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Review Article

Recent updates and perspectives on approaches for the development of vaccines against visceral leishmaniasis

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Abstract

Visceral leishmaniasis (VL) is one of the most important tropical diseases worldwide. Although chemotherapy has been widely used to treat this disease, problems related to the development of parasite resistance and side effects associated with the compounds used have been noted. Hence, alternative approaches for VL control are desirable. Some methods, such as vector control and culling of infected dogs, are insufficiently effective, with the latter not ethically recommended. The development of vaccines to prevent VL is a feasible and desirable measure for disease control; for example, some vaccines designed to protect dogs against VL have recently been brought to market. These vaccines are based on the combination of parasite fractions or recombinant proteins with adjuvants that are able to induce cellular immune responses; however, their partial efficacy and the absence of a vaccine to protect against human leishmaniasis underline the need for characterization of new vaccine candidates. This review presents recent advances in control measures for VL based on vaccine development, describing extensively studied antigens, as well as new antigenic proteins recently identified using immuno-proteomic techniques.

Keywords: Vaccine. Visceral leishmaniasis. Recombinant proteins. Immuno-proteomic approach. Hypothetical proteins.

INTRODUCTION

Leishmaniasis is a disease complex caused by different species of protozoan parasites of the genus *Leishmania*[1]. The disease causes high levels of morbidity and mortality worldwide, where approximately 1-1.5 million cases of tegumentary leishmaniasis (TL) and 0.2-0.5 million cases of visceral leishmaniasis (VL) are registered annually[2]. VL is caused by parasites of the *Leishmania donovani* complex, including the species *L. donovani* and *Leishmania infantum*[3]. In the Americas, VL is a zoonotic disease caused by *L. infantum*, where dogs are considered the main domestic reservoirs of the parasites[4] [5]. In human VL, the outcomes of infection can vary from an asymptomatic and/or subclinical disease to a form with acute symptoms; the disease carries a high risk of mortality in the absence of an adequate treatment[6] [7].

Chemotherapy based on the administration of pentavalent antimonials has been used to treat VL; however, these products present problems related to their toxicity[8] [9] [10]. Other drugs, such as pentamidine, miltefosine, and amphotericin B also present issues of toxicity and/or high cost[11] [12] [13] [14]. Early diagnosis of VL could allow for more effective treatment of the disease; however, parasitological diagnosis, based on the direct observation of amastigote forms has low sensitivity and requires invasive collection procedures[15]. The detection of *Leishmania* deoxyribonucleic acid (DNA) using the polymerase chain reaction (PCR) technique is highly specific; however, its sensitivity is variable[16] [17]. Serological tests based on the detection of antileishmanial antibodies in patient serum samples are also employed for the diagnosis of VL; however, these are also associated with issues related to sensitivity and/or specificity, depending on the antigens targeted[18] [19].

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Evidence of life-long immunity to leishmaniasis has also inspired the development of prophylactic vaccination protocols against the disease, although few have progressed beyond the experimental stage[14]. An ideal vaccine candidate against leishmaniasis should be safe, affordable to the population, and able to induce both cluster of differentiation 4^+ ($CD4^+$) and cluster of differentiation 8^+ ($CD8^+$) T cell responses and long-term immunological memory, which could be boosted by natural infections, thus reducing the number of vaccine doses required. In addition, an ideal vaccine should be effective against different *Leishmania* species and stable at room temperature or at 4°C, to eliminate the need for storage at -20°C or -80°C[20]. However, the induction and maintenance of long-lasting immunity and protection against different *Leishmania* species are very difficult to achieve, since the majority of candidate vaccines are composed of antigens that only offer species-specific protection[21] [22] [23] [24] [25] [26].

