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Immunoinformatics Analyses of a Selection of Important *Leishmania major* Vaccine Epitopes: an *in silico* Approach

Hamed Rezayatmand[1], Golnoush Farahzadeh[2], Iraj Alipourfard[3], Arash Rezaeirad[4], Mahsa Alem[5], Sasan Khazaei[6], Davood Siamian[7], Negar Jahani[8], Faezeh Kalantarzadeh[9], Mohammad Arad Zandieh[8], Seyyed Amir Hosseini[10], Saeed Heydarian[10], Hamidreza Majidiani*[11,12], Azar Najafi*[13]

1. Department of Pharmacy and Technology of Organic Substances, Industrial Pharmacy, Ukrainian state, Chemical Technology University, Dnipro, Ukraine. 2. Pharmaceutical chemistry, faculty of chemistry, Azad Tehran north university, Tehran, Iran. 3. Institute of physical chemistry, Polish Academy of Science ،Warsaw, Poland. 4. Institute of science, Molecular Biology, Üsküdar University, Istanbul, Türkiye. 5. Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. 6. Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran. 7. Department of Biology, Faculty of Basic Science, Islamic Azad University, Tonekabon Branch, Mazandaran, Iran. 8. Faculty of Veterinary Medicine, Islamic Azad University, Science and Research Branch, Tehran, Iran. 9. Faculty of Veterinary Medicine, Islamic Azad University, Shushtar Branch, Shushtar, Iran. 10. Department of Internal Medicine, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. 11. Healthy Aging Research Centre, Neyshabur University of Medical Sciences, Neyshabur, Iran. 12. Department of Basic Medical Sciences, Faculty of Medicine, Neyshabur University of Medical Sciences, Neyshabur, Iran. 13. Deputy of Research and Technology, Alborz University of Medical Sciences, Karaj, Iran.

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***Corresponding Authors:**
Hamidreza Majidiani (DVM, PhD);
Department of Basic Medical Sciences, Faculty of Medicine, Neyshabur University of Medical Sciences, Neyshabur, Iran.
Email: majidianih1@nums.ac.ir
Tel/Fax: (+98) 5143306391

Azar Najafi;
Deputy of Research and Technology, Alborz University of Medical Sciences, Karaj, Iran.
Email: azarnajafi5772@gmail.com
Tel/Fax: (+98) 2145987237

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ABSTRACT

Introduction: Cutaneous leishmaniasis (CL) due to *Leishmania major* (*L. major*) is a widespread vector-borne parasitic infection in subtropical areas. Numerous studies have been conducted to present possible vaccination candidates to address CL. In the present study, 18 *L. major* vaccine candidate antigens, namely gp46, CatL, CatB, grp78, H1, H2A, H2B, and H4, HSP60, HSP70, HSP83 (HSP90), HSP100, rP0, KMP11, STI-1, TSA, LeIF, and LACK were evaluated by *in silico* methods to find novel immunogenic epitopes. **Methods:** online predictions were performed regarding physicochemical, solubility, antigenicity, allergenicity, signal peptide, and transmembrane domains. Since four proteins (*i.e.*, CatB, CatL, gp46, and grp78) were shown to possess signal peptides and transmembrane domains, they were further analyzed along with two antigenic proteins (STI-1 and H2A), regarding post-translational modifications (PTMs), structural (secondary and tertiary) predictions, and epitope mapping for B-cells, cytotoxic T-lymphocyte (CTL) and helper T-lymphocyte (HTL) epitopes against human leukocyte antigen (HLA) reference sets. **Results:** The world coverage of the CTL and HTL allele-epitope compositions were 96.34% and 41.78%, respectively. Finally, potentially-immunogenic CTL (n = 8) and antigenic HTL (n = 8) epitopes, which were strong IFN-γ inducers, along with 6 B-cell epitopes were selected. **Conclusion:** These epitopes are potential immunodominant regions among these antigens that upon further evaluations could be considered for a multi-epitope vaccine construction against CL.

INTRODUCTION

Cutaneous leishmaniasis (CL) is a prevalent vector-borne parasitic infection in the Middle East, North Africa, and South America, which in across the Old World is mostly caused by *Leishmania major* and *L. tropica* [1]. The necessity for the development of novel, effective vaccination candidates exist more than ever since the control methods, such as control over the reservoirs (*i.e.*, people, rodents, or sandfly vectors) and the use of harmful treatments have proven to be ineffective [2]. Several *L. major* antigens have been presented and employed in immunization trials against CL in the past. Among these, few have shown promising preventive effects; namely, membrane glycoprotein 46 (gp46), cathepsin L-like cysteine protease (CatL), cathepsin B-like cysteine protease (CatB), glucose-regulated protein 78 (grp78), histone proteins (H1, H2A, H2B,

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and H4), heat shock proteins (HSPs; HSP60, HSP70, HSP83 (HSP90), HSP100), ribosomal protein P0 (rP0), kinetoplast membrane protein 11 (KMP11), stress-inducible protein-1 (STI-1), thiol-specific antioxidant (TSA), *Leishmania* elongation initiation factor (LeIF), and *Leishmania* activated C-kinase antigen (LACK) [3]. Therefore, the present investigation was performed to accurately determine the bioinformatics features (antigenicity, allergenicity, physico-chemical properties, post-translational modification (PTM) sites, signal peptides, transmembrane domains, subcellular localization and structural analysis) of multiple antigenic *L. major* proteins (*i.e.*, gp46, CatL, CatB, grp78, H1, H2A, H2B, and H4, HSP60, HSP70, HSP83 (HSP90), HSP100, rP0, KMP11, STI-1, TSA, LeIF, and LACK) to predict the cytotoxic T-lymphocyte (CTL) and helper T-lymphocyte (HTL) epitopes of these proteins through comprehensive immunoinformatics approaches.

MATERIALS AND METHODS

Overview

A schematic representation of the present study has been illustrated in Fig. 1.

