 0.5 and

2%, respectively. All the parameters for this server were stored as defaults. In addition, MHC-I epitopes were analyzed for frequent HLA subtypes in Iran, including HLA-A*02, B*35 and C*12, based on previous studies. These subtypes were shown to cover approximately 88% of the total ethnic population. For helper T-lymphocyte (HTL) epitopes that play key roles in induction of MHC-II regulated cellular immune responses, epitopes were predicted using netMHC2pan-3.2 (https://services.healthtech.dtu.dk/service.php?NetMHCIIpan-3.2). Thresholds for strong and weak binders were reported as 2 and 10%, respectively. All the parameters for this server were stored as defaults. Additionally, MHC-II epitopes were analyzed for frequent HLA subtypes in Iran, including HLA-DQA1*01, DQB1*03 and DRB1*11, based on previous studies. These subtypes were demonstrated to cover approximately 88% of the total ethnic population (10).

Prediction of B-lymphocyte (BL) epitopes

Naturally, B-cell epitopes include important roles in inducing humoral immune responses against infectious agents. In the current study, Bepipred2 (http://www.cbs.dtu.dk/services/BepiPred//) was used for linear B-cell epitope predictions. The threshold was set as 0.5. This server allowed users to predict B-cell epitopes through their physicochemical characteristics (e.g., flexibility/mobility, hydrophilicity, polarity, accessibility, exposed surface and turns) and a combination of these characteristics.

Construction of the multiple-epitope vaccine

To construct an efficient multiple-epitope vaccine, a pool of epitopes were extracted from the highlighted sequences and epitopes with binding capacity to their respective HLA alleles were selected as joined via appropriate linkers. The linker sequences were selected based on the previous studies (11). Furthermore, the PAPAPA linker was used to connect adjuvant sequences to N and C terminals of the multiple-epitope peptide. The AAYKK linker was used to connect the CTL epitopes. This linker can be cleaved by cathepsin B, producing double lysine (KK) site and AAY motif (12). The AAY motif is appropriate for binding to TAP transporter, which includes significant roles in epitope presentation to the host immune system (3). Glycine-rich linkers such as GSGSGS can improve flexibility and solubility. These were used to join the HTL epitopes. The B-cell epitopes were connected to each other using KK rigid linkers.

Cleavage site prediction

Cleavage sites of the peptide sequences from human proteasome multi-complex were predicted using NetChop 3.1 (https://services.healthtech.dtu.dk/service.php?NetChop-3.1). In this study, threshold was reported as 0.5. The server was able to produce reliable cleavage-site predictions for the mammalian proteasomes.

Physicochemical characteristic assessment and immunogenicity, allergenicity and toxicity predictions

Immunogenicity is the ability of antigens to bind to T and B-cell receptors, inducing immune responses against infections in hosts. In this study, antigenicity of the vaccine construct was predicted using VaxiJen 2.0 (http://www. ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). Allergenicity of the vaccine construct was analyzed using AllerTOP v2.0 (http://www.ddg-pharmfac.net/AllerTOP/). Suggested epitopes might be allergen, causing serious problems. Toxicity of the epitopes was investigated using ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/algo.php). Furthermore, assessment of physicochemical characteristics of the designed vaccine such as molecular weight (MW), net charge and half-life was carried out using ProtParam (http://web.expasy.org/protparam/).

Structure analysis, modeling and validation

Secondary structure of the final multiple-epitope vaccine sequence was predicted using PSIPred (http://bioinf.cs.ucl.ac.uk/psipred/). The 3D-structure prediction of the designed vaccine was assessed using I-TASSER (https://zhanggroup.org/I-TASSER/). The 3D-structure model subjected to Ramachandran plot server (https://swift.cmbi.umcn.nl/servers/html/ramaplot.html) to validate its tertiary structure.