Protective immunity against VL is based on the development of an antigen- and parasite-specific T-helper 1 (Th1)-type cellular response, primed by the production of interferon-gamma (IFN-γ), interleukin-2 (IL-2), interleukin-12 (IL-12), granulocyte macrophage colony-stimulating factor (GM-CSF), and other cytokines[27] [28] [29] [30] [31] [32]. The induction of $CD4^+$ Th1 cell responses against parasite antigens is crucial in controlling primary infection, when cytokines such as IFN-γ induce nitric oxide production by activated phagocytic cells able to kill internalized parasites[33] [34]. Concomitantly to the role of $CD4^+$ T cells, $CD8^+$ T cells also contribute to protection against disease, and have an important role in controlling primary infections by increasing the Th1 response through a mechanism dependent on IFN-γ production[35]. In contrast, cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-13 (IL-13), interleukin-18 (IL-18), and transforming growth factor beta (TGF-β) represent disease promoting molecules which inhibit the Th1 response, contributing to the deactivation of infected macrophages and, consequently, to the development of disease[36] [37] [38].

In recent years, proteomic screening studies have revealed a number of antigenic proteins specific to the *Leishmania* genus and frequently annotated as hypothetical proteins in genome databases. This review explores recent developments and discusses the prospects for vaccine development against VL, focusing on well-known antigens described in the literature, as well as the discovery of new antigens by immuno-proteomic approaches.

SECOND-GENERATION VACCINES AGAINST VISCERAL LEISHMANIASIS

Advances in recombinant DNA technology have led to the extensive study of several species- or stage-specific *Leishmania* molecules as candidate vaccines in the form of recombinant proteins. The major advantages of these candidates are their purity and the production yields achievable. Several proteins have been frequently investigated as candidate vaccines for the cutaneous form of leishmaniasis; however, few of these have been evaluated in mammalian VL models[39]. Recombinant proteins have been evaluated as second-generation vaccines for VL with variable degrees of success, usually depending on the vaccine formulation and associated immune adjuvants, as well as the animal model used for testing. Amastigote and promastigote parasite antigens are the most common vaccine candidates tested.

Among amastigote-specific antigens tested for induction of immune protection against VL, the A2 antigen has emerged as an effective candidate. It is encoded by a multigene family that is abundantly expressed in the amastigote forms of some *Leishmania* species able to cause VL[40]. Studies of the administration of recombinant A2 protein associated with immune adjuvants[41] [42] or as a DNA vaccine[43], as well as in attenuated non-replicative viruses[44], non-pathogenic bacteria[45], or non-virulent *Leishmania tarentolae*[46], have provided evidence of its protective efficacy in mammalian models. In general, anti-A2 protective immunity is associated with the generation of parasite-specific IgG2a antibodies, as well as with the production of high levels of antileishmanial IFN-γ and low levels of IL-10 by T cells in recall response to the A2 protein or parasite extracts[40]. Other amastigote-specific antigens that have been considered promising candidates for VL prevention include the cysteine proteinases (CP). These enzymes belong to the papain super-family, three classes of which (CPA, CPB, and CPC) have been identified in *Leishmania* parasites. Studies have shown that recombinant CPB protein, in combination with an immune adjuvant or as a DNA vaccine, induced protection against *Leishmania major* infection in BALB/c mice[47]. In another study, recombinant CPA/CPB polyprotein vaccine was administered in association with poloxamer 407 as an adjuvant, and induced a protective response against *L. major* in BALB/c mice, which was more robust than the response induced by recombinant CPA and CPB proteins administered as separate individual antigens[48]; however, these antigens were not tested as vaccines against VL.