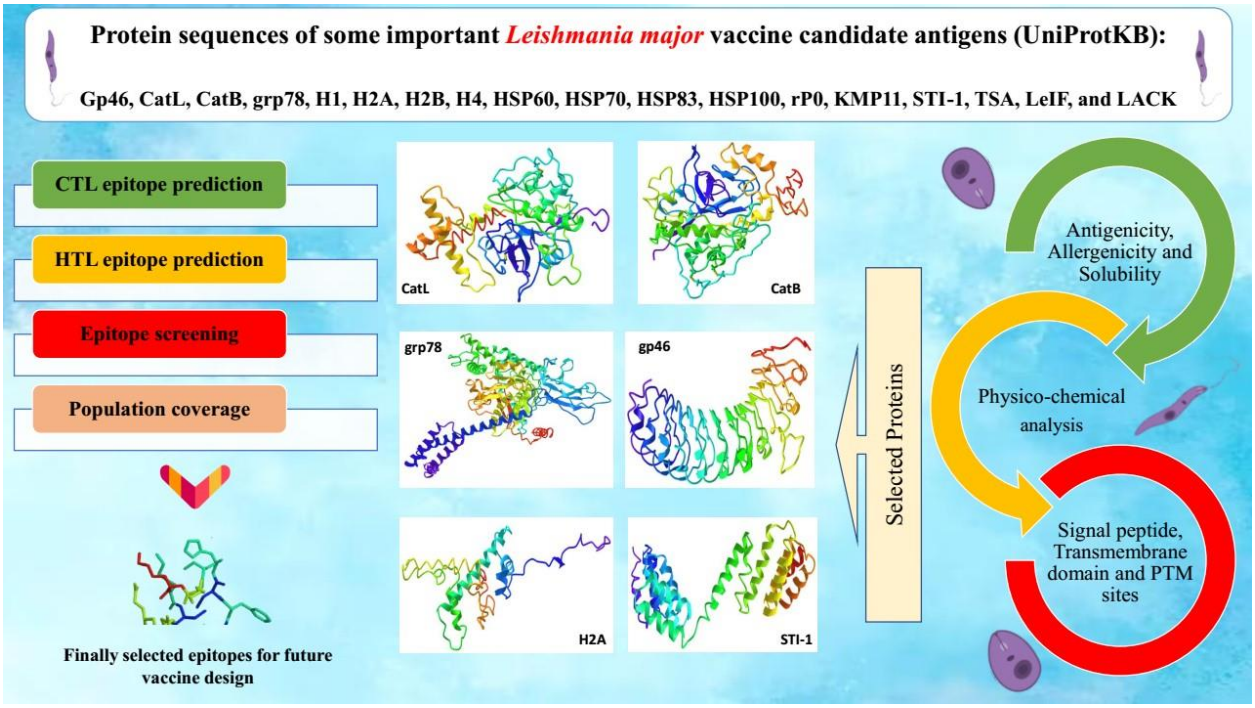

Fig. 1. Schematic representation of *in silico* analyses performed on 18 *L. major* vaccine candidate antigens with subsequent epitope mapping for the selected proteins.

Amino Acid Sequence Retrieval

The amino acid sequences of 18 selected *L. major* proteins were retrieved through a leading high quality, comprehensive and freely-accessible resource of protein sequences and functional information, UniProt Knowledge Base [4], available at https://www.uniprot.org/, with the following accession numbers: Q4Q6B6 (gp46), P90627 (CatB), Q4QI62 (CatL), Q4Q8E6 (grp78), Q9TVI8 (H1), Q4QEG6 (H2A), Q4Q8P9 (H2B), Q4QFI3 (H4), Q94596 (HSP60), P14834 (HSP70), Q4Q4I6 (HSP83), Q25317 (HSP100), A0A8J9XFU7 (KMP-11), Q25306 (LACK), W5XL77 (LeIF), E9ADB9 (rP0), A9LJZ6 (TSA) and Q4Q271 (STI-1).

Forecasting Antigenic, Allergenic, Solubility and Physicochemical Characteristics of the Proteins

Some of the preliminary physico-chemical properties of the proteins were predicted using the ExPASy ProtParam web tool (https://web.expasy.org/protparam/) [5]. The protein solubility was evaluated using the Protein-Sol web tool, developed by the University of Manchester (https://protein-sol.manchester.ac.uk/) [6]. The antigenicity of the proteins was demonstrated by using the VaxiJen v2.0 web server, available at http://www.ddg pharmfac.net/vaxijen/VaxiJen/VaxiJen.html [7]. Finally, the allergenicity of the protein was determined using Multiple Em for Motif Elicitation (MEME)/ Motif Alignment and Search Tool (MAST) approach of the AlgPred online server (https://webs.iiitd.edu.in/raghava/algpred/) [8].

Signal Peptide, Transmembrane Domain, Subcellular Localization and Post-Translational Modification (PTM) Sites Prediction

A number of PTM sites were predicted, including palmitoylation [9], phosphorylation [10], O-glycosylation [11] and N-glycosylation [11] as well as lysine acetylation [12]. For this aim, multiple online tools from DTU Health Tech Services (NetPhos 3.1, NetOGlyc 4.0, and NetNGlyc 1.0) (https://services.healthtech.dtu.dk) and the Cuckoo workgroup (CSS-Palm and GPS-Pail 2.0) (http://biocuckoo.org/) were used. Furthermore, regarding prediction of signal peptide, transmembrane domain and subcellular localization of eukaryotic proteins, SingalP-6.0 [13], Deep TMHMM [14] and DeepLoc2.0 [15] online tools, available at https://services.healthtech.dtu.dk, were utilized, respectively.

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Secondary and Tertiary Structure Predictions

The structural analyses of the proteins were initially done using secondary structure prediction by the NetSurfP-3.0 server (https://services.healthtech.dtu.dk/service.php?NetSurfP-3.0) [16]. Subsequently, a fully automated protein homology modeling tool, Iterative Threading ASSEmbly Refinement (I-TASSER), was used to predict the top-five three-dimensional (3D) models of the protein using derived structural templates by multiple threading approach of the Local Meta-Threading Server, LOMETS. (https://zhanggroup.org/I-TASSER/).

Continuous B-cell Epitopes Predictions and Screening

Linear B-cell epitopes for the 6 selected proteins were predicted using the ABCpred (https://webs.iiitd.edu.in/raghava/abcpred/ABC_method.html) and SVMTriP (http://sysbio.unl.edu/SVMTriP/) web servers. Next, those sequences shared between two servers were extracted and further screened in terms of antigenicity, allergenicity, and water solubility, using VaxiJen v2.0, AllergenFP v1.0, and PepCalc (https://pepcalc.com/peptide-solubility-calculator.php) web tools, respectively.

Prediction and Screening of Human HTL and CTL Epitopes

Major histocompatibility complex class-II (MHC-II) binders, the so-called HTL epitopes, were predicted using the MHC-II epitope prediction method of the IEDB web server using the recommended method (http://tools.iedb.org/mhcii/), "Human" as the target host, and the "HLA reference set alleles" option (population coverage over 97%) [17]. The top-ten high-ranked (lower percentile rank) epitopes were then screened regarding antigenicity and interferon gamma (IFN-γ) induction by using VaxiJen v2.0 and IFNepitope (http://crdd.osdd.net/raghava/ifnepitope/) online tools, respectively.

Those 9-10-mer CTL epitopes (MHC-I binders) specific to humans were predicted using the IEDB MHC-I epitope prediction tool, available at http://tools.iedb.org/mhci/, and the IEDB recommended method 2020.09 (NetMHCpan EL 4.1) [17], with the selection of reference HLA allele set [18]. The top-ten high-affinity epitopes having a percentile rank <1 were screened in terms of immunogenicity and IFN-γ induction using the specific tool on the IEDB server (http://tools.iedb.org/immunogenicity/) and the IFNepitope server.

