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# Immunoinformatics Approach for The Prediction of Novel Peptide-Based Epitope Vaccine Design Against Leishmania Donovani: A Computational Biology Approach

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**Abstract**

**INTRODUCTION:**

A devastating vector-borne parasitic disease that primarily affects tropical and subtropical areas is visceral leishmaniasis (VL). As a neglected tropical disease with rising drug resistance and no approved vaccine, VL falls into this category. Traditional vaccine production methods are frequently exceedingly time-consuming and difficult. Designing multi-epitope vaccines with anticipated immuno-dominant epitopes of various antigenic proteins is now easier thanks to advancements in bioinformatics and their use in immunology. In order to construct an in-silico chimeric vaccine, we have chosen four antigenic proteins from *Leishmania donovani* and determined their T-cell and B-cell epitopes. The different physicochemical properties of the vaccine have been investigated, and it is anticipated that the tertiary structure of the chimaera construct will facilitate docking investigations and molecular dynamics simulations.

**RESULT:**

The epitopes are connected with particular linkers to create the vaccine construct. The vaccine's expected tertiary structure has been confirmed to be accurate, and docking experiments have shown that the construct has a strong affinity for the TLR-4 receptor. An examination of population coverage reveals that vaccination can protect the vast majority of people on the planet. The vaccination raises a pro-inflammatory response that results in the multiplication of activated T and B cells, according to in-silico immunological simulation experiments.

**CONCLUSION:**

The bioinformatics data cited above provide evidence that the design may function as a potential vaccination. To verify vaccine potency, additional wet lab production of the vaccine and in vivo studies in animal models must be carried out.

**Keywords:** *Leishmania donovani*, B-cell & T-cell epitopes, adjuvant, Disulfide engineering, Molecular Docking

**Introduction:**

Phlebotomus sandflies carry the protozoan infection known as leishmania, which is spread by these insects. Today, most authorities divide all cases into two categories: cutaneous leishmaniasis, which is caused by *Leishmania tropica* or *Leishmania braziliensis*, and visceral leishmaniasis, which is an infection with *Leishmania donovani*. According to estimates from the WHO, species of the genus Leishmania, a neglected and primarily tropical illness, pose a threat to the health of 350 million people in 88 countries worldwide **[1]**. Although related, *Leishmania donovani* is the source of visceral leishmaniasis as seen in both newborns and adults. This evidence and the diverse geographic regions where *Leishmania tropica* and *Leishmania donovani* infections occur support the idea that these agents are not similar. The liver, spleen, and bone marrow are all infected by *Leishmania donovani*, which causes a severe and perhaps fatal systemic illness. A prominent humoral reaction compromised cell-mediated immunity (CMI), and an inability to manage infection are the hallmarks of human visceral leishmania. Successful treatment results in a positive Leishmanin skin test, which measures DTH reactivity, and the restoration of in vitro proliferative responses to parasite antigens **[2]**.

Small, ovoid, or spherical, unicellular *Leishmania donovani* are seen in macrophages and reticuloendothelial cells. Their diameter ranges from 2 to 4 micrometers. The parasites are distinguished by a kinetoplast that appears as either a tiny dot or a thin, oblique rod, and a relatively bigger, rounded nucleus that is often found on one side of the body **[3]**. The few approved treatments for kala-azar in India include miltefosine, paromomycin, pentavalent antimonials (sodium stibogluconate), and liposomal/free amphotericin B. In the long run, all have one or more shortcomings or restrictions. Global demand for the VL vaccine has become a top public health priority due to factors including rising resistance to pentavalent antimonials, the prohibitive cost of the available lipid formulations of amphotericin B, toxicity, hospitalization requirements, HIV-coinfection, and an increase in reports of relapse. The ability to resist infection for the rest of one's life suggests that vaccination against VL can be done successfully. There isn't currently a human-use anti-leishmanial vaccine available. Unprecedented hurdles for VL vaccination arise from the parasite's exceptional host evasion mechanism, significant inter-specific variation in lipophosphoglycan (LPG) structure, and the variety of the human population **[4]**.

The parasites transform dramatically throughout their life cycle, going from motile, flagellated promastigote forms that live in the alkaline sand-fly vector's midgut to sessile amastigotes that develop in the acidic *parasitophorous vacuole* of the host macrophage. Leishmania must adjust to variations in the food supply in addition to environmental changes in pH, temperature, and osmotic tension **[5]**. The female sand fly consumes carbohydrates (mostly sucrose) from plants or aphid honeydew in addition to intermittent blood feeding. In the insect midgut, leishmania parasites thrive in an environment rich in sugar and amino acids and preferentially use simple sugars as a form of energy. *Leishmania promastigotes* can also use proline as an energy source when glucose is scarce **[6]**. Proline concentrations in the haemolymph of some bloodsucking insects can reach 60 mm and are used as an energy source for flight; however, little is known regarding proline concentrations in sand flies **[7]**.

The majority of the first-generation Leishmania vaccines used live/attenuated parasites, suicide cassettes, and dead vaccinations **[8]**. Following are some examples of novel antigens that have been

purified and identified thanks to advances in molecular biology, including L. major homologues of the thiol-specific-antioxidant (TSA) and stress-inducible protein-1 (LmSTI1) in eukaryotes, *Leishmania homologue* of receptor for activated C kinase (LACK antigen), elongation and initiation factor (LeIF), promastigote surface antigen 2 (PSA-2), Leishmania salivary protein 15 (SP15), chimeric protein Q, Leish-111F, fucose-mannose ligand (FML) and glycoprotein 63 (gp63) **[9]**.

The Leishmania parasite contains numerous significant enzymes that are thought to be promising targets for the creation of novel therapeutics. Metalloproteases are among the Leishmania enzymes, particularly those that belong to metzincins, which have zinc atoms in their structural makeup, such as the so-called leishmanolysin **[10]**. Glycoprotein 63 (Gp63), also known as leishmanolysin, is a 63 kDa metalloprotease from the M8 family (subclan MA(M), metzincins), which is present on the surface of the Leishmania parasite and contributes to its virulence and pathogenesis **[11]**. It was first discovered in 1980, and genetic and biochemical analyses revealed that it was a surface antigen expressed in Leishmania promastigotes with a variety of substrates, including casein, gelatin, albumin, haemoglobin, and fibrinogen **[12]**.

The resistance to complement-mediated lysis, inactivation of transcription factors, down-regulation of macrophage protein synthesis, and activation of protein tyrosine phosphatases are only a few of the roles this protein plays to help the parasite survive and spread within infected host cells **[13]**. The antigenic compound Gp63 is most frequently utilized in Leishmania vaccination studies. Yet, reports on the protective effect of the pure protein have produced contradictory findings when they used various adjuvants and parasite strains **[14]**.

## Materials and Methods:

### Selection of *Leishmania donovani* Proteins for Potential Epitope Screening:

In order to find the proteins to constitute a subunit vaccine, an in-depth literature survey was performed. Leishmanolysin (Uniport ID – P23223) protein of the organism *Leishmania donovani* were selected and their amino acid sequences were retrieved in FASTA format from UniProtKB database (https://www.uniprot.org/) **[15]**.

### Linear B-Lymphocyte (LBL) Epitope Prediction:

B-cell epitopes are significant in the development of vaccines because B-cells are essential for producing antibodies that neutralize antigens during infection and for stimulating the humoral immune response. The ABCPred server was used to predict the consecutive B-cell epitopes (http://crdd.osdd.net/raghava/abcpred/). Using a default threshold of 0.5, ABCPred employs ANN to predict B-cell epitopes using fixed-length patterns **[16]**.