In an evaluation of antigens expressed in promastigote forms of *Leishmania* parasites as vaccine candidates against VL, parasite surface antigen-2 (PSA-2), which comprises three polypeptides with molecular weights ranging from 50.0 to 96kDa[49], showed satisfactory results. This immunogen was able to induce protection against a *Leishmania* challenge in mice, through the development of a Th1-type response, when administered associated with *Corynebacterium parvum* as an adjuvant[50]. Kinetoplastid membrane protein-11 (KMP-11), a highly conserved protein expressed in different *Leishmania* species, was also verified as protective against *L. donovani* infection in hamsters[51] [52]. In addition, the nucleoside hydrolase 36kDa (NH36) antigen was shown to be protective against *Leishmania infantum, Leishmania mexicana,* and *Leishmania amazonensis* species in BALB/c mice, indicating its potential as a heterologous vaccine to protect against different *Leishmania* species[53] [54].

It has been postulated that a formulation containing different *Leishmania* proteins expressed in both parasite stages should provide better results, in terms of a more effective and protective vaccine against VL[42] [55]. The use of vaccines combining different proteins could provide the benefits of increased simplicity and reduced production costs, since it would only be necessary to produce a single vaccine to protect against different *Leishmania* species[56]. However, few studies have evaluated chimeric

vaccines aimed at protection against VL[25][57], since the majority of reports have been of investigations of single antigens[14][58][59][60].

The development of a multi-antigenic vaccine requires an appropriate choice of the biological targets for use in its composition. In a recent study, a polyprotein vaccine formulated with monophosphoryl lipid A, KSAC, was shown to be immunogenic and effective in inducing protection against *L. infantum* and *L. major* in mice. KSAC is a chimeric protein composed of the *Leishmania* homolog of the receptor for activated C kinase (LACK), glycoprotein 63 kDa (gp63), thiol-specific-antioxidant (TSA), hydrophilic acylated surface protein B (HASPB), sterol 24-*c*-methyltransferase (SMT), KMP-11, A2, and CPB proteins. In models challenged with both *Leishmania* species, the protective response was associated with the production of high levels of IFN-γ, combined with low levels of IL-4 and a decreased antileishmanial IgG1 response[61]. Another chimeric protein, Leish-111f, which is composed of a combination of TSA, stress inducible protein 1 (LmSTI-1), and the *Leishmania* homolog of the eukaryotic translation initiation factor (eIF4A), was also able to protect BALB/c mice against *Leishmania* infection, when administered in association with immune adjuvants[62].

Another field that could be developed in relation to the discovery of new candidate VL vaccines is based on vector salivary proteins. To date, evidence indicates that salivary molecules able to induce a Th1-type response in immunized animals could create a protective immunological environment at the bite site, which could influence when parasites are injected, allowing control of the disease and concomitant promotion of *Leishmania*-specific immunity[63]. The Th1-type immunological environment in response to these antigens at the bite site could promote a protective immune response against the parasite challenge. In this context, PdSP15, a 15-kDa salivary protein, which is a member of the family of small odorant binding proteins from *Phlebotomus duboscqi*, was evaluated as a candidate antigen against leishmaniasis in non-human primates[64]. In addition, LJM19, an 11-kDa salivary protein of unknown function and LJL143, a 38-kDa salivary protein with anticoagulant activity[65], both of which are present in the saliva of *Lutzomyia longipalpis*, were shown to be protective against VL[66]. **Table 1**

TABLE 1
Summary of vaccines against visceral leishmaniasis based on individual recombinant proteins or polyproteins.