Population Coverage Analysis of the Allele-Epitope Compositions

The predicted MHC-binding epitopes together with the associated HLA alleles (both Class I and Class II), were submitted to the population coverage analysis tool in the IEDB database using the default parameters [19].

RESULTS

Basic Physicochemical Characteristic of the Examined Proteins

The highest number of amino acids and molecular weight (Mw) belonged to HSP100 protein with 867 residues and 96.91 kDa, respectively, whereas KMP-11 had the lowest number of residues (92) and Mw (11.23). The theoretical pI ranged from 5.01 in rP0 to 12.20 in H1 protein. In most of the proteins, including HSP60, 70, and 90, CatL, CatB, grp78, rP0, KMP-11, LeIF, and LACK, the negatively-charged residues (Asp and Glu) were dominant; of note, H1 protein had no negatively charged residues. All of the examined proteins were predicted to possess an estimated half-life of 30 h in mammalian reticulocytes. Regarding the instability index, 7 proteins (H2B, H4, HSP90, KMP-11, STI-1, TSA, and LeIF) were found to be unstable (threshold: >40), among which, H4 had the highest index (76.55) and LeIF was borderline unstable (40.94) while the remaining were stable. Aliphatic indices of the proteins were found mostly in the range of 60-90, while only KMP-11 had a lower index of 33.04. Moreover, all the proteins were shown to be hydrophilic in nature, as substantiated by the GRAVY scores.

Prediction of Antigenicity, Allergenicity and Solubility

All 18 examined *L. major* proteins were found to be antigenic in nature, using the VaxiJen v2.0 server predictions using "Parasite" option and a 0.4 threshold. The highest and lowest antigenic index belonged to H2A (H2A) and grp78 (40.44) proteins, respectively. Allergenicity prediction performed using MEME/MAST motifs and IgE epitopes in the AlgPred server, and the results were shown to be negative for all the proteins with an exception of positive IgE epitopes found in HSP100. Also, solubility prediction by the Protein-Sol server demonstrated that all the proteins, except for CatL, CatB, gp46, TSA, LACK, and LeIF, were soluble in nature (Table 1).

Table 1. Prediction of antigenicity, allergenicity, and solubility of 18 *L. major* vaccine candidate antigens.

No.	Vaccine candidate antigens	Antigenicity score (VaxiJen v2.0)	Allergenicity (AlgPred)		Protein solubility (Protein-Sol)
			IgE epitopes	MEME/MAST motifs	
1.	**Histone H1**	0.5875 (Probable antigen)	-	-	0.905 (Soluble)
2.	**Histone H2A**	0.9951 (Probable antigen)	-	-	0.848 (Soluble)
3.	**Histone H2B**	0.5406 (Probable antigen)	-	-	0.795 (Soluble)
4.	**Histone H4**	0.6962 (Probable antigen)	-	-	0.785 (Soluble)
5.	**HSP60**	0.5073 (Probable antigen)	-	-	0.552 (Soluble)
6.	**HSP70**	0.4918 (Probable antigen)	-	-	0.522 (Soluble)
7.	**HSP83 (HSP90)**	0.5791 (Probable antigen)	-	-	0.583 (Soluble)
8.	**HSP100**	0.4922 (Probable antigen)	+	-	0.476 (Soluble)
9.	**Cathepsin L**	0.6111 (Probable antigen)	-	-	0.224 (Insoluble)
10.	**Cathepsin B**	0.4624 (Probable antigen)	-	-	0.385 (Insoluble)
11.	**Glucose-regulated protein 78 (GRP78)**	0.4044 (Probable antigen)	-	-	0.711 (Soluble)
12.	**Ribosomal protein (P0)**	0.4637 (Probable antigen)	-	-	0.670 (Soluble)

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13.	**Kinetoplast membrane protein 11 (KMP-11)**	0.6018 (Probable antigen)	-	-	0.735 (Soluble)
14.	**Membrane glycoprotein 46 (gp46)**	0.6426 (Probable antigen)	-	-	0.284 (Insoluble)
15.	**Stress-inducible-1 (STI-1)**	0.6438 (Probable antigen)	-	-	0.527 (Soluble)
16.	**Thio-specific antioxidant (TSA)**	0.4776(Probable antigen)	-	-	0.448 (Insoluble)
17.	***Leishmania* elongation initiation factor (LeIF)**	0.4566 (Probable antigen)	-	-	0.334 (Insoluble)
18.	***Leishmania*-activated C-kinase antigen (LACK)**	0.6096 (Probable antigen)	-	-	0.319 (Insoluble)

Forecasting Signal Peptide, Transmembrane Domain, Subcellular Localization and PTM sites

Based on the DeepLoc server, among *L. major* proteins, HSP60 was destined for the mitochondrion, cathepsin enzymes were allocated to the lysosome or vacuole, grp78 was appointed to the endoplasmic reticulum, and gp46 was directed towards extracellular. Moreover, the rest of the proteins were predicted to possess cytoplasmic or nucleus (histone proteins) localization. A putative signal peptide and transmembrane domain were only predicted for CatL, CatB, grp78 and gp46 proteins. Henceforth, the remaining predictions were performed on these 4 proteins in addition to two highly antigenic proteins (H2A and STI-1) (data not shown). The N-glycosylation sites were absent in H2A and STI-1 proteins, while no O-glycosylation was predicted for gp46. The highest number of N- and O-glycosylation regions were found in CatL and H2A, respectively. No palmitoylation sites were predicted for H2A, grp78 and gp46. Moreover, the H2A protein was highly lysine-acetylated (52 sites) and the highest phosphorylation sites, based on the amino acid sequence length, were found in the gp46 protein, with 18 serine, 18 tyrosine, and 6 threonine sites (data not shown).

Structural Analysis of the Selected Proteins

Based on the results from the NetSurfP secondary structure analysis tool coils were the predominant secondary structure, constituent in 4 proteins (CatL, CatB, gp46, and H2A), whereas helices were frequently found in the STI-1 protein. Furthermore, a significant proportion of the grp78 protein secondary structure was formed by extended strands and helices. In addition, H2A possessed a significant proportion of disordered residues, while disordered regions were only found at the N-termini of CatL, CatB, and gp46 as well as the N-terminus and C-terminus of grp78. More details of the secondary structure prediction are provided in Fig. 2. Based on the I-TASSER predictions, those models with C-scores of -0.34 (CatB), -0.68 (CatL), 0.47 (gp46), -0.05 (grp78), -2.47 (H2A), and 0.13 (STI-1) were finally selected (Fig. 3).



Fig. 2. Secondary structure prediction for 6 selected *L. major* vaccine candidate antigens (CatL, CatB, grp78, gp46, H2A and STI-1) using NetSurfP web server.