### Cytotoxic T-Lymphocyte (CTL) Epitope Prediction:

The antigenic peptide is presented to the T-cell receptor in a highly selective manner by the interaction of the MHC molecule. As a result, the NetMHCpan 4.1 server (https://services.healthtech.dtu.dk/service.php?NetMHCpan-4.1) predicted CTL epitopes. For 12 HLA class I supertypes, it forecasts epitopes. Each supertype consists of a collection of functionally related HLA alleles that have similar structural characteristics in their peptide-binding grooves, allowing them to bind to the same panel of peptides with similar specificity. All epitope lengths were set to 9mers prior

to prediction, and conserved epitopes with scores equal to or below 3.0 percentile ranks were chosen **[17]**.

**Prediction of Helper T-Lymphocyte (HTL) Epitope / MHC-II:**

The chosen protein was entered into the IEDB (Immune Epitope Database) MHC-II binding tool to predict (15mer) HTL. Using the entire HLA (Human Leukocyte Antigen) reference set, the HTL epitopes were predicted. It includes HLA-DR, HLA-DQ, and HLA-DP as well as all other HLA class II alleles. The forecast was made using the IEDB's recommended criteria, which comprised a full set of 27 human alleles that encompassed 99% of the population. The peptides were sorted based on the adjusted rank while maintaining a window length of 15. HTL epitopes were chosen based on their IC50 values, with low IC50 values indicating a high affinity. The IC50 value is the least concentration of a medication needed to provide 50% inhibition in vitro **[18]**.

**Antigenicity, Allergenicity, Immunogenicity, & Toxicity Prediction:**

The ability of a chemical structure to bind precisely with a particular subset of products with adaptive immunity-T cell receptors or antibodies and B cell receptors is known as antigenicity. With the use of the VaxiJen server (http://www.ddg-pharmfac.net/vaxijen/), the antigenicity of the epitopes was predicted. Whereas the AllerTop v2.0 server (https://www.ddg-pharmfac.net/AllerTOP/) assessed the allergenicity of the vaccination design. The AllerTOP v2.0 server uses machine learning techniques to sort the allergens using nearest neighbours, auto, cross-variance modification, and amino acid E descriptors. Also, we conducted a toxicity study using the Toxinpred server (https://webs.iiitd.edu.in/raghava/toxinpred/). The immunogenicity score of epitopes was predicted using the IEDB Analytic Resource's Class-I immunogenicity tool (http://tools.iedb.org/immunogenicity/) **[19]**.

**Construction of Multi-Epitopic Vaccine:**

A chemical compound known as an adjuvant enhances the immune response to the proteins or carbohydrates in the vaccination. B-cell, HTL, and CTL epitopes from the envelope and surface glycoprotein were used to create a multi-epitopic vaccination. The KK linker, the AAY linker, and the GPGPG linker were used to connect the B-cell epitopes, the CTL epitopes, and the HTL epitopes. To increase the vaccine's immunogenic potential, TLR-4 was added as an adjuvant with an EAAAK linker to the N terminus. TLR-4 (PDB ID: 4G8A) agonist 50S ribosomal L7/L12 with accession no. P9WHE3 was chosen as an adjuvant to enhance antigen-specific immune responses because TLR-4 expression rises during the *L. donovani* infection in human immune cells **[20]**.

**Population Coverage:**

The next crucial step is to calculate the population coverage rates for the predicted epitopes. Because HLA molecules are extremely polymorphic in the peptide-binding regions, population coverage analysis is critical in the design and development of epitope-based vaccines. The IEDB (http://tools.iedb.org/population/) server's web-based population coverage analysis tool with default parameters was used to calculate population coverage. The population coverage tool provides HLA allele frequencies for 78 different populations divided into 11 geographical areas. The population coverage analysis tool computes the percentage of people who are expected to respond to a set of

epitopes with known MHC restrictions. As a result, it is advantageous to select only epitopes with a high response rate for different populations **[21]**.

**Physiochemical Properties of the Constructed Vaccine:**

The final vaccine's physical and chemical properties, such as its amino acid composition, theoretical pI, instability index, in-vitro and in-vivo half-life, aliphatic index, molecular weight, and grand average of hydropathicity (GRAVY), were determined using the ProtParam server (https://web.expasy.org/protparam/) **[22]**.

**Secondary Structure Prediction:**

The secondary structure of the multi-epitope vaccine construct was calculated using position-specific iterated prediction (PSIPRED) analysis on outputs from PSI-BLAST (http://bioinf.cs.ucl.ac.uk/psipred/) **[23]**.

**Predicting Tertiary Structure & Validation:**

The tertiary structure of the vaccine's assembly was predicted using Robetta, which is available online at (https://robetta.bakerlab.org/). PROCHECK, ERRAT, and SAVES Verify 3D servers were used to validate the 3D model that was retrieved from the server. By viewing the Ramachandran plot created using the PROCHECK server (http://servicesn.mbi.ucla.edu/PROCHECK/), the best-generated model was assessed. In order to compare trustworthy high-resolution crystallographic structures to non-bonded atom-atom interactions, the ERRAT server (http://services.mbi.ucla.edu/ERRAT/) was employed.

**Disulfide Engineering for the Stability of the Vaccine:**

Disulfide by Design v2.0 (http://cptweb.cpt.wayne.edu/DbD2/index.php) was used for disulfide engineering to achieve stability of the simulated structure of the final vaccine build **[24]**.

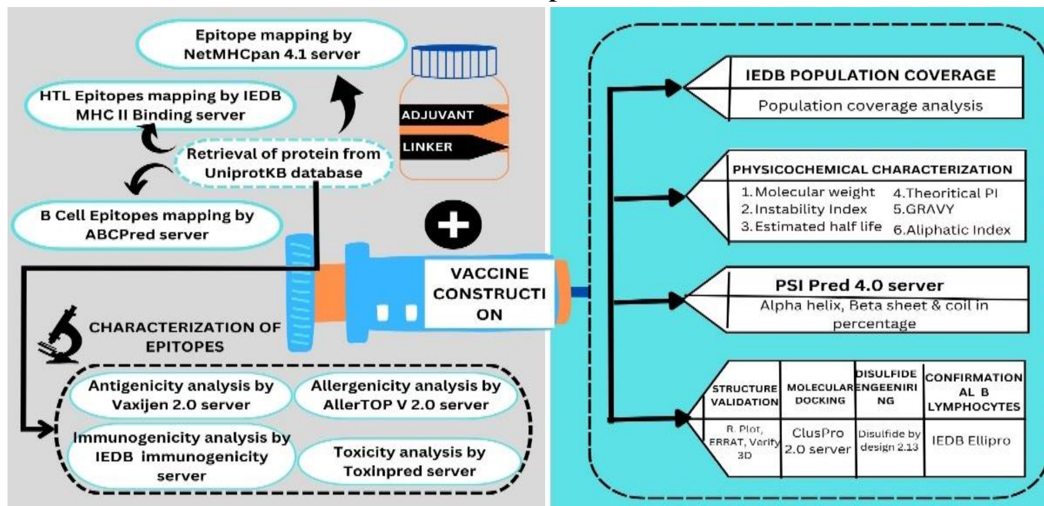
**Discontinuous B-Cell Epitope Prediction:**

The final validated 3D structure of the vaccine was predicted using the ElliPro server (http://tools.iedb.org/ellipro/) with the default parameters (Minimum residue score 0.5 and Maximum distance 6). Each epitope is given a score, which is the PI (Protrusion Index) value averaged over the residues of the epitope. With this method, ellipsoids are used to calculate the protein's 3D structure. An ellipsoid contains 90% of the protein's residues, according to a 0.9 PI score, whereas the remaining 10% are outside of it. The mass center of each residue, which is outside the largest practical ellipsoid, determines the PI number. The PI value is used to predict discontinuous epitopes, and the distance R (defined as the separation between the residue's mass center) is used to group them. A higher R-value implies that more predicted discontinuous epitopes are present **[25]**.