Protein	Infective species	Model	Remarks	Reference
A2	*L. infantum*	Beagle dog	Partial protection	Fernandes et al[40]
Aldolase	*L. donovani*	Hamster	Partial protection	Gupta et al[67]
Cysteine-peptidases	*L. infantum*	Beagle dog	No protection	Poot et al[68]
Cysteine-proteinase III	*L. infantum*	BALB/c mice	Partial protection	Khoshgoo et al[69]
Cyclophilin 1	*L. infantum*	BALB/c mice	High protection	Santos-Gomes[70]
dp72	*L. infantum*	BALB/c mice	Partial protection	Jaffe et al[71]
eIF2	*L. donovani*	Hamster	65% protection	Kushawcha[72]
HASPB1	*L. donovani*	BALB/c mice	70%–90% protection	Stager et al[26]
LCR1	*L. infantum*	BALB/c mice	Partial protection	Wilson et al[73]
LdSir2HP	*L. donovani*	Hamster	High protection	Baharia et al[60]
LeishH1	*L. infantum*	BALB/c mice	High protection	Agallou et al[27]
L3/L5	*L. infantum*	BALB/c mice	High protection	Ramírez et al[74]
NH36	*L. infantum*	BALB/c mice	80% protection	Aguilar-Be et al[54]
ORFF	*L. donovani*	BALB/c mice	Partial protection	Tewary et al[75]
78 kDa protein	*L. donovani*	BALB/c mice	High protection	Joshi & Kaur[59]
A2/CPA/CPB*	*L. infantum*	BALB/c mice	High protection	Saljoughian et al[57]
KSAC*	*L. infantum*	C57BL/6 mice	High protection	Goto et al[61]
Leish-111f*	*L. infantum*	Beagle dog	No protection	Gradoni et al[76]
NS protein*	*L. donovani*	BALB/c mice	High protection	Coler et al[77]
Q protein*	*L. infantum*	Beagle dog	90% protection	Molano et al[78]
8E/p21/SMT*	*L. donovani*	C57BL/6 mice	High protection	Duthie et al[79]

dp72: 72 kDa *L. donovani* **protein**; **eIF2**: eukaryotic initiation factor-2; **HASPB1**: hydrophilic acylated surface protein B; **LCR1**: complete conservation of an immunogenic gene; **LdSir2HP**: NAD^+-dependent silent information regulatory-2 (SIR2 family or sirtuin) protein; **LeishH1**: *Leishmania (L.) infantum* histone H1; **L3/L5**: *Leishmania major* ribosomal proteins L3 (LmL3)/L5 (LmL5); **NH36**: nucleoside hydrolase 36 kDa; **ORFF**: open-reading frame; **A2/CPA/CPB**: A2/cysteine proteinase type II/ cysteine proteinase type I; **KSAC**: polyprotein vaccine formulated with monophosphoryl lipid A; **NS protein**: nucleoside hydrolase and a sterol 24-c-methyltransferase; **8E/p21/SMT**: 8E/p21/sterol methyltransferase; ***L.***: *Leishmania*. *Indicates polyprotein or chimeric vaccines.

shows a summary of relevant vaccine candidates evaluated as individual recombinant protein or polyprotein vaccines against VL[67] [68] [69] [70] [71] [72] [73] [74] [75] [76] [77] [78] [79].

THE APPLICATION OF IMMUNO-PROTEOMIC APPROACHES TO THE IDENTIFICATION OF NEW *LEISHMANIA* ANTIGENS WITH POTENTIAL TO BE USED AS VISCERAL LEISHMANIASIS VACCINES

Immuno-proteomic approaches have been developed to identify new *Leishmania* proteins with distinct biological functions, such as new diagnostic markers, vaccine candidates, and/or potential drug targets[80] [81] [82]. The use of antileishmanial antibodies obtained from infected mammalian hosts contributed to the refinement of these analyses, by assisting in the identification of antigens recognized by the immune system during active disease[82] [83]. Immuno-proteomic approaches usually involve protein preparation and separation by bi-dimensional electrophoresis, followed by immunoblotting experiments, and subsequent identification of protein spots by mass spectrometry (**Figure 1**). In a recent immuno-proteomic study, several antigenic parasite proteins were identified from serum samples of dogs developing VL[83]. These proteins were analyzed *in silico* for epitope identification, and the best antigenic determinants were employed in enzyme-linked immunosorbent assay (ELISA) assays aiming to identify antigenic peptides of interest for the serodiagnosis of canine disease. The authors speculated about the use of these candidates as vaccines in future assays, owing to the existence of putative-T cell motifs in the antigens. In another immuno-proteomic approach, developed using protein extracts from the stationary promastigote and amastigote-like stages of *L. infantum*, several specific promastigote (**Table 2**) and amastigote (**Table 3**) hypothetical proteins were identified in serum samples from dogs with asymptomatic and/or symptomatic VL[82].