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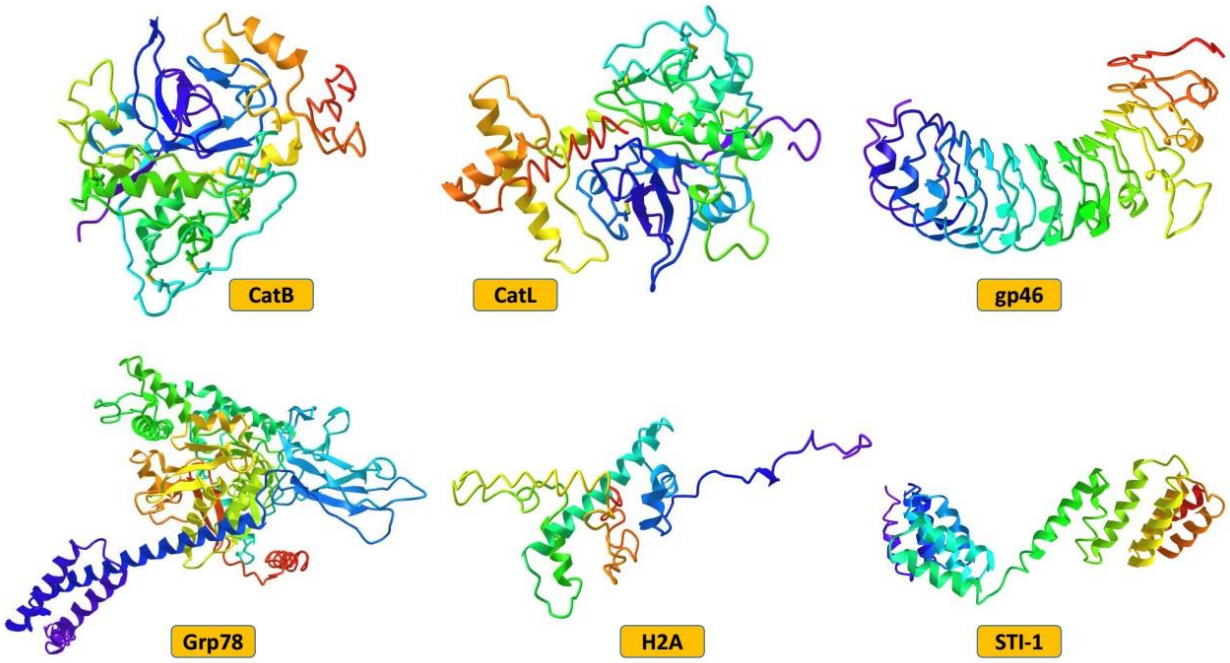

Fig. 3. The homology-modeling of tertiary structures of 6 selected *L. major* vaccine candidate antigens (CatL, CatB, grp78, gp46, H2A and STI-1) using I-TASSER server. Protein visualization was done using "iCn3D" as a web-based 3D structure viewer.

Prediction and Screening of Linear B-cell Epitopes

Out of the 6 selected *L. major* vaccine candidate antigens (H2A, CatL, CatB, grp78, gp46, and STI-1), several continuous B-cell epitopes were predicted using ABCpred and SVMTriP web servers, among which the most antigenic, water-soluble, and allergenic peptide was selected for each protein, including "KTGGKAGRRD" (H2A), "VSMESSERVMTAWLAK" (CatL), "SYSVKGEKELMI" (CatB), "TKDSGKIAGL" (grp78), "TSKGVNLYLDER" (gp46), and "AEFYTRAIELQTE" (STI-1) (Table 2).

Table 2. Final screening of the shared continuous B-cell epitopes of 6 important *L. major* vaccine candidate antigens, predicted using ABCpred and SVMTriP web servers.

Bradyzoite Marker	Epitope	VaxiJen score	Allergenicity	Water Solubility
H2A	HSRLKDGLYRKQ	1.0150	Yes	Good
	RHLLLAIRGDEELNQ	0.9478	No	Good
	GVVPNVHKALEKK	0.2412	No	Good
	RCGASAAIYCAALLE	0.8542	No	Poor
	SATADQTSIVSGGK	1.1331	No	Good
	KTGGKAGRRD*	1.3586	No	Good
	GRRDRMSRAA	0.8126	Yes	Good
	IELSGAAAKAQKT	0.7564	No	Good
Cathepsin L	SSFMSYHSGVLT	0.3275	Yes	Poor
	LSEAEFAARYLN	0.6792	Yes	Good
	VSMESSERVMTAWLAK*	0.6079	No	Good
	YVRVTMGVNA	1.2185	Yes	Poor
	SRAALCAVAVVCV	-0.1185	No	Poor

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	VGYNMTGEVPYW	0.1155	No	Poor
	QRLANFERNL	0.5541	Yes	Good
	RADLSAVPDAVDWR	0.0783	No	Good
	NIESQWAVAGHKL	0.3996	No	Poor
	GGGLMLQA	0.4984	Yes	Poor
Cathepsin B	LSCCFICGL	1.2012	No	Poor
	MGVTDMSTEAV	0.0464	Yes	Good
	SYSVKGEKELMI*	0.9566	No	Good
	MQVYSDFVGYKS	0.2441	No	Poor
	AAEHWPMCLTI	0.3439	No	Poor
	PLLGKSFVAE	0.4157	Yes	Good
	WLWWVWVGIATE	1.9580	Yes	Poor
	YFLIQRGNNECKIES	0.4480	No	Good
GRP78	TIYAIKRLIGRK	-0.5032	No	Good
	SLLTIDEGFFEVVA	-0.5871	No	poor
	QIEVAFDVDENS	1.0777	No	Good
	MTKDNRLLGKFD	0.7903	No	Good
	LVEGYDFSEKITRA	0.2120	No	Good
	NVDISNDQKALARLRK	0.5831	No	Good
	EDAKLKKSDIDEI	-0.1622	No	Good
	TKDSGKIAGL*	1.4181	No	Good
Gp46	HTFLYGIRVDD	1.3659	Yes	Good
	TFLYGIRVDDSLLAA	1.0315	No	Poor
	TSKGVNLYLDER*	0.8497	No	Good
	DTVGKYVLVTS	-0.0654	Yes	Poor
	PTWGSLRKVSSIT	-0.6618	No	Poor
	TGTIPEAWSSLR	0.1750	No	Poor
STI-1	WLKGYFRLGVAMESM	1.0019	No	Poor
	HMYSLMVDDCNA	0.8939	Yes	Poor
	NGEASGALYS	1.8326	No	Poor
	AEFYTRAIELQTE*	1.4402	No	Good
	EEVMDKLHAINTKVR	-0.1901	No	Good
	GIAYEGMEKWKLA	-0.4108	Yes	Good

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	SWQNLNNFEKAAAD	-0.7689	No	Good
	GVAGASQGILRCQ	0.9527	No	Poor
	DYKAKGNDAFKAKRYQ	1.2638	No	Good

* Potent shared, antigenic and non-allergenic linear B-cell epitopes with good water solubility.