Codon optimization and in-silico cloning

EMBOSS Backtranseq(https://www.ebi.ac.uk/Tools/services/web/toolresult.ebi?jobId=emboss_backtranseq-I20230206-103555-0550-44267292-p1m) was used for the reverse translation and JCAT (https://www.jcat.de/Start.jsp) for codon optimization and quantitative analysis. The server calculated codon adaptation index and GC content as essential parameters for cloning. These parameters play critical roles for high quantity and quality expressions of the construct in *Escherichia coli* hosts. Open reading frames (ORFs) of the highlighted sequences were investigated using ORFfinder database with default *E. coli* (https://www.ncbi.nlm.nih.gov/orffinder). Moreover, BamHI and HindIII restriction enzyme sites were added to two terminals of the multiple-epitope DNA sequence for cloning in pET-28b (+) vectors.

Results

Epitopes of CTL, HTL and BL were predicted using netMHCnpan-4.1, netMHC2pan-3.2 and Bepipred 2, respectively. Amino-acid sequences of the chosen epitopes are listed in Table 1.

Table 1: Amino acid sequences of the selected epitopes

L. m	*L. i*	*CTL epitope*	*HTL epitope*	*BL epitope*
Gp63	Gp63	KHLIPQALQLAVA KAREQYMPWGR NAGCAF	SNTDFVMYVALTM AIFQDVKHLIPQAL	QLHTERLKVRQVQDKWKVTGMGDDVCS DFKVPPAHITDGLCQGNVQAAKDGGNAA AGRRGPRAAA
rk39	rk39	SQLEATAAAKML ASQLEATAAAKM SARAAELASQL	LASQLEATAAAKM SAARTAELASRLKA TAA	RERMRVTLEERLRVAELRAAELAGVLEATA AAKTSAEKGALEQQLRESEASAAELAGVLE ATSAAKTAVEEDLEKIKG

L. m, Leishmania major; *L. I, Leishmania infantum*; CTL, cytotoxic T-lymphocyte; HTL, helper T-lymphocyte; BL, B-lymphocyte

Sequence of the multiple-epitope vaccine construct

Sequence of the multiple-epitope vaccine construct was as follows:

MIKLKFGVFFIVLLS-
SAYAHGTPQNITDLCAEDHNTQIHTLND-
KIFSYTESLAGKREMAIITFKNGAT-
FQVEVPGSQHIDSQKKAIERMKDTLRI-
AYLTEAKVEKLCVWNNKTPHAIAAIS-
MANPAPA-
PASQLEATAAAKMAAYKKRAAELASQLAA
YKKLASQLEATAAAKMSAAAYKKKHLIPQ
ALQLAAYKKMPWGRNAGCAFAAYKKA-
VAKAREQYGSGSGSARTAELASRLKA-
TAAGSGSGSLASQLEATAAAKMSAGSGSGSS
NTDFVMYVGSGSGSALT-
MAIFQDGSGSGSVKHLIPQALKKQLH-
TERLKVRQVQDKWKVTGMGDDVCSDFKV
PPAHITDGLKKCQGNVQAAKDGGNAAA-
GRRGPRAAAKKRERMRVTLEER-
LRVAELRAAELAGVLEATAAAK-
TSAEKKKGALEQQLRE-
SEASAAELAGVLEATSAAK-
TAVEEDLEKIKGPAPAPAMI-
KLKFGVFFIVLLS-
SAYAHGTPQNITDLCAEDHNTQIHTLND-
KIFSYTESLAGKREMAIITFKNGAT-
FQVEVPGSQHIDSQKKAIERMKDTLRI-
AYLTEAKVEKLCVWNNKTPHAIAAISMAN

Cleavage site

Cleavage sites were predicted using NetChop 3. Servers have predicted 162 cleavage sites for the multiple-epitope protein (data not shown). The proteasome multi-complex

consisted of enzymes that cut the proteins via the peptide bonds, altering the proteins into peptides. Peptides from proteasome cleavage connect to class MHC-I molecules, move to the cell membrane and then are presented to cytotoxic T-cells.