Some of these proteins have already been validated as candidate VL vaccines (**Table 4**). These antigens were selected because they are conserved among different *Leishmania* species, but are not present in other Trypanosomatidae or in mammalian hosts. In addition, the selected antigens contain specific $CD4^+$ and $CD8^+$ T cell epitopes. In this context, the protective efficacy against *L. infantum* infection of LiHyp1, a *Leishmania* protein belonging to the super-oxygenase family, was evaluated in BALB/c mice. Immunization using the recombinant LiHyp1 protein

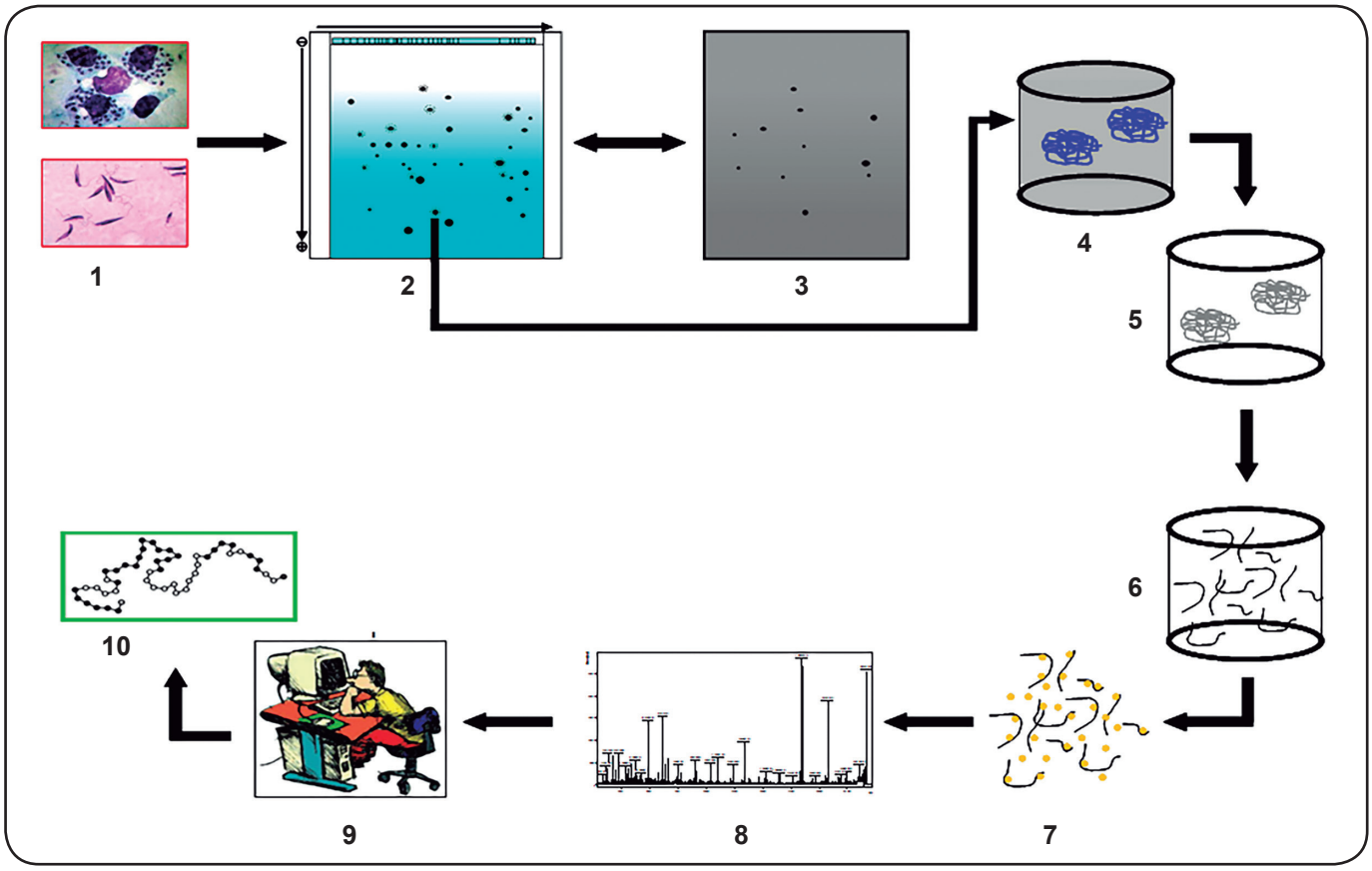

FIGURE 1. Experimental workflow for accurate identification of antigenic proteins using bi-dimensional (2-DE) immunoblotting assays. The technical steps usually performed when an immuno-proteomic approach is applied are: 1) Preparation of total protein extracts from the parasite. 2) Isoelectric focusing and bi-dimensional SDS-PAGE. 3) Immunoblotting experiment. 4, 5, 6) Protein digestion and peptide extraction. 7, 8) Prepare and identify spots using MALDI-TOF-TOF peptide mass mapping. 9) Database search to identify the antigenic proteins. 10) Identified proteins. **SDS-PAGE:** Polyacrylamide gel electrophoresis. **MALDI-TOF-TOF:** matrix-assisted laser desorption ionization/time-of-flight mass spectrometer.

TABLE 2

Hypothetical proteins identified in *Leishmania infantum* stationary promastigotes by an immuno-proteomic approach using serum samples from dogs with asymptomatic and symptomatic VL.