Prediction and Screening of Potent HTL and CTL Epitopes

With the exception of CatL protein, potent antigenic HTL epitopes capable of inducing IFN-γ were predicted in H2A (CAALLEYLTTEVIEL), CatB (LCLVAVFALLLATTV, AVFALLLATTVSGLY, CLVAVFALLLATTVS, LVAVFALLLATTVSG, VAVFALLLATTVSGL), grp78 (AVAYGAAVQAAVLTG, EAVAYGAAVQAAVLT), gp46 (HVEYISLYSNSLTGT, VEYISLYSNSLTGTL, SLTHVEYISLYSNSL) and STI-1 (DWLKGYFRLGVAMES) (Table 3). Among CTL epitopes, 6 potential binders were shown to possess high immunogenicity, encompassing "YLTTEVIEL" (H2A), "SEAEFAARY" (CatL), "STEAVPPRNF" (CatB), "EPTAAAIAY" (grp78), "LTGTIPEAW" (gp46) and "AEFYTRAIEL" (STI-1) (Table 4). Regarding IFN-γ induction, 9 epitopes were eligible, including "RAARAELNF" (H2A), "SEAEFAARY", "SAVPDAVDW" and "SAVGNIESQW" (CatL), "GEKELMIEL" (CatB), "EPTAAAIAY" (Grp78), "LTGPLPEEW" and "RPRAALLAV" (gp46) as well as "EPVKEKAVY" (STI-1).

Table 3. Helper T-lymphocyte specific epitope prediction for 6 selected *L. major* vaccine candidate antigens and subsequent screening regarding antigenicity and IFN-γ induction.

Protein	Allele	Start-End	HTL epitope	Method	Percentile rank	Antigenicity	IFN-γ inducing	
							Result	Score
Histone H2A	HLA-DQA1*05:01/DQB1*02:01	42 - 56	AALLEYLTTEVIELS	Consensus (comb.lib./smm/nn)	0.14	0.1767	Negative	1
	HLA-DQA1*05:01/DQB1*02:01	41 - 55	CAALLEYLTTEVIEL	Consensus (comb.lib./smm/nn)	0.14	0.0573	**Positive**	0.4390
	HLA-DQA1*05:01/DQB1*02:01	43 – 57	ALLEYLTTEVIELSG	Consensus (comb.lib./smm/nn)	0.16	0.1851	Negative	1
	HLA-DQA1*05:01/DQB1*02:01	44 – 58	LLEYLTTEVIELSGA	Consensus (comb.lib./smm/nn)	0.17	0.0644	Negative	1
	HLA-DQA1*05:01/DQB1*02:01	45 – 59	LEYLTTEVIELSGAA	Consensus (comb.lib./smm/nn)	0.21	0.1995	Negative	1
	HLA-DQA1*01:02/DQB1*06:02	30 – 44	KQRCGASAAIYCAAL	Consensus (comb.lib./smm/nn)	0.56	0.8481	Negative	2

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	HLA-DQA1*01:02/DQB1*06:02	31 – 45	QRCGASAAIYCAALL	Consensus (comb.lib./smm/nn)	0.56	0.9368	Negative	2
	HLA-DQA1*01:02/DQB1*06:02	32 – 46	RCGASAAIYCAALLE	Consensus (comb.lib./smm/nn)	0.58	0.8542	Negative	2
	HLA-DPA1*01:03/DPB1*04:01	42 – 56	AALLEYLTTEVIELS	NetMHCIIpan	0.95	0.1767	Negative	1
	HLA-DQA1*01:02/DQB1*06:02	29 - 43	RKQRCGASAAIYCAA	Consensus (comb.lib./smm/nn)	0.98	0.8097	Negative	2
Cathepsin L	HLA-DPA1*01:03/DPB1*04:01	18 – 32	HARFGITKFFDLSEA	NetMHCIIpan	0.07	-0.4792	Negative	-0.3282
	HLA-DPA1*01:03/DPB1*04:01	17 – 31	PHARFGITKFFDLSE	NetMHCIIpan	0.1	-0.4191	Negative	-0.4224
	HLA-DPA1*01:03/DPB1*04:01	19 – 33	ARFGITKFFDLSEAE	NetMHCIIpan	0.12	-0.7247	Negative	-0.5605
	HLA-DPA1*01:03/DPB1*04:01	16 – 30	NPHARFGITKFFDLS	NetMHCIIpan	0.12	-0.1432	Negative	-0.3223
	HLA-DPA1*01:03/DPB1*04:01	15 – 29	RNPHARFGITKFFDL	NetMHCIIpan	0.18	-0.0368	Negative	-0.4443
	HLA-DRB1*13:02	16 – 30	AFEWVLRNMNGTVFT	Consensus (smm/nn/sturniolo)	0.22	0.3137	Negative	1
	HLA-DRB1*13:02	17 - 31	FEWVLRNMNGTVFTE	Consensus (smm/nn/sturniolo)	0.23	0.6973	Negative	1
	HLA-DRB1*13:02	15 – 29	QAFEWVLRNMNGTVF	Consensus (smm/nn/sturniolo)	0.23	0.2437	Negative	1
	HLA-DRB1*13:02	18 – 32	EWVLRNMNGTVFTEK	Consensus (smm/nn/sturniolo)	0.31	0.8513	Negative	1
	HLA-DPA1*01:03/DPB1*04:01	20 - 34	RFGITKFFDLSEAEF	NetMHCIIpan	0.32	-0.7129	Negative	-0.7353
Cathepsin B	HLA-DRB1*01:01	13- 27	AVFALLLATTVSGLY	Consensus (comb.lib./smm/nn)	0.01	0.4028	**Positive**	0.7449

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	HLA-DRB1*01:01	10 – 24	CLVAVFALLLATTVS	Consensus (comb.lib./smm/nn)	0.01	0.3264	**Positive**	0.4844
	HLA-DRB1*01:01	9 – 23	LCLVAVFALLLATTV	Consensus (comb.lib./smm/nn)	0.01	0.1915	**Positive**	0.5971
	HLA-DRB1*01:01	11 – 25	LVAVFALLLATTVSG	Consensus (comb.lib./smm/nn)	0.01	0.7546	**Positive**	0.5059
	HLA-DRB1*01:01	12 – 26	VAVFALLLATTVSGL	Consensus (comb.lib./smm/nn)	0.01	0.4544	**Positive**	0.8028
	HLA-DRB1*07:01	3 – 17	LCLVAVFALLLATTV	Consensus (comb.lib./smm/nn)	0.14	0.1915	**Positive**	0.5971
	HLA-DQA1*03:01/DQB1*03:02	15 – 29	CGSCWAIAAVEAISD	Consensus (comb.lib./smm/nn)	0.16	-0.0601	Negative	1
	HLA-DRB1*01:01	14 – 28	AKSALCLVAVFALLL	Consensus (comb.lib./smm/nn)	0.16	0.1647	Negative	2
	HLA-DRB1*01:01	5 – 19	ALCLVAVFALLLATT	Consensus (comb.lib./smm/nn)	0.16	0.1066	**Positive**	0.4627
	HLA-DQA1*04:01/DQB1*04:02	8 - 22	CGSCWAIAAVEAISD	Consensus (comb.lib./smm/nn)	0.19	-0.0601	Negative	1
Grp78	HLA-DQA1*05:01/DQB1*03:01	38 – 52	AVAYGAAVQAAVLTG	Consensus (comb.lib./smm/nn)	0.09	1.0518	**Positive**	0.6594
	HLA-DQA1*05:01/DQB1*03:01	36 – 50	DEAVAYGAAVQAAVL	Consensus (comb.lib./smm/nn)	0.09	0.8555	Negative	1