**Molecular Docking:**

Molecular docking was performed for the constructed multi-epitope vaccine with TLR-4, receptor (PDB ID: 4G8A). The PDB files of receptor and ligand were submitted to the ClusPro2.0 (https://cluspro.bu.edu/login.php) server and the default settings were used accordingly. ClusPro utilizes the Fourier correlation algorithm and sorts out the models with the amalgamation of desolvation and

electrostatic energies. Using PyMol v2.4 software, the best docking pose in the top-rank cluster was utilized for visualization, and the outcomes were interpreted **[26]**.




**[Figure.1: The multi-epitope vaccination against *Leishmania donovani* was designed using a schematic representation of the immunoinformatics]**

**Results & Discussion:**

**Retrieval of Protein Sequence:**

UniProt database (http://www.uniprot.org/) was used to obtain the amino acid sequence of Leishmanolysin protein from *Leishmania donovani* (Accession ID: P23223). A manual search in the UniProt database's search bar generated the protein's accession ID **[27]**.

**Linear B-Cell Epitope Prediction:**

The ABCPred server was used to predict 16-mer B-cell epitopes from nine potential vaccine proteins with an 85% specificity. For the prediction, a threshold value of 0.51 was used. Most epitopes were predicted to exit. Some of the linear B-cell epitopes predicted were also T-cell epitopes. The serial numbers of overlapping B-cell and T-cell epitopes are the same **[28]**.

| Uniprot ID | B-cell epitope | Position | Score | Antigenicity score | Toxicity | Hydrophobicity | Hydropathicity | Hydrophilicity | charge | Mol. Wt. |
|---|---|---|---|---|---|---|---|---|---|---|
| P23223 | AGSAGSHIKMRNAQDE | 315 | 0.93 | 1.7274 | Non-toxin | -0.27 | -1.01 | 0.49 | 0.5 | 1672.03 |
| P23223 | GGSGYANCTPGLRVEL | 516 | 0.92 | 2.0697 | Non-toxin | -0.06 | -0.09 | -0.17 | 0 | 1594.01 |

**[Table.1: Final selected B-cell epitopes from *Leishmania donovani* of leishmanolysin protein and their corresponding immunogenic properties]**

**Cytotoxic T-Lymphocyte (CTL) Epitopes Mapping:**

The NetMHCpan4.1 (http://www.cbs.dtu.dk/services/NetMHCpan-4.1/) server was used to predict CTL epitopes of the target protein. The epitope identification threshold for a strong binder was set at 0.5%,

while it was set at 2% for a weak binder. The server generates epitope predictions for 12 HLA class I supertypes. Each supertype's epitopes were predicted. The option 'Sort by Prediction score' was chosen. Prior to prediction, all epitope lengths were set to 9mers, which are conserved epitopes that bind to a wide range of HLA alleles **[29]**.

| **Uniprot ID** | **Epitope** | **Immunogenicity** | **NetMHCpan 4.1 allele** | **Position** | **% Rank** | **Antigenicity score** | **Toxicity** | **Hydrophobicity** | **Hydropathicity** | **Hydrophilicity** | **Charge** | **Mol. wt.** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P23223 | IPLPPYWQY | 0.09022 | HLA-A*01:01 | 419 | 0.817 | 2.1124 | Non-toxin | 0.09 | -0.39 | -1.27 | 0 | 1176.51 |
| P23223 | QLHTERLKV | 0.06042 | HLA-A*02:01 | 153 | 1.426 | 1.2237 | Non-toxin | -0.35 | -0.83 | 0.36 | 1.5 | 1123.46 |
| P23223 | VTMRCHTGR | 0.02559 | HLA-A*03:01 | 398 | 2.097 | 1.4636 | Non-toxin | -0.36 | -0.6 | 0.1 | 2.5 | 1060.38 |
| P23223 | HFKVPPAHI | 0.00222 | HLA-A*24:02 | 180 | 0.694 | 1.5445 | Non-toxin | 0.01 | -0.02 | -0.48 | 2 | 1045.38 |
| P23223 | DTATRTYSV | 0.03892 | HLA-A*26:01 | 504 | 0.422 | 1.3473 | Non-toxin | -0.27 | -0.69 | 0.09 | 0 | 1013.18 |
| P23223 | STRDGRFAI | 0.2425 | HLA-B*07:02 | 120 | 0.627 | 0.9597 | Non-toxin | -0.33 | -0.59 | 0.46 | 1 | 1022.24 |
| P23223 | SSTHRHRSV | 0.07302 | HLA-B*08:01 | 6 | 0.804 | 0.855 | Non-toxin | -0.53 | -1.59 | 0.44 | 3 | 1066.26 |
| P23 | GRRG | 0.1447 | HLA- | 569 | 2. | 1.476 | Non | -0.48 | -1.17 | 0.83 | 3 | 911 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 223 | PRAAA | | B*27:05 | | 283 | 3 | -toxin | | | | | .14 |
| P23223 | AHALGFSVV | 0.02924 | HLA-B*39:01 | 254 | 0.096 | 0.8671 | Non-toxin | 0.25 | 1.58 | -0.94 | 0.5 | 900.17 |
| P23223 | LEMEDQGGA | 0.00471 | HLA-B*40:01 | 307 | 3.043 | 1.7506 | Non-toxin | -0.14 | -0.81 | 0.62 | -3 | 949.13 |
| P23223 | TTETVTNSY | 0.04618 | HLA-B*58:01 | 486 | 1.814 | 0.8296 | Non-toxin | -0.19 | -0.86 | -0.21 | -1 | 1015.16 |
| P23223 | KMRNAQDEL | 0.01657 | HLA-B*15:01 | 323 | 0.983 | 0.7069 | Non-toxin | -0.5 | -1.66 | 0.98 | 0 | 1104.37 |

**[Table.2: Predicted CTL epitopes from *Leishmania donovani* of leishmanolysin protein to design multi-epitope vaccine construct with their corresponding MHC Class I alleles and their immunogenic properties]**

**Helper T-Lymphocyte (HTL) Epitopes Mapping:**

The selected protein was used as input in the IEDB MHC-II binding tool to predict (15mer) HTL. The entire HLA (Human leukocyte antigen) reference set was used to predict the HTL epitopes. It includes all HLA class II alleles, such as HLA-DR, HLA-DQ, and HLA-DP. To make the prediction, the suggested IEDB parameters were used, which included a complete set of 27 human alleles that covered 99% of the population. The window length was kept at 15, and the peptides were ordered by adjusted rank. HTL epitopes were chosen based on their IC50 (Inhibition concentration 50) values, with low IC50 values (the minimal concentration of a drug required for 50% inhibition in vitro) demonstrating strong affinity **[30]**.