Sera class[a]	Identified species[b]	Accession number[c]	*Mr* (kDa) (exp/pred)[d]	*pI* (exp/pred)[e]
Asymptomatic	*L. major*	CAJ09012.1	23/24	5.88/6.44
Asymptomatic	*L. braziliensis*	XP_001567688.1	36/72	6.21/6.33
Asymptomatic	*L. infantum*	XP_001466647.1	42/41	5.05/4.89
Symptomatic	*L. infantum*	XP_001463668.1	18/23	4.81/5.97
Symptomatic	*L. braziliensis*	XP_001564693.1	21/24	5.55/10.64
Symptomatic	*L. major*	XP_001684884.1	21/21	6.20/6.60
Symptomatic	*L. braziliensis*	XP_001565846.1	22/35	6.45/7.68
Symptomatic	*L. major*	XP_001686061.1	24/37	4.52/9.17
Symptomatic	*L. braziliensis*	XP_001568689.1	27/24	6.80/8.89
Symptomatic	*L. major*	XP_888524.1	50/54	5.35/5.20
Symptomatic and asymptomatic	*L. braziliensis*	XP_001568117.1	13/35	5.23/5.25
Symptomatic and asymptomatic	*L. major*	XP_001682223.1	12/59	5.85/5.64
Symptomatic and asymptomatic	*L. major*	XP_001684096.1	18/19	4.65/9.75
Symptomatic and asymptomatic	*L. infantum*	XP_001468309.1	25/82	5.82/6.41
Symptomatic and asymptomatic	*L. braziliensis*	XP_001568364.1	68/71	4.85/5.89

VL: visceral leishmaniasis; ***Mr* (Kda):** molecular weight; ***pI:*** isoelectric point; **exp/pred**: expected/predicted; ***L.:*** *Leishmania;* **NCBI:** National Center for Biotechnology Information. [a]Serum samples from dogs with VL. [b]Identified species. [c]NCBI Accession number. [d]Expected/predicted *M*r. [e]Expected/predicted *pI.*

TABLE 3

Hypothetical proteins identified in amastigote-like *Leishmania infantum* by an immuno-proteomic approach using serum samples from dogs with asymptomatic and symptomatic VL.