Physicochemical characteristics

In general, number of the AAs was 588, MW was 62752.09 Da and the theoretical isoelectric point was 9.55. The total numbers of negatively charged AAs (Glu and Asp) and positively charged AAs (Arg and Lys) residues were 55 and 80, respectively. The instability index was computed as 37.33. Protein structure included a stable form. Half-life of the protein was estimated nearly 30 h in mammalian cells, more than 20 h in yeasts and more than 10 h in *E. coli*. The aliphatic index was 80.58 and the grand mean of hydropathicity (GRAVY) was -0.244.

Prediction of immunogenicity, allergenicity and toxicity

Results of the servers showed that the multiple-epitope vaccine was not allergenic. Vaxigen server predicted possibility of the multiple-epitope vaccine antigenicity as 0.7 and scores indicated that the currently designed vaccine could produce effective immune responses. All the selected epitopes in the scaffold were predicted as non-allergenic and nontoxic epitopes.

Secondary and tertiary structure prediction

Secondary and tertiary structure prediction of the designed vaccine are shown in Figs. 1, 2.

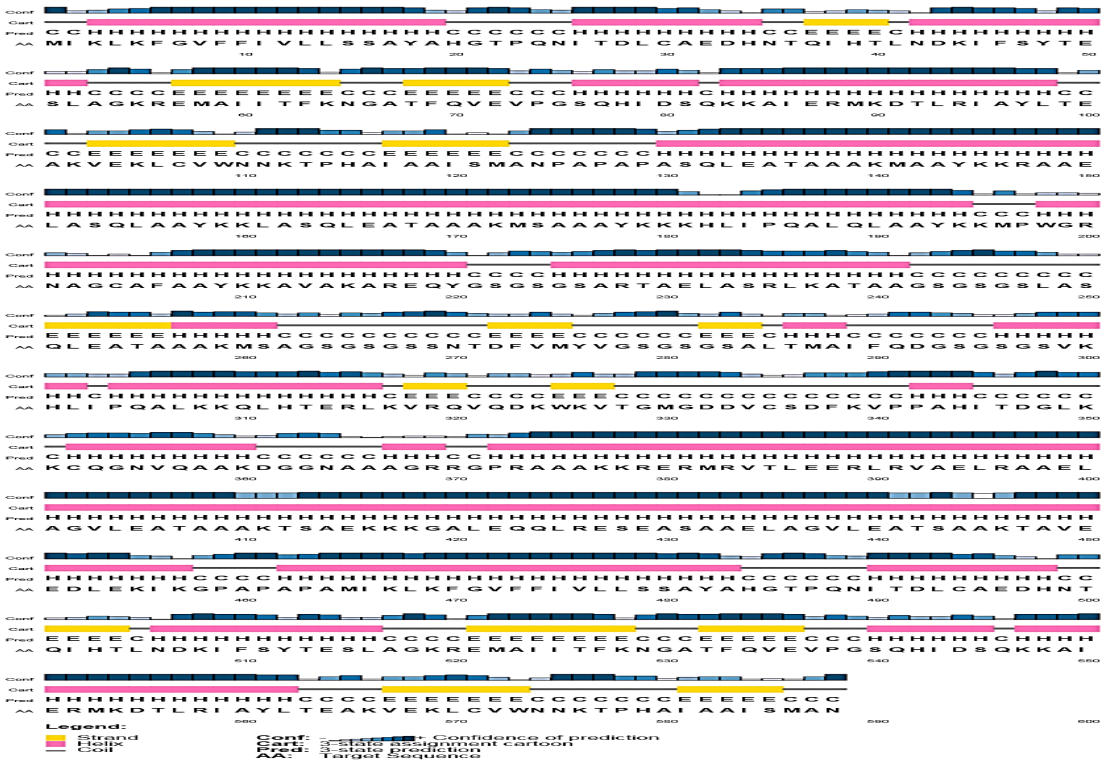

Fig. 1: Graphical output from the secondary structure prediction of the designed vaccine. Prediction was carried out using PSIPRED server. Blue bars show confidence of the prediction