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	HLA-DQA1*05:01/DQB1*03:01	37 – 51	EAVAYGAAVQAAVLT	Consensus (comb.lib./smm/nn)	0.09	1.0503	**Positive**	0.6721
	HLA-DQA1*05:01/DQB1*03:01	35 – 49	PDEAVAYGAAVQAAV	Consensus (comb.lib./smm/nn)	0.09	0.8104	Negative	1
	HLA-DQA1*01:02/DQB1*06:02	11 – 25	AVCLVSAILVVSAAA	Consensus (comb.lib./smm/nn)	0.11	0.4557	Negative	2
	HLA-DQA1*01:02/DQB1*06:02	12 – 26	VCLVSAILVVSAAAV	Consensus (comb.lib./smm/nn)	0.11	0.3309	Negative	1
	HLA-DQA1*01:02/DQB1*06:02	13 – 27	CLVSAILVVSAAAVP	Consensus (comb.lib./smm/nn)	0.12	0.3708	Negative	1
	HLA-DQA1*01:02/DQB1*06:02	10 – 24	MAVCLVSAILVVSAA	Consensus (comb.lib./smm/nn)	0.12	0.5555	Negative	2
	HLA-DQA1*01:02/DQB1*06:02	9 – 23	LMAVCLVSAILVVSA	Consensus (comb.lib./smm/nn)	0.14	0.5999	Negative	2
	HLA-DQA1*01:02/DQB1*06:02	25 - 39	INEPTAAAIAYGLNK	Consensus (comb.lib./smm/nn)	0.16	-0.0735	Negative	1
Gp46	HLA-DRB1*01:01	136 – 150	HVEYISLYSNSLTGT	Consensus (comb.lib./smm/nn)	0.1	0.9449	**Positive**	1
	HLA-DRB1*01:01	135 – 149	THVEYISLYSNSLTG	Consensus (comb.lib./smm/nn)	0.1	0.9790	Negative	-0.2514
	HLA-DRB1*01:01	137 - 151	VEYISLYSNSLTGTL	Consensus (comb.lib./smm/nn)	0.1	0.9697	**Positive**	1
	HLA-DRB1*04:05	134-148	LTHVEYISLYSNSLT	Consensus (smm/nn/sturniolo)	0.12	0.5979	Negative	-0.0820
	HLA-DRB1*04:05	135 – 149	THVEYISLYSNSLTG	Consensus (smm/nn/sturniolo)	0.15	0.9790	Negative	-0.02514
	HLA-DRB1*01:01	134 – 148	LTHVEYISLYSNSLT	Consensus (comb.lib./smm/nn)	0.16	0.5979	Negative	-0.0820
	HLA-DRB1*01:01	133 – 147	SLTHVEYISLYSNSL	Consensus (comb.lib./smm/nn)	0.16	0.4518	**Positive**	0.0511
	HLA-DRB1*04:05	136 – 150	HVEYISLYSNSLTGT	Consensus (smm/nn/sturniolo)	0.21	0.9449	**Positive**	1

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	HLA-DRB1*09:01	152 – 166	PPEWAKMKSAKWFLL	Consensus (comb.lib./smm/nn)	0.3	0.4666	Negative	-0.2440
	HLA-DRB1*04:05	133 - 147	SLTHVEYISLYSNSL	Consensus (smm/nn/sturniolo)	0.32	0.4518	Negative	-0.1187
STI-1	HLA-DPA1*02:01/DPB1*14:01	13 – 27	KGYFRLGVAMESMVK	NetMHCIIpan	0.02	0.5969	Negative	-0.5074
	HLA-DPA1*02:01/DPB1*14:01	14 – 28	GYFRLGVAMESMVKY	NetMHCIIpan	0.03	0.4143	Negative	-0.3416
	HLA-DPA1*02:01/DPB1*14:01	12 – 26	LKGYFRLGVAMESMV	NetMHCIIpan	0.03	0.9526	Negative	-0.3270
	HLA-DPA1*02:01/DPB1*14:01	15 – 29	YFRLGVAMESMVKYD	NetMHCIIpan	0.07	0.5341	Negative	-0.2952
	HLA-DPA1*02:01/DPB1*14:01	11 – 25	WLKGYFRLGVAMESM	NetMHCIIpan	0.1	1.0019	Negative	-0.3327
	HLA-DPA1*02:01/DPB1*14:01	10 – 24	DWLKGYFRLGVAMES	NetMHCIIpan	0.73	0.8179	**Positive**	0.0948
	HLA-DRB1*04:05	10 – 24	DWLKGYFRLGVAMES	Consensus (smm/nn/sturniolo)	0.86	0.8179	**Positive**	0.0948
	HLA-DRB1*04:05	13- 27	KGYFRLGVAMESMVK	Consensus (smm/nn/sturniolo)	0.86	0.5969	Negative	-0.5076
	HLA-DRB1*04:05	12 – 26	LKGYFRLGVAMESMV	Consensus (smm/nn/sturniolo)	0.86	0.9526	Negative	-0.3279
	HLA-DRB1*04:05	11 - 25	WLKGYFRLGVAMESM	Consensus (smm/nn/sturniolo)	0.86	1.0019	Negative	-0.4636

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Table 4. Cytotoxic T-lymphocyte specific epitope prediction for 6 selected *L. major* vaccine candidate antigens and subsequent screening regarding immunogenicity and IFN-γ induction.