Sera class[a]	Identified species[b]	Accession number[c]	*Mr* (kDa) (exp/pred)[d]	*pI* (exp/pred)[e]
Asymptomatic	*L. infantum*	XP_001467126.1	19/21	5.25/6.60
Asymptomatic	*L. infantum*	XP_001468941.1	37/36	6.53/5.72
Symptomatic	*L. infantum*	XP_001568117.1	13/35	5.23/5.25
Symptomatic	*L. infantum*	AF398369_1	14/13	6.55/5.27
Symptomatic	*L. major*	XP_001684096.1	18/19	4.65/9.75
Symptomatic	*L. infantum*	XP_001468309.1	25/82	5.82/6.41
Symptomatic	*L. braziliensis*	XP_001564596.1	28/94	5.02/5.58
Symptomatic	*L. infantum*	XP_001467567.1	25/25	6.40/8.40
Symptomatic	*L. major*	XP_843545.1	41/45	6.18/6.21
Symptomatic	*L. braziliensis*	XP_001564657.1	46/52	6.25/10.09
Symptomatic	*L. infantum*	XP_001465588	50/45	6.09/4.04
Symptomatic	*L. braziliensis*	XP_001564657.1	60/52	6.12/10.09
Symptomatic	*L. infantum*	XP_001463134.1	75/72	5.40/8.95
Symptomatic	*L. infantum*	XP_001466114.1	72/108	6.25/6.12
Symptomatic and asymptomatic	*L. major*	XP_888524.1	50/54	5.35/5.20
Symptomatic and asymptomatic	*L. infantum*	XP_001469969.1	58/86	5.25/5.52
Symptomatic and asymptomatic	*L. braziliensis*	XP_001568364.1	68/71	4.85/5.89

VL: visceral leishmaniasis; ***Mr* (Kda):** molecular weight; ***pI:*** isoelectric point; **exp/pred**: expected/predicted; ***L.:*** *Leishmania;* **NCBI:** National Center for Biotechnology Information. [a]Serum samples from dogs with VL. [b]Identified species. [c]NCBI Accession number. [d]Expected/predicted *M*r. [e]Expected/predicted *pI.*

TABLE 4

Leishmania-specific hypothetical proteins validated as vaccine candidates for visceral leishmaniasis.

Protein	Accession number	*Mr* (kDa)/*pI*	Biological application	Reference(s)
LiHyp1	XP_001468941.1	36.6/5.72	Vaccine/Diagnosis	[14]
LiHyp6	XP_001467126.1	21.4/6.60	Vaccine/Diagnosis	[55]
LiHyD	XP_001468360.1	36.5/9.49	Vaccine/Diagnosis	[31] [91]
LiHyT	XP_001465138.1	36.4/10.58	Vaccine	[32] [55]
LiHyV	XP_888524.1	54.0/5.16	Vaccine/Diagnosis	[84]

***Mr*:** molecular weight; ***pI*:** isoeletric point. **LiHyp1**: *Leishmania infantum* hypothetical protein 1; **LiHyp6**: *Leishmania infantum* hypothetical protein 6; **LiHyD**: *Leishmania infantum* hypothetical protein D; **LiHyT**: *Leishmania infantum* hypothetical protein T; **LiHyV**: *Leishmania infantum* hypothetical protein V.