| Uniprot_ID | MHC II Epitope | Alleles | Pos | IC50 value | Percentile Rank | Antigenicity Score | Toxicity | Hydrophobicity | Hydropathicity | Hydrophilicity | Charge | Mol wt. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P23223 | AAAGAAVIAAVGTAA | HLA-DQA1*05:01/DQB1*03:01 | 22-36 | 3 | 0.03 | 0.4894 | Non-toxin | 0.28 | 1.84 | -0.65 | 0 | 1184.55 |
| | PVINSSTAVAKAREQ | HLA-DQA1*01:02/DQB1*06:02 | 284-298 | 38 | 0.66 | 0.4359 | Non-toxin | -0.2 | -0.3 | 0.22 | 1 | 1570.97 |

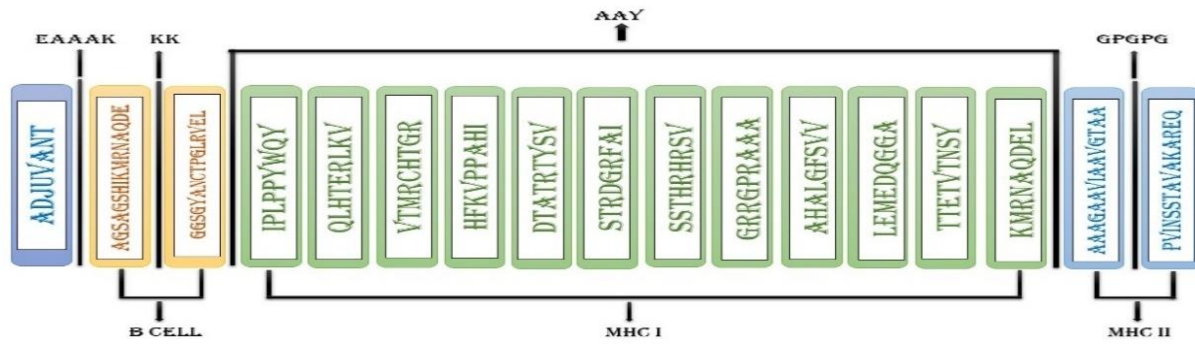
**[Table.3: Predicted HTL epitopes from *Leishmania donovani* of leishmanolysin protein design multi-epitope vaccine construct with their corresponding MHC Class II alleles and their immunogenic properties]**

**Antigenicity, Allergenicity & Toxicity Assessment:**

The vaccine must be antigenic and non-allergic, as well as induce humoral and cell-mediated immune responses against the pathogen of interest. Further investigation revealed that the epitopes were antigenic, as predicted by the VaxiJen v2.0 server, with a high probability score. The epitopes were also identified as non-allergen by the AllerTOP v2.0 server. Using the ToxinPred server, toxicity predictions revealed that none of the selected epitopes were toxic to humans. The IEDB Class I immunogenicity tool predicted the immunogenicity score of the selected CTL epitopes. All of the epitopes were highly immunogenic **[31]**.

**Construction of Multi-Epitope Vaccine:**

The vaccine construct was invented with high immunogenicity, non-toxicity, non-allergenic, and strong binding affinity T-cell and B-cell epitopes predicted by various epitope prediction tools. The KK linker connected the two B-cell epitopes, the AAY linker connected the CTL epitopes, and the GPGPG linker connected the HTL epitopes. TLR-4 antagonist was added to the N-terminal end with an EAAAK linker as an adjuvant to improve the immunogenicity of the vaccine construct **[32]**.




**[Figure.2: The final multi-epitope vaccine construct's structural arrangement of B and T-cell epitopes, as well as linkers and adjuvant]**

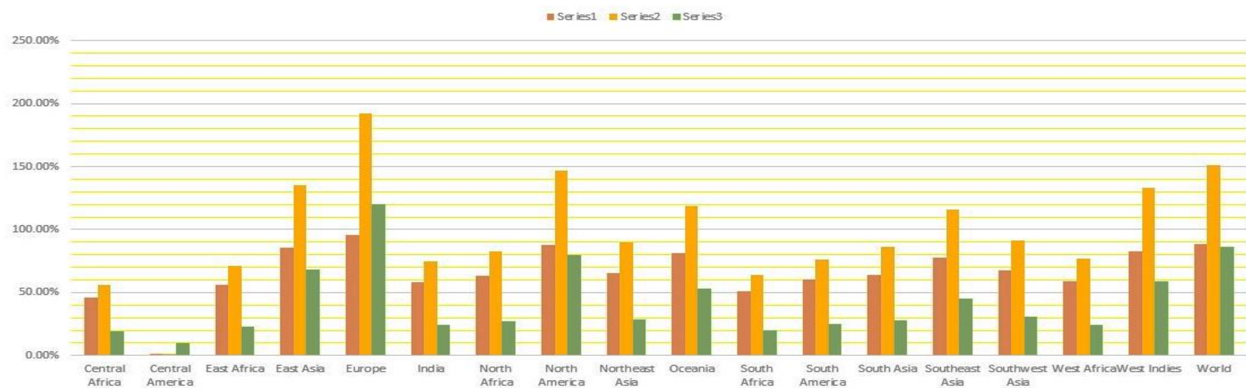
**Estimation of Population Coverage:**

The analysis of how many people will be covered by the respective HLA alleles of the predicted epitopes is a crucial component of designing an effective vaccine because different MHC-I and MHC-II HLA alleles are exposed at a different frequency (population/individual) in different ethnicities throughout the world. About 88.42% of the population was protected by the intended vaccine construct, according to population coverage analysis data. The highest coverage rates were seen in Europe (95.66%), East Africa (55.74%), West Africa (58.76%), Central Africa (45.98%), and North America (87.54%) **[33]**.

| Geographical Area | Population Coverage | Average Hit | PC90 |
|---|---|---|---|
| World | 88.42% | 1.51 | 0.86 |
| East Asia | 85.20% | 1.35 | 0.68 |
| Northeast Asia | 65.65% | 0.9 | 0.29 |
| South Asia | 64.17% | 0.86 | 0.28 |
| India | 58.34% | 0.75 | 0.24 |
| Southeast Asia | 77.71% | 1.16 | 0.45 |
| Southwest Asia | 67.89% | 0.91 | 0.31 |
| Europe | 95.66% | 1.92 | 1.2 |
| East Africa | 55.74% | 0.71 | 0.23 |
| West Africa | 58.76% | 0.77 | 0.24 |
| Central Africa | 45.98% | 0.56 | 0.19 |
| North Africa | 63.03% | 0.83 | 0.27 |
| South Africa | 51.22% | 0.64 | 0.2 |
| West Indies | 82.95% | 1.33 | 0.59 |

| North America | 87.54% | 1.47 | 0.8 |
|---|---|---|---|
| Central America | 1.40% | 0.01 | 0.1 |
| South America | 60.53% | 0.76 | 0.25 |
| Oceania | 81.26% | 1.19 | 0.53 |

**[Table.5: Population coverage analysis of the final vaccine construct using the population coverage analysis tool of the IEDB database by keeping the default parameters]**




**[Figure.3: Graph of population coverage]**

**Determination of Physicochemical Features, Solubility:**

This was determined that the ultimate molecular weight of the vaccine was 36680.52 kDa. The vaccine was shown to be an easy-to-handle, purifiable small-scale construct for testing. The vaccine candidate was discovered to be slightly basic in nature according to its theoretical pI value of 7.10. This protein has 39 positively charged and 39 negatively charged amino acid residues. The vaccine's half-life in mammalian cells was 30 hours; in yeast cells, it was over 20 hours; and in E. coli cells, it was over 10 hours. The created vaccine has an extraordinarily high instability index (II) of 28.20, indicating that it is stable. The vaccine's thermal stability is determined by the protein aliphatic index (AI), which has a score of 79.38. The vaccine's grand average of hydropathicity (GRAVY) was (-0.087) **[34]**.