Protein name	MHC-I allele	Start	End	Length	Peptide	Percentile rank	Immunogenicity	IFN-γ production
Histone H2A	HLA-B*07:02	9	17	9	KPRHLLLAI	0.02	0.04161	Negative
	HLA-A*68:01	25	33	9	QVVKATISR	0.02	-0.1133	Negative
	HLA-A*02:01	47	55	9	YLTTEVIEL	0.02	0.35884	Negative
	HLA-A*03:01	22	30	9	RLKDGLYRK	0.01	0.002	Negative
	HLA-A*02:06	47	55	9	YLTTEVIEL	0.04	0.35884	Negative
	HLA-B*08:01	7	15	9	RIKPRHLLL	0.02	-0.01615	Negative
	HLA-A*02:01	43	52	10	ALLEYLTTEV	0.04	0.21055	Negative
	HLA-A*68:01	24	33	10	NQVVKATISR	0.11	-0.06476	Negative
	HLA-B*58:01	6	14	9	RAARAELNF	0.09	0.18399	**Positive**
	HLA-A*31:01	47	55	9	KSKKKSAKR	0.04	-0.74571	Negative
Cathepsin L	HLA-B*44:03	30	38	9	SEAEFAARY	0.01	0.32754	**Positive**
	HLA-B*44:02	30	38	9	SEAEFAARY	0.01	0.32754	**Positive**
	HLA-A*24:02	37	45	9	RYLNGAAYF	0.01	0.09058	Negative
	HLA-A*23:01	37	45	9	RYLNGAAYF	0.01	0.09058	Negative

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	HLA-B*58:01	6	14	9	SSERVMTAW	0.03	0.0151	Negative
	HLA-B*57:01	6	14	9	SSERVMTAW	0.06	0.0151	Negative
	HLA-A*02:01	30	38	9	LLTGYPVSV	0.03	-0.02916	Negative
	HLA-A*01:01	24	33	10	AVDASSFMSY	0.02	-0.41029	Negative
	HLA-B*58:01	4	12	9	SAVPDAVDW	0.05	0.10847	**Positive**
	HLA-B*57:01	34	43	10	SAVGNIESQW	0.09	0.05775	**Positive**
Cathepsin B	HLA-B*40:01	2	10	9	GEKELMIEL	0.01	0.02547	**Positive**
	HLA-B*44:03	21	29	9	TEAVPPRNF	0.01	0.0729	Negative
	HLA-B*44:02	21	29	9	TEAVPPRNF	0.01	0.0729	Negative
	HLA-A*68:01	21	29	9	TTVSGLYAK	0.03	-0.11077	Negative
	HLA-A*11:01	21	29	9	TTVSGLYAK	0.01	-0.11077	Negative
	HLA-A*01:01	8	16	9	NTDWGDKGY	0.02	0.12177	Negative
	HLA-A*24:02	24	32	9	KYPPCPSTI	0.02	-0.19464	Negative
	HLA-B*15:01	49	58	10	LVKYKGSTSY	0.01	-0.47035	Negative
	HLA-B*35:01	25	33	9	YPPCPSTIY	0.03	-0.11386	Negative
	HLA-B*44:02	20	29	10	STEAVPPRNF	0.02	0.13073	Negative

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Grp78	HLA-A*11:01	31	39	9	SVTNPIIQK	0.01	0.16521	Negative
	HLA-B*15:01	38	46	9	KMKEISETF	0.01	0.1118	Negative
	HLA-A*68:01	13	21	9	ETVGGVMTK	0.01	-0.00616	Negative
	HLA-A*68:01	30	38	9	EVSAMVLQK	0.02	-0.22351	Negative
	HLA-A*03:01	31	39	9	SVTNPIIQK	0.01	0.16521	Negative
	HLA-B*35:01	27	35	9	EPTAAAIAY	0.02	0.26208	**Positive**
	HLA-B*40:01	22	31	10	RERVEAKNSL	0.02	-0.11118	Negative
	HLA-B*35:01	44	52	9	QPSVLIQVF	0.02	0.02868	Negative
	HLA-B*57:01	44	52	9	TTYSVAGVW	0.05	-0.03792	Negative
	HLA-A*02:03	21	29	9	KLIERNTQI	0.01	0.15334	Negative
Gp46	HLA-B*58:01	3	11	9	LTGPLPEEW	0.01	0.1216	**Positive**
	HLA-B*58:01	39	47	9	LTGTLPPTW	0.01	0.04214	Negative
	HLA-B*57:01	39	47	9	LTGTLPPTW	0.01	0.04214	Negative
	HLA-B*57:01	3	11	9	LTGPLPEEW	0.01	0.1216	**Positive**
	HLA-B*58:01	27	35	9	LTGTLPPEW	0.01	0.07796	**Positive**
	HLA-B*07:02	13	21	9	RPRAALLAV	0.01	0.09733	**Positive**

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	HLA-B*57:01	27	35	9	LTGTLPPEW	0.01	0.07796	**Positive**
	HLA-B*58:01	15	23	9	LTGTLPSSW	0.01	-0.20746	Negative
	HLA-B*58:01	51	59	9	LTGTIPEAW	0.01	0.27658	Negative
	HLA-B*57:01	15	23	9	LTGTLPSSW	0.02	-0.20746	Negative
STI-1	HLA-B*58:01	34	42	9	IAYEGMEKW	0.01	-0.07425	Negative
	HLA-B*57:01	34	42	9	IAYEGMEKW	0.01	-0.07425	Negative
	HLA-A*68:02	47	55	9	EVMDKLHAI	0.01	-0.20496	Negative
	HLA-B*35:01	20	28	9	VAMESMVKY	0.02	-0.37381	Negative
	HLA-B*35:01	46	54	9	EPVKEKAVY	0.02	-0.25196	**Positive**
	HLA-A*68:01	35	44	10	EASGALYSNR	0.04	-0.13918	Negative
	HLA-A*02:03	51	59	9	KLHAINTKV	0.03	0.08014	Negative
	HLA-B*44:03	15	24	10	EEAKQLGNSF	0.04	-0.39812	Negative
	HLA-B*44:02	15	24	10	EEAKQLGNSF	0.03	-0.39812	Negative
	HLA-B*40:01	34	43	10	AEFYTRAIEL	0.07	0.33013	Negative

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Allele-Epitope Population Coverage Predictions

The findings of the IEDB population coverage analysis tool showed that the CTL allele-epitope compositions predicted for the selected six Leishmania proteins in this study would provide a high coverage for about 96.34% of the global population. This coverage would extend to 109 countries across 16 distinct geographic regions. Among these, the highest CTL epitope coverage belonged to the Europe (98.77%), North America (96.67%), East Asia (95.55%), and West Indies (95.11%). In addition, HTL allele-epitopes compositions demonstrated a relatively low population coverage for the whole world (41.87%); such result was retrieved while some of the HLA alleles were not available by the IEDB coverage analysis tool for the calculation (data not shown).