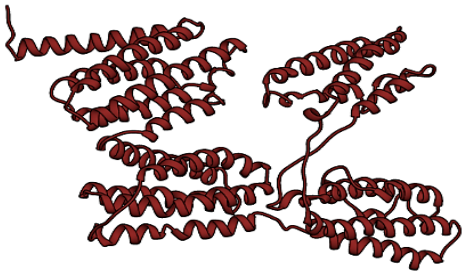

Fig. 2: Predicted tertiary structure of the associated protein using I-TASSER server

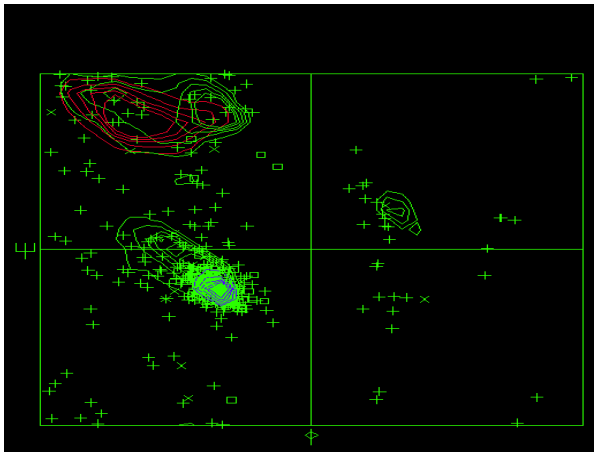

Fig. 3: Ramachandran plot analysis of the peptide sequence

Geometric quality of the primary model was analyzed using Ramachandran plot (Fig. 3), showing that a majority of the peptide bonds have rotated near axes of the helix. The 95% residues were in the most favored allowed regions. Therefore, geometry quality of the predicted vaccine was validated based on the Ramachandran plot.

Protein reverse translation and construct design for recombinant expression

Technically, it is necessary to reverse translate AA sequences into nucleotide sequences to construct cassette in plasmid vectors for the expression of the protein. A 1764-bp optimized codon sequence of the vaccine construct was achieved using JCAT server. The codon optimization index was reported as 1.0 and 50% of the GC content of the improved nucleotides, revealing its higher expression possibility in *E. coli* cells, cloned into the expression vector pET28a (+) virtually. However, further analyses and laboratory orientations such as macrophage loading and EVs purification are necessary.

Discussion

In this study, a multiple-epitope peptide vaccine was designed for *Leishmania* species via monocyte-derived EVs preparation. For this purpose, 11 class-I and class-II HLA epitopes from *GP63* and *rk39* proteins of *L. major* and *L. infantum* were selected to construct a multiple-epitope candidate vaccine. In recent years, use of dendritic cell-derived exosomes as vaccines has demonstrated promising results, compared to dendritic cell-based vaccines. Studies have shown that mice administrated with APC-derived exosomes as a

prophylactic strategy were completely protected against pathogens (13, 14). Presence of MHC classes I and II on exosomes with high numbers of costimulatory molecules and adjuvant effects of the exosome lipid composition make the exosome effective as good immunotherapy vehicles (15). Dendritic cell-derived exosomes used in antitumor vaccines are effective for boosting prophylactic immunity in animal models. However, this study have shown less effective immune responses of the hosts in clinical trials (15).