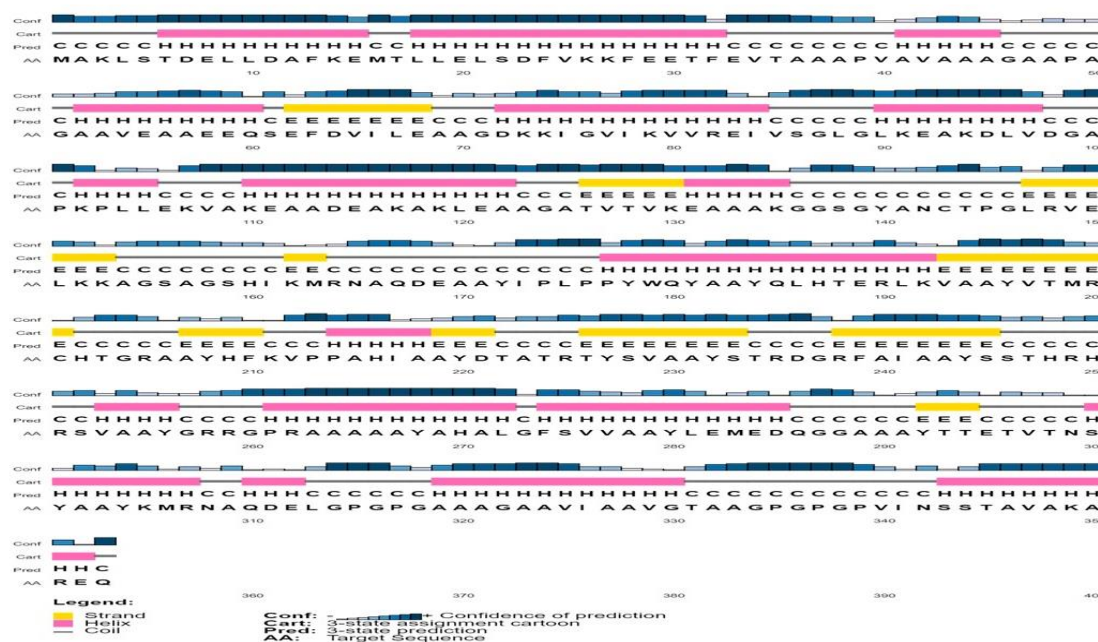
| **Parameters** | **Vaccine Construction Sequence** |
|---|---|
| Number of amino acids | 353 |
| Molecular weight | 36680.52 |
| Theoretical pI | 7.10 |
| Total number of negatively charged residues (Asp + Glu) | 39 |
| Total number of positively charged residues (Arg + Lys) | 39 |

| Parameter | Value | | |
|---|---|---|---|
| Instability index | 28.20 | | |
| Aliphatic index | 79.38 | | |
| Grand average of hydropathicity (GRAVY) | -0.087 | | |
| The estimated half-life | 30 hours (mammalian reticulocytes, in vitro).<br>>20 hours (yeast, in vivo)<br>>10 hours (Escherichia coli, in vivo). | | |
| Amino acid composition | Ala (A) | 81 | 22.98% |
| | Arg (R) | 16 | 4.5% |
| | Asn (N) | 5 | 1.4% |
| | Asp (D) | 13 | 3.7% |
| | Cys (C) | 2 | 0.6% |
| | Gln (Q) | 7 | 2.0% |
| | Glu (E) | 26 | 7.4% |
| | Gly (G) | 29 | 8.2% |
| | His (H) | 8 | 2.3% |
| | Ile (I) | 10 | 2.8% |
| | Leu (L) | 21 | 5.9% |
| | Lys (K) | 23 | 6.5% |
| | Met (M) | 6 | 1.7% |
| | Phe (F) | 8 | 2.3% |
| | Pro (P) | 16 | 4.5% |
| | Ser (S) | 16 | 4.5% |
| | Thr (T) | 21 | 5.9% |
| | Trp (W) | 1 | 0.3% |
| | Tyr (Y) | 17 | 4.8% |
| | Val (V) | 27 | 7.6% |
| | Pyl (O) | 0 | 0% |
| | Sec (U) | 0 | 0% |
| Atomic composition | Carbon | C | 1625 |
| | Hydrogen | H | 2573 |
| | Nitrogen | N | 453 |
| | Oxygen | O | 498 |
| | Sulfur | S | 8 |

| | |
|---|---|
| Secondary structure analysis | Alpha helix (Hh) – 45.89%<br>Extended strand (Ee) – 16.72%<br>Random coil (Cc) – 37.39% |
| Solubility (SOLpro Server) | SOLUBLE with probability 0.942830 |

**[Table.6: Physicochemical properties, secondary structure, and solubility analysis of final vaccine construct as predicted by ProtParam tool, PSIPRED, and SOLpro server]**

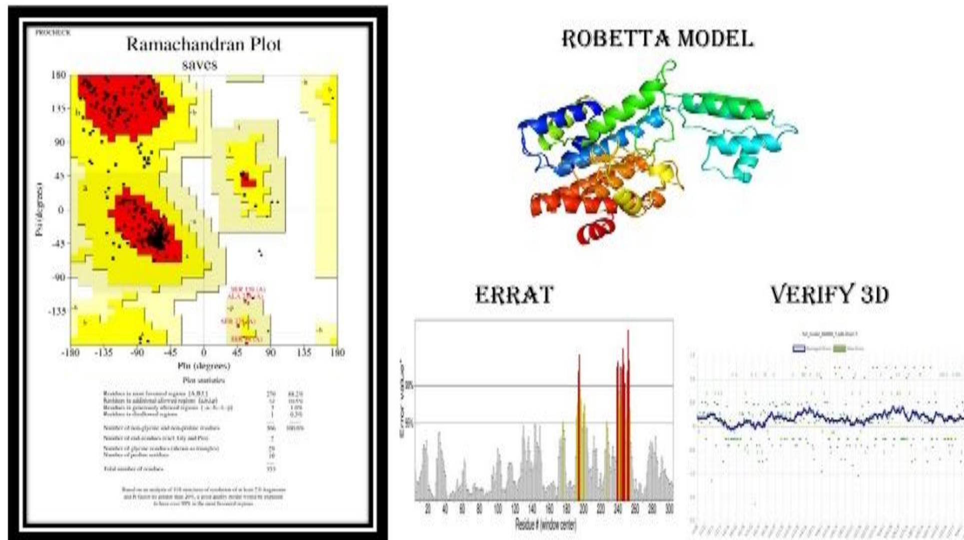
**Secondary Structure Prediction:**
The secondary structure of the final subunit vaccine construct was predicted using PSIPRED. The sequence is made up of 45.89 percent alpha helices, 16.72 percent beta strands, and 37.39 percent random coils. These results suggest that interactions between and within chains may have contributed to the secondary structure of the protein. Since greater solubility suggests better purification during downstream processing, solubility is an important consideration in post-production vaccine development. The SolPro servers found that the vaccination protein was soluble when it was overexpressed in *E. coli* **[35]**.