plus saponin adjuvant induced a Th1 immune response in the vaccinated animals, which was primed by protein- and parasite-specific IFN-γ, IL-12, and GM-CSF production, combined with the presence of low levels of IL-4 and IL-10. In addition, the protected animals displayed significant reductions in the number of parasites in their livers, spleens, bone marrow, and draining lymph nodes, compared with that in control groups. The protection was correlated with parasite-specific and dependent IFN-γ production, mainly by $CD4^+$ T cells, which were the major source of IFN-γ in these animals[14].The same immune profile was found when the hypothetical LiHyD[31], LiHyT[32], LiHyp6[55], and LiHyV[84] proteins were evaluated as vaccine candidates. In all cases, the antigens were shown to be protective against infection, since vaccinated and challenged animals presented significantly lower parasite levels in evaluated organs compared with control groups. In addition, vaccinated and challenged animals demonstrated predominantly IL-12 driven IFN-γ production (also mediated mainly by $CD4^+$ T cells) against parasite proteins, whereas unprotected controls showed high levels of anti-*Leishmania* IgG1 antibodies and a parasite mediated IL-4 and IL-10 response.

An aspect that should be considered when evaluating the efficacy of a vaccine is the use of adjuvants. Although recombinant protein-based vaccines offer considerable advantages in terms of safety, standardization, purity, and production costs, they generally present limited immunogenicity and require the use of immune adjuvants[85]. It is generally accepted that the adjuvants used in leishmaniasis vaccine formulations should be able to induce a Th1 response, and some adjuvants are capable of this, including recombinant IL-12, saponin, BCG, monophosphoryl lipid A (MPL), CpG, recombinant virus, and others[86] [87]. The induction of IL-12 is critical for vaccine efficiency and many of these adjuvants activate the innate immune response via Toll-like receptors (TLR), also influencing acquired immune responses[88]. Significant protection is not usually achieved when animals are immunized with recombinant proteins in the absence of adjuvants. These findings have been corroborated by studies evaluating other well-known protective antigens against leishmaniasis[31] [32] [40] [54].

The concept of cross-protective vaccines is based on the presence of common antigens among pathogens and on the ability of formulated vaccines to elicit cellular immunity[89]. Since multiple *Leishmania* species are distributed in common geographical areas, it would be desirable to develop vaccines capable of inducing protection against more than one parasite species[90]. In this context, LiHyT, which was firstly identified, in *L. infantum*[32] as protective against this species, was also shown to confer protection in BALB/c mice against *L. major* and *L. braziliensis*. Mice immunized with LiHyT and saponin as an adjuvant, developed a robust Th1 immune response, which was responsible for the induction of significant reductions of parasite load in the tissues and organs evaluated[84]. This cross-protective immunity was also found when another hypothetical protein, rLiHyD, was used as an antigen with saponin as an adjuvant[91].

As described, the use of chimeric vaccines containing multiple proteins and/or polypeptides could provide more robust protective efficacy against various *Leishmania* species[61] [76] [78]. In this context, three recombinant proteins were combined in a vaccine and tested for their protective effects against *L. infantum* infection. These proteins are expressed in both the promastigote and amastigote stages of the parasites, and their combination was able to induce pronounced parasite-specific IFN-γ, IL-12, and GM-CSF responses in immunized mice, which was maintained after challenge. The infected and vaccinated animals showed significant reductions in parasite burden in the various organs evaluated compared with control mice, the protection being associated with IL-12-dependent IFN-γ production against parasite extracts, and correlated with the induction of antileishmanial nitrite production. More importantly, this polyprotein vaccine was able to induce a more robust Th1 response associated with better control of parasite dissemination in the organs of the vaccinated animals, compared with the use of the individual recombinant proteins[55].

CONCLUSIONS

Effective prophylactic measures to control VL are imperative. Such measures include the design of vaccines, which is the most economical way to control neglected diseases. An ideal vaccine candidate should be able to induce robust antileishmanial Th1 immunity, be parasite-specific (to avoid adverse effects in mammalian hosts), and exhibit a high degree of homology between different *Leishmania* species. Hypothetical proteins, considered unknown molecules until their recognition by the immune system of infected mammalian

hosts, could be considered for this purpose and explored for use in the prevention of VL. In addition, the development and use of new technologies, such as reverse vaccinology, to identify novel candidate VL vaccines should be also considered.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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