DISCUSSION

In the present study, the amino acid sequences of 18 *L. major* vaccine candidates were initially obtained from the UniProt database. Antigenicity analysis showed that all proteins were probable antigens (threshold: 0.4), while HSP100 was shown to be allergenic (had IgE epitopes) in nature while other vaccine candidates lacked any IgE epitopes or MEME/MAST motifs. It has been known that the lack of allergenicity is a prominent feature of good vaccine candidates destined to be used in subunit vaccines [20, 21]. Additionally, protein stability and solubility are both important biophysical parameters to consider in the design of rational multi-epitope vaccines [22]. The proteins with the highest solubility (threshold: 0.45) among the 18 examined candidates belonged to H1 (0.905), H2A (0.848), and H2B (0.795), while few proteins, namely CatL (0.224), CatB (0.385), gp46 (0.284), TSA (0.448), LeIF (0.334), and LACK (0.319) were found to be insoluble. Moreover, acceptable stability values were estimated for all proteins, except for seven (H2B, H4, HSP83, KMP-11, STI-1, TSA, and LeIF), which were shown to be unstable. With a negative GRAVY score, all of the examined proteins were more hydrophilic and could interact with a water-based environment. KMP-11 had the highest GRAVY among all, and as a result, the highest hydrophilicity, scoring -1.447. All other *L. major* antigenic compounds showed significant thermostability across a wide temperature range, with the exception of KMP-11. Molecules with Mw greater than 5–10 kDa are strong immunogens and here we observed that all examined vaccine candidates had a suitable Mw and a long half-life (30 h) in mammalian cells. For further purification considerations, all of these factors may aid researchers in evaluating the proteins more accurately.

Among the 18 proteins, only 4 (i.e., CatL, CatB (lysosome/vacuole), grp78 (endoplasmic reticulum) and gp46 (extracellular) were found to possess a putative signal peptide and transmembrane domain, respectively. Since these molecular targets are more accessible to the immune cells, we continued the rest of analyses on these 4 proteins along with the 2 (i.e., H2A and STI-1) highly antigenic ones. The PTMs are a class of biological events that can be either reversible or irreversible. They involve the addition of modifying groups, such as methyl, glycosyl, phosphoryl, acetyl to amino acid residues which can then alter the dynamics and structure of the proteins. As revealed in the current study for CatL, CatB, grp78, and gp46, such events are commonly reported in secretory and/or membrane proteins [23]. Altogether, such modifications would impact the biological processes, including gene expression, cell cycle control and signal transduction [24]. We observed that H2A, gp46, CatB, and CatL proteins had a high prevalence of coils in their secondary structure. Additionally, we predicted helices for STI-1 and grp78 and extended strands for grp78.

Humoral immune responses can play significant role during leishmaniasis. For this aim, we predicted common B-cell-associated epitopes for 6 *L. major* vaccine candidate antigens (i.e., H2A, gp46, CatB, CatL, grp78, and STI-1) using two web servers, comprising “KTGGKAGRRD” (H2A), “VSMESSERVMTAWLAK” (CatL), “SYSVKGEKELMI” (CatB), “TKDSGKIAGL” (grp78), “TSKGVNLYLDER” (gp46), and “AEFYTRAIELQTE” (STI-1). Most CD8+ T cells in the lesion site exhibit cytolytic function, without killing the intracellular parasites [25], promoting inflammation; hence, selection of IFN-γ inducing epitopes from this subset of T cells could be assumed beneficial to combat CL. Given the intracellular nature of Leishmania parasites, the induction of IFN-γ cytokine, whether by CD4+ Th1 and/or CD8+ T-cells, is a pivotal function for the selected epitopes, resulting in macrophage activation and downstream parasite clearance mechanisms [26]. Interestingly, no IFN-γ-inducing HTL epitopes were found among the top-ten HTL epitopes predicted for the CatL protein. Regarding H2A, the only inducer epitope (CAALLEYLTTEVIEL) was not antigenic enough (VaxiJen score: 0.0573), while other proteins showed some antigenic epitopes capable of inducing this important cytokine, encompassing CatB11-25 (LVAVFALLLATTVSG; VaxiJen score: 0.7546), CatB12-26 (VAVFALLLATTVSGL; VaxiJen score: 0.4544), grp7838-52 (AVAYGAAVQAAVLTG; VaxiJen score: 1.0518), grp7837-51 (EAVAYGAAVQAAVLT; VaxiJen score: 1.0518), gp46136-150 (HVEYISLYSNSLTGT; VaxiJen score: 0.9449), gp46137-151 (VEYISLYSNSLTGTL; VaxiJen score: 0.9697), gp46133-147 (SLTHVEYISLYSNSL; VaxiJen score: 0.4518) and STI-110-24 (DWLKGYFRLGVAMES; VaxiJen score: 0.8179). The top-ten human CTL epitopes (predicted using the IEDB HLA reference set covering over 97% of the global population), were further screened in terms of immunogenicity and the ability to induce IFN-γ cytokine. With the exception for STI-146-54 IFN-γ-inducing epitope (EPVKEKAVY; immunogenicity: -0.25196) that was not shown to possess adequate immunogenicity, at least one potent immunogenic IFN-γ-inducing epitope was predicted regarding other examined proteins, including H2A6-14 (RAARAELNF; immunogenicity: 0.18399), CatL30-38 (SEAEFAARY; immunogenicity: 0.32754), CatL4-12 (SAVPDAVDW; immunogenicity: 0.10847) and CatL34-43 (SAVGNIESQW; immunogenicity: 0.05775), CatB2-10 (GEKELMIEL; immunogenicity: 0.02547), grp7827-35 (EPTAAAIAY; immunogenicity: 0.26208), gp463-11 (LTGPLPEEW; immunogenicity: 0.1216), gp4627-35 (LTGTLPPEW; immunogenicity: 0.07796), and gp4613-21 (RPRAALLAV; immunogenicity: 0.09733). An estimated CTL epitope world coverage of 96.34%, based on the population coverage analysis of the IEDB server was obtained. Also, a high coverage percentage was estimated for the areas endemic for leishmaniasis, such as Southwest Asia, South America and West Africa. Meanwhile, the global HTL epitope-HLA allele coverage was relatively low (41.87%).

In conclusion, the strength of this study was the selection of a wide range of *L. major* vaccine candidate antigens (n = 18) and immunoinformatics analyses using a variety of computer-based methods as the basic step for epitope selection. Among these, the gp46 protein had the highest CTL (n=3) and HTL (n=3) epitopes, which can be emphasized in both subunit and multi-epitope

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vaccines against CL. Finally, 8 CTL and 8 HTL epitopes capable of eliciting IFN-γ cytokine, which were shown to be highly immunogenic or antigenic, respectively, predicted for human HLA reference alleles, along with six antigenic B-cell epitopes introduced in the current study could be further utilized in the experimental vaccinology research against CL. The strength of this study was the selection of a wide range of *L. major* vaccine candidate antigens (n = 18) and immunoinformatics analyses using a variety of computer-based methods as the basic step for epitope selection. Evidently, the only goal of the current study was the selection of those potent antigens and their immunodominant regions that may be eligible for a multi-epitope vaccine design. The design and construction of a multi-epitope vaccine based on identified antigens and epitopes, along with the analysis of its potential interactions with related immune components such as TLRs, represent promising avenues for future exploration.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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