**[Figure.4: Secondary structure prediction of the final multi-epitope vaccine construct by using PSIPRED tool]**

**Tertiary Structure Prediction & Validation of the Vaccine Construct:**
For the creation of the vaccine, the Robetta server was used to predict five tertiary structures, which were then examined using a Ramachandran plot to determine the best model. Because Model 1 had the finest arrangement of residues in permitted zones, it was chosen. The Ramachandran plot revealed that the preferred (81.4%), allowed (12.9%), and generously allowed (2.1%) regions contained the majority of the residues, while the disallowed zone had 3.6% of the residues. The Verify-3D score of 95.93 and the model's ERRAT quality factor of 92.661, respectively, show that this structure is energetically

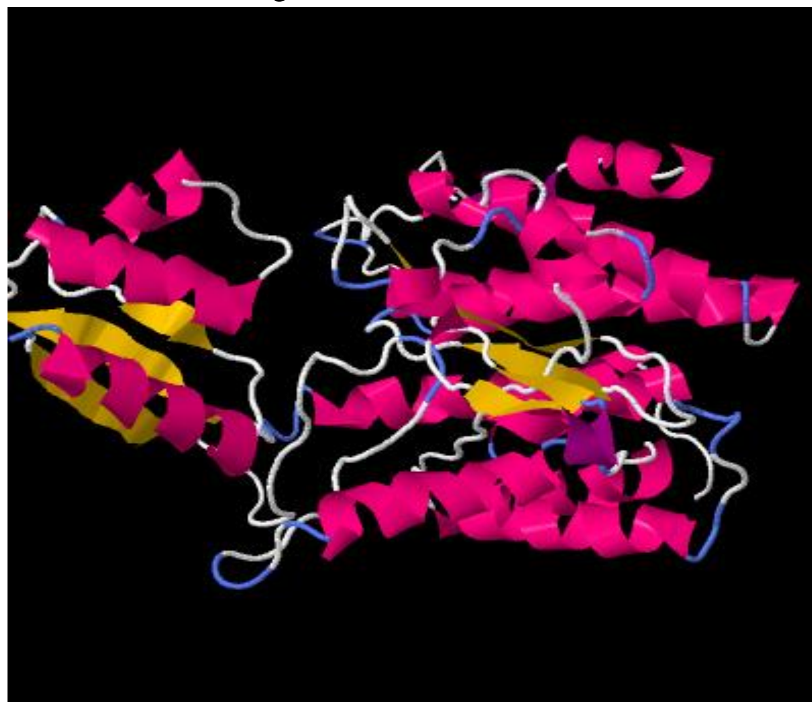
stable. Fig. 5 displays the multi-epitope peptide structure's Ramachandran plot, ERRAT, and verify3D **[36]**.




**[Figure.5: Several structure validations tools results confirmed the modeled multi-epitope vaccine structure to be reliable & accurate]**

**Disulfide Engineering for Vaccine Protein Stability:**

In a wide range of biological applications, protein stability is crucial, and it is an alluring technique to mimic molecular interaction stabilization in nature. Target proteins are very stable because of the covalent disulfide bonds, and disulfide engineering has been very successful in many different applications. Two pairs of residues, ALA55-ALA85 and ASN184-PHE197, were selected for the disulfide engineering process based on the requirements that the bond energy score should be less than 2.2 kcal/mol and the "3 value" should range from "87 to +97" **[37]**.



**[Figure.6: Vaccine protein disulfide engineering. In order to create a disulfide bridge between the pair of residues represented in the purple (ALA55, ASN184) and olive (ALA85, PHE197) spheres, respectively, they were changed to cysteine residues]**

**Mapping of Discontinuous B-Cell Epitopes in the Vaccine Protein:**

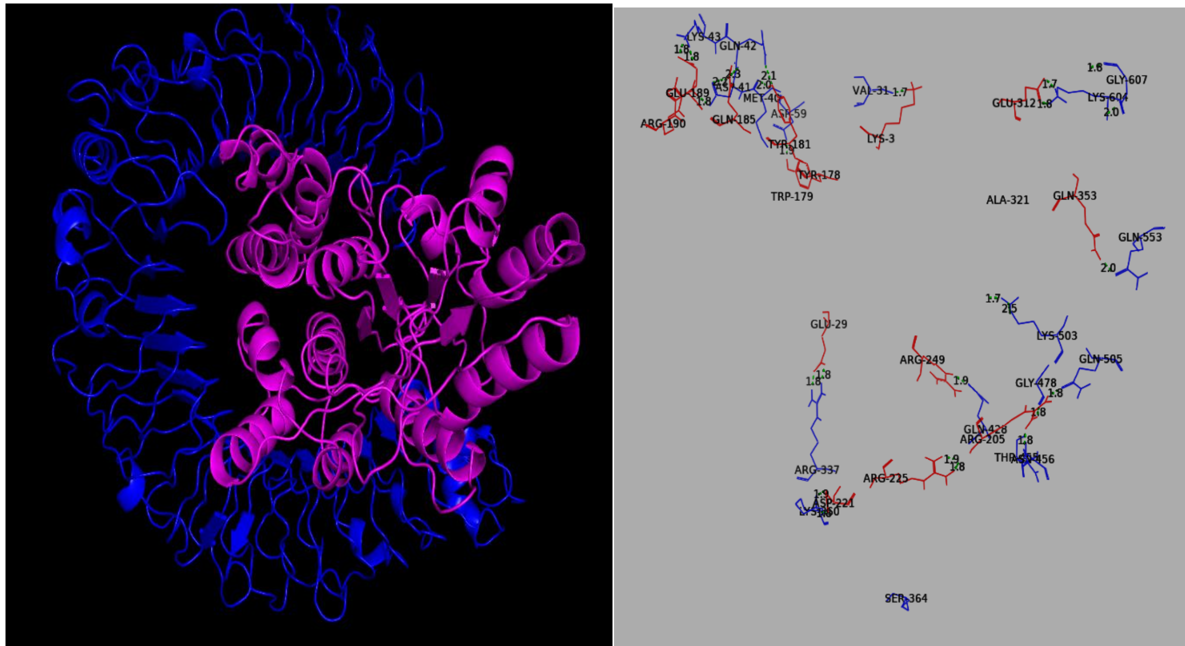
By secreting antibodies and cytokines that neutralize foreign antigens, B cells enhance humoral immunity. Hence, the protein domain must have a sufficient number of B-cell epitopes. The ElliPro tool of the IEBD server was used to investigate the existence of conformational B-cell epitopes **[38]**.

| No. | Residues | Number of residues | Ellipro Score |
|---|---|---|---|
| 1 | A:I66, A:L67, A:E68, A:A69, A:A70, A:G71, A:D72, A:K73, A:K74, A:I75, A:G76, A:V77, A:I78, A:K79, A:V80, A:V81, A:R82, A:E83, A:S86, A:G87, A:L88, A:G89, A:L90, A:K91, A:E92, A:A93, A:K94, A:D95, A:L96, A:V97, A:D98, A:G99, A:A100, A:P101, A:K102, A:P103, A:L104, A:A109, A:K110, A:D114, A:E115, A:A116, A:K117, A:A118, A:K119, A:L120, A:E121, A:A122, A:A123, A:G124, A:A125, A:T126, A:V127, A:T128, A:V129, A:K130 | 56 | 0.767 |
| 2 | A:E284, A:D285, A:Q286, A:G287, A:G288, A:A289, A:A290, A:T293, A:Y304, A:R307, A:N308, A:A309, A:Q310, A:D311, A:E312, A:L313, A:G314, A:P315, A:G316, A:P317, A:G318, A:A321, A:A332, A:A333, A:G334, A:P335, A:G336, A:P337, A:G338, A:P339, A:V340, A:I341, A:N342, A:S343, A:S344, A:T345, A:A346, A:V347, A:A348, A:K349, A:A350, A:R351, A:E352 | 43 | 0.724 |
| 3 | A:M1, A:A2, A:K3, A:E15, A:M16, A:T17, A:L19, A:E20, A:D23, A:F24, A:K26, A:K27, A:F28, A:E29, A:E30, A:T31, A:F32, A:P174, A:P176, A:Y178, A:W179, A:Y181, A:A182, A:A183, A:Y184, A:Q185, A:L186, A:H187, A:T188, A:E189, A:R190, A:L191, A:V193 | 33 | 0.693 |
| 4 | A:E33, A:V34, A:T35, A:A36, A:A37 | 5 | 0.612 |
| 5 | A:E59, A:Q60, A:E62, A:E131, A:A132, A:A133, A:A134, A:K135, A:G136, A:G137, A:S138, A:G139, A:E150, A:L151, A:K152, A:K153, A:A154, A:G155, A:S156, A:A157, A:G158, A:S159, A:K162 | 23 | 0.588 |
| 6 | A:E169, A:A171, A:Y172, A:I173, A:R200, A:C201, A:H202, A:T203, A:G204, A:A207, A:Y208, A:F210, A:V212, A:A219, A:Y220, A:D221, A:T222, A:A223, A:T224, A:R225, A:T226, A:Y227, A:S245, A:S246, A:T247, A:H248, A:R249, A:H250 | 28 | 0.587 |
| 7 | A:L4, A:S5, A:E8 | 3 | 0.558 |