In personalized immunotherapy, isogenic exosomes derived from the patients' APC, including isogenic MHC-I and MHC-II haplotypes, are loaded with antigens (e.g., *Leishmania* lysate). This can be incorporated by *Leishmania* associated peptides, shifting leishmanial cells into immunogenic targets. Furthermore, activation of CTL clone and efficient anti-parasite cellular responses occur through release of interferon γ (IFN-γ) and promotion of specific parasite-cell lysis. This process is significantly further efficient when exosomes are derived from mature lipopolysaccharide-treated APCs (2). DC-secreted exosomes pulsed with *T. gondii*-derived antigens *in vitro* could induce strong activation of systemic and mucosal immune responses in syngeneic and allogeneic mice (14).

Practically, vaccines with broad ranges of reactivity for approximately 90% of the ethnic populations may be admissible for public use due to the highly polymorphic nature of MHC molecules. This could be achieved using 11 uniquely described HLA-restricted $CD8^+$ epitopes (16, 17,18). They suggested that extraction of four $CD8^+$ epitopes or more might provide at least 90% coverages for Asian and African ethnic groups. As a solution, a combination of class-I and class-II epitopes could be used for a wider population coverage of the vaccine. Anam Naz et al. used a combination of class-I and class prioritized epitopes for designing multiple-epitope vaccines against COVID-19 using MHC restricted alleles (A*01:01, A*03:01, A*02:01, A*24:02, A*26:01, B*07:02, B*08:01, B*15:01,B*27:05, B*40:01,B*39:01, B*58:01, HLA-DRB1*03:01, and HLA- DRB1*15:01, DRB1*07:01) and reported a population coverage score of nearly 94% (19). Doolan et al. identified HLA degenerated T-cell epitopes from *Plasmodium falciparum* validated for developing broadly efficacious epitope-based vaccines against infections. They designed a multiple-epitope vaccine that its epitopes bound to 5–11 various HLA-DR molecules, covering of nearly 90% of the ethnically diverse population (20).

In this study, 11 class-I and class-II HLA epitopes from *GP63* and *rk39* proteins of *L. major* and *L. infantum* were selected to construct a multiple-epitope vaccine candidate. The *V. cholerae* β-subunit was used as an adjuvant. Due to lack of an epitope prediction server for canine MHC, multiple sequence alignments were carried out and similar human and canine MHC alleles were identified. In the recent study, frequent human MHC alleles were replaced for canine MHC alleles to predict the epitopes. Similar allogenic MHC alleles are suggested to include capacity for the induction of canine immune responses. Thus, EVs derived from the associated monocytes loaded with a recombinant multiple-epitope vaccine candidate may be useful to fight *L. major* and *L. infantum* infections in humans and dogs. Theoretically, exosomal vaccines can activate T-lymphocytes in recipients via three various mechanisms of i) exosomal MHC-peptide complexes binding to T-lymphocytes (direct activation); ii) recycling of the exosomal vaccine MHC onto the surface of the recipient APCs (cross dressing); and iii) peptide loading on endogenous MHC molecules via exosomal vaccine degradation and processing by the recipient APCs. In the latter mechanisms, MHC match between the patients and exosome donors is not necessary and hence allogenic exosomes can be used with further viabilities (21).

Exosomes lacking class-I MHC and those with class-I and class-II MHC mismatches induced promising results for cancer treatment (22). Although MHC- identical exosomes can directly activate T cells via presenting peptide-MHC complexes to T-cells, their study revealed that exosome-induced immune responses could be independent of MHC molecules. These findings have verified that antigen delivery and presentation by the host APCs are more important than direct T-cell activation by exosomal MHC-peptide complexes. Therefore, syngeneic and allogeneic exosomes can induce stimulation of immune cells and exosome immunotherapy can be applied using allogeneic APC-derived exosomes (22).

Conclusion

A multiple-epitope peptide vaccine with high antigenicity, no allergenesity and no toxicity was designed for monocyte-derived EV preparation against the zoonotic parasite of *Leishmania*, the agent of severe visceral and cutaneous leishmaniosis. In conclusion, this vaccine candidate can be used for further developments.

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Conflict of interest

The authors declare no conflict of interest.

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