**[Table.8: Discontinues B-cell epitopes with their scores predicted by Ellipro]**

**Molecular Docking of Vaccine with TLR-4 Receptor:**

Through controlling the stimulation of polymorphonuclear neutrophils, the production of adhesion molecules, chemotaxis, and chemoattractant receptors, as well as the detection of ligands and phagocytosis, among other professional phagocyte tasks, TLR-4 plays a crucial role in staphylococcal disease. Increasing data suggest that leishmanolysin is essential for Leishmania donovani to activate TLR-4. TLR-4 was consequently selected as the immunological receptor. The TLR-4 immune receptor (PDB ID: 4G8A) and the final vaccination design were molecularly docked using the open-source website ClusPro. On the basis of the consensus method, the best-docked complex from each server was chosen **[39]**.




**[Figure.7: Molecular docking of the final vaccine construct with human TLR-4 receptor]**

## Conclusion:

*Leishmania* species are the etiological agents of the complex tropical disease leishmaniasis. Visceral Leishmaniasis is one of the leading causes of death worldwide in tropical and subtropical areas. Lack of vaccine and several limitations associated with the existing treatment regimens underline to develop novel therapeutics against leishmaniasis. The first step of the drug discovery process is the identification of drug targets. In this study, we developed a new multi-epitope vaccination against VL using immunoinformatics methods. Our multi-epitope subunit vaccine appears to be immunogenic and non-allergic, with the ability to elicit cellular and humoral immune responses, according to the data. Although in silico results demonstrated this vaccine's efficacy, experimental validation of the vaccine's immunogenic potency is still required.

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## Conflict of Interest:

Nil

## References

1. Alexander, J., & Russell, D. G. (1992). The interaction of Leishmania species with macrophages. *Advances in parasitology*, *31*, 175-254. DOI-10.1016/S0065-308X (08)60022-6.
2. Liew, F. Y., & O'donnell, C. A. (1993). Immunology of leishmaniasis. *Advances in parasitology*, *32*, 161-259. DOI-10.1016/S0065-308X (08)60208-0.
3. Fairlamb, A. H., Gow, N. A. R., Matthews, K. R., & Waters, A. P. (2016). Drug resistance in eukaryotic microorganisms. Nature Microbiology. DOI-10.1038/nmicrobiol.2016.92.

4. Beverley, S. M., & Turco, S. J. (1998). Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoan parasite Leishmania. *Trends in microbiology*, *6*(1), 35-40. DOI-10.1016/S0966-842X (97)01180-3.
5. Stuart, K., Brun, R., Croft, S., Fairlamb, A., Gürtler, R. E., McKerrow, J., & Tarleton, R. (2008). Kinetoplastids: related protozoan pathogens, different diseases. *The Journal of clinical investigation*, *118*(4), 1301-1310. DOI-10.1172/JCI33945.
6. Krassner, S. M., & Flory, B. (1972). Proline metabolism in Leishmania donovani promastigotes. *The Journal of Protozoology*, *19*(4), 682-685. DOI- 10.1111/j.1550-7408. 1972.tb03560.x.
7. Koslowsky, D. J., Göringer, H. U., Morales, T. H., & Stuart, K. (1992). In vitro guide RNA/mRNA chimaera formation in Trypanosoma brucei RNA editing. *Nature*, *356*(6372), 807-809. DOI-10.1038/356807a0.
8. Gillespie, P. M., Beaumier, C. M., Strych, U., Hayward, T., Hotez, P. J., & Bottazzi, M. E. (2016). Status of vaccine research and development of vaccines for leishmaniasis. *Vaccine*, *34*(26), 2992-2995. DOI-10.1016/j.vaccine.2015.12.071.
9. Nagill, R., & Kaur, S. (2011). Vaccine candidates for leishmaniasis: a review. *International immunopharmacology*, *11*(10), 1464-1488. DOI-10.1016/j.intimp.2011.05.008.
10. Passalacqua, T. G., Torres, F. A., Nogueira, C. T., de Almeida, L., Del Cistia, M. L., dos Santos, M. B., & Zottis, A. (2015). The 2′, 4′-dihydroxychalcone could be explored to develop new inhibitors against the glycerol-3-phosphate dehydrogenase from Leishmania species. *Bioorganic & Medicinal Chemistry Letters*, *25*(17), 3564-3568. DOI-10.1016/j.bmcl.2015.06.085.
11. Yao, C., Donelson, J. E., & Wilson, M. E. (2003). The major surface protease (MSP or GP63) of Leishmania sp. Biosynthesis, regulation of expression, and function. *Molecular and biochemical parasitology*, *132*(1), 1-16. DOI- 10.1016/S0166-6851(03)00211-1.
12. Mercado-Camargo, J., Cervantes-Ceballos, L., Vivas-Reyes, R., Pedretti, A., Serrano-García, M. L., & Gómez-Estrada, H. (2020). Homology modeling of leishmanolysin (gp63) from Leishmania panamensis and molecular docking of flavonoids. *ACS omega*, *5*(24), 14741-14749. DOI-10.1021/acsomega.0c01584.
13. Chang, C. S., Inserra, T. J., Kink, J. A., Fong, D., & Chang, K. P. (1986). Expression and size heterogeneity of a 63 kilodalton membrane glycoprotein during growth and transformation of Leishmania mexicana amazonensis. *Molecular and biochemical parasitology*, *18*(2), 197-210. DOI-10.1016/0166-6851(86)90038-1.
14. Rivier, D., Bovay, P., Shah, R., Didisheim, S., & Mauel, J. (1999). Vaccination against Leishmania major in a CBA mouse model of infection: role of adjuvants and mechanism of protection. *Parasite immunology*, *21*(9), 461-473.
15. Saha, S., Vashishtha, S., Kundu, B., & Ghosh, M. (2022). In-silico design of an immunoinformatics-based multi-epitope vaccine against Leishmania donovani. *BMC Bioinformatics*, *23*(1), 1-28. DOI-10.1186/s12859-022-04816-6.
16. Reynisson, B., Alvarez, B., Paul, S., Peters, B., & Nielsen, M. (2020). NetMHCpan-4.1 and NetMHCIIpan-4.0: improved predictions of MHC antigen presentation by concurrent motif deconvolution and integration of MS MHC eluted ligand data. *Nucleic acids research*, *48*(W1), W449-W454. DOI-10.1093/nar/gkaa379.
17. Nielsen, M., Lund, O., Buus, S., & Lundegaard, C. (2010). MHC class II epitope predictive algorithms. *Immunology*, *130*(3), 319-328. DOI-10.1111/j.1365-2567.2010. 03268.x.

18. Nielsen, M., Lund, O., Buus, S., & Lundegaard, C. (2010). MHC class II epitope predictive algorithms. *Immunology*, *130*(3), 319-328. DOI-10.1111/j.1365-2567.2010. 03268.x.
19. Dimitrov, I., Flower, D. R., & Doytchinova, I. (2013, April). AllerTOP-a server for in silico prediction of allergens. In *BMC bioinformatics* (Vol. 14, No. 6, pp. 1-9). BioMed Central. DOI-10.1007/s00894-014-2278-5.
20. Khatoon, N., Pandey, R. K., & Prajapati, V. K. (2017). Exploring Leishmania secretory proteins to design B and T cell multi-epitope subunit vaccine using immunoinformatics approach. *Scientific reports*, *7*(1), 1-12. DOI-10.1038/s41598-017-08842-w.
21. Misra, N., Panda, P. K., Shah, K., Sukla, L. B., & Chaubey, P. (2011). Population coverage analysis of T-Cell epitopes of Neisseria meningitidis serogroup B from Iron acquisition proteins for vaccine design. *Bioinformation*, *6*(7), 255. DOI-10.6026/97320630006255.
22. Dong, R., Chu, Z., Yu, F., & Zha, Y. (2020). Contriving multi-epitope subunit of vaccine for COVID-19: immunoinformatics approaches. *Frontiers in immunology*, *11*, 1784. DOI-10.3389/fimmu.2020.01784.
23. McGuffin, L. J., Bryson, K., & Jones, D. T. (2000). The PSIPRED protein structure prediction server. *Bioinformatics*, *16*(4), 404-405. DOI-10.1093/bioinformatics/16.4.404.
24. Narang, P. K., Dey, J., Mahapatra, S. R., Roy, R., Kushwaha, G. S., Misra, N., & Raina, V. (2022). Genome-based identification and comparative analysis of enzymes for carotenoid biosynthesis in microalgae. *World Journal of Microbiology and Biotechnology*, *38*, 1-22. DOI-10.1007/s11274-021-03188-y.
25. Ponomarenko, J., & Bui, H. H. LiW., Fusseder N., Bourne PE, Sette A. & Peters B. 2008. ElliPro: a new structure-based tool for the prediction of antibody epitopes. *BMC Bioinformatics*, *9*(514), 10-1186.DOI-10.1186/1471-2105-9-514.
26. Bhattacharya, M., Sharma, A. R., Mallick, B., Sharma, G., Lee, S. S., & Chakraborty, C. (2020). Immunoinformatics approach to understand molecular interaction between multi-epitopic regions of SARS-CoV-2 spike-protein with TLR4/MD-2 complex. *Infection, Genetics and Evolution*, *85*, 104587. DOI-10.1016/j.meegid.2020.104587.
27. Vishnu, U. S., Sankarasubramanian, J., Gunasekaran, P., & Rajendhran, J. (2017). Identification of potential antigens from non-classically secreted proteins and designing novel multitope peptide vaccine candidate against Brucella melitensis through reverse vaccinology and immunoinformatics approach. *Infection, Genetics and Evolution*, *55*, 151-158. DOI-10.1016/j.meegid.2017.09.015.
28. Saadi, M., Karkhah, A., & Nouri, H. R. (2017). Development of a multi-epitope peptide vaccine inducing robust T cell responses against brucellosis using immunoinformatics based approaches. *Infection, Genetics and Evolution*, *51*, 227-234. DOI-10.1016/j.meegid.2017.04.009.
29. Reynisson, B., Alvarez, B., Paul, S., Peters, B., & Nielsen, M. (2020). NetMHCpan-4.1 and NetMHCIIpan-4.0: improved predictions of MHC antigen presentation by concurrent motif deconvolution and integration of MS MHC eluted ligand data. *Nucleic acids research*, *48*(W1), W449-W454. DOI-10.1093/nar/gkaa379.
30. Fleri, W., Paul, S., Dhanda, S. K., Mahajan, S., Xu, X., Peters, B., & Sette, A. (2017). The immune epitope database and analysis resource in epitope discovery and synthetic vaccine design. Front Immunol 8: 278. *CRITICAL REVIEWS IN MICROBIOLOGY*, *505*. DOI-10.3389/fimmu.2017.00278.

31. Bui, H. (2006). H, Sidney J, Dinh K, Southwood S, Newman MJ, Sette A. Predicting population coverage of T-cell epitope-based diagnostics and vaccines. *BMC Bioinformatics*, *7*(1), 153. DOI-10.1186/1471-2105-7-153.
32. Shey, R. A., Ghogomu, S. M., Esoh, K. K., Nebangwa, N. D., Shintouo, C. M., Nongley, N. F., & Souopgui, J. (2019). In-silico design of a multi-epitope vaccine candidate against onchocerciasis and related filarial diseases. *Scientific reports*, *9*(1), 4409. DOI-10.1038/s41598-019-40833-x.
33. Ayyagari, V. S., TC, V., & Srirama, K. (2022). Design of a multi-epitope-based vaccine targeting M-protein of SARS-CoV2: an immunoinformatics approach. *Journal of Biomolecular Structure and Dynamics*, *40*(7), 2963-2977. DOI-10.1080/07391102.2020.1850357.
34. Baseer, S., Ahmad, S., Ranaghan, K. E., & Azam, S. S. (2017). Towards a peptide-based vaccine against Shigella sonnei: A subtractive reverse vaccinology-based approach. Biologicals, 50, 87-99. DOI-10.1016/j.biologicals.2017.08.004.
35. Dey, J., Mahapatra, S. R., Lata, S., Patro, S., Misra, N., & Suar, M. (2022). Exploring Klebsiella pneumoniae capsule polysaccharide proteins to design multiepitope subunit vaccine to fight against pneumonia. *Expert Review of Vaccines*, *21*(4), 569-587. DOI-10.1080/14760584.2022.2021882.
36. Dey, J., Mahapatra, S. R., Singh, P., Patro, S., Kushwaha, G. S., Misra, N., & Suar, M. (2021). B and T cell epitope-based peptides predicted from clumping factor protein of Staphylococcus aureus as vaccine targets. *Microbial Pathogenesis*, *160*, 105171. DOI-10.1016/j.micpath.2021.105171.
37. Dombkowski, A. A., Sultana, K. Z., & Craig, D. B. (2014). Protein disulfide engineering. *FEBS letters*, *588*(2), 206-212. DOI-10.1016/j.febslet.2013.11.024.
38. Hossan, M. I., Chowdhury, A. S., Hossain, M. U., Khan, M. A., Mahmood, T. B., & Mizan, S. (2021). Immunoinformatics aided-design of novel multi-epitope-based peptide vaccine against Hendra henipavirus through proteome exploration. *Informatics in Medicine Unlocked*, *25*, 100678. DOI-10.1016/j.imu.2021.100678.
39. Lari, A., Lari, N., & Biabangard, A. (2022). Immunoinformatics approach to design a novel subunit vaccine against visceral leishmaniasis. *International Journal of Peptide Research and Therapeutics*, *28*, 1-14. DOI-10.1007/s10989-021-10344